CULTIVOS HERBACEOS

17th INTERNATIONAL SUNFLOWER CONFERENCE

Vol. 1

JUNTA DE ANDALUCIA

Consejería de Agricultura y Pesca

Proceedings of the

17th International Sunflower Conference

Vol. 1

Córdoba, Spain

June 8-12, 2008

Sponsored by The International Sunflower Association, Paris, France

Instituto de Investigación y Formación Agraria y Pesquera
CONSEJERÍA DE AGRICULTURA Y PESCA
CSIC
International Sunflower Association

Título: Proc. 17th International Sunflower Conference, Córdoba, Spain (2008), Vol. 1

© Edita: JUNTA DE ANDALUCÍA - Consejería de Agricultura y Pesca

Publica: Secretaría General Técnica. Servicio de Publicaciones y Divulgación

Colección: Congresos y Jornadas

Serie: Cultivos Herbáceos

Depósito Legal: SE-3054-08

Impresión: German López - Servicios Gráficos

Proceedings of the 17th International Sunflower Conference
Córdoba, Spain. June 8-12, 2008

Editor: Leonardo Velasco

Photography: *Spanish landrace of confectionary sunflower collected by L. Velasco and B. Pérez-Vich in Villarta de San Juan, Ciudad Real, Spain, on October 10, 2007.*

Foreword

The proceedings of the *17th International Sunflower Conference* contain 142 contributions from scientists of 24 countries. They include plenary lectures in several disciplines and regular communications presented in posters during the conference and discussed in the corresponding workshops. The manuscripts are classified by disciplines. They offer a good picture of the current state of the art of sunflower research and cultivation around the world.

The manuscripts in the *Proceedings* have been reviewed by an editorial committee with the main objective of helping the authors to improve their manuscripts through a critical reading. The authors received the edited manuscripts together with the comments of the reviewers and then went on to draft their final version. All the manuscripts received have been published in the *Proceedings*. The contents of the manuscripts are the responsibility of the authors. They should be considered as being privileged communications that require the express consent of the authors to be reprinted in part or as a whole. We wish to thank both the members of the Editorial Committee for their dedication to the task of editing such a large number of manuscripts, as well as all the authors for their collaboration throughout the whole edition process.

The Organizing Committee would also like to thank Diana Badder and José A. Palacios for their excellent editorial assistance in the preparation of these *Proceedings*. We are indebted to the Spanish Association of Sunflower Breeders (Asociación Española de Mejoradores de Girasol), which collaborated actively in the organization of the conference, and, very especially, to Juan Parejo, who was in charge of the financial side.

Finally, we would like to thank all the participants in the conference, who have contributed to its success by a careful preparation and revision of manuscripts and posters, presentation of their research in the workshops, and stimulating discussions throughout the conference on the scientific and technical aspects of sunflower research and cultivation in the world.

The Organizing Committee
17th International Sunflower Conference
Córdoba, Spain. June 8-12, 2008

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PLENARY

DISEASE RESISTANCE AND PATHOLOGY

CROP PRODUCTION – PHYSIOLOGY

Research progress in sunflower diseases and their management

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ABSTRACT

Sunflower diseases are of major concern in the production of this crop worldwide. This is due to the regular and quite often severe attack by different pathogens. As a result considerable yield losses occur or the quality of product lessens. Though the number of pathogens known to attack sunflower is relatively high, only a handful to a dozen are considered important ones depending on region and cultivar. In this review, I am focusing particularly on these significant pathogens. The emphasis will be on new findings and results obtained by researchers related to pathogen biology, ecology, genetics, host resistance, and control. It was interesting to note a considerable shift in the relative dominance of diseases over the last four years as reflecting in the number of publications available. The scientists' efforts have resulted in better understanding of individual diseases and underline their significance in the improvement of sunflower management.

Key words: ecology – diseases – genetics – host resistance – pathogen biology

INTRODUCTION

Sunflower diseases are one of the major constraints in influencing production stability of this crop worldwide. Though there are more than a dozen of pathogens that may attack cultivated sunflower resulting yield losses, just a few are of concern in a particular country or region. In a literature survey of the past four years (2004 to 2007), I found a great number of publications regarding sunflower diseases. Similarly to Felicity Vear's findings reported at the 16th International Sunflower Conference in Fargo (Vear, 2004), there were significant differences in the number of papers dealing with individual pathogens and/or originating from different countries and regions. An overview of reference sources obtained from the Web of Science or kindly provided by individual scientists served as a basis of this present review. A total of 13 sunflower pathogens were the subject of papers available and there were major differences in the proportion of these references (Table 1). Most of the publications dealt with downy mildew (*Plasmopara halstedii*), and broomrape (*Orobanche cumana*), followed by white rot (*Sclerotinia sclerotiorum*), stem canker (*Diaporthe helianthi*), Alternaria blight (*Altermaria helianthi*, *A. helianthinficiens*), rust (*Puccinia helianthi*) and black stem (*Phoma macdonaldii*) in a decreasing sequence of order. Some diseases of local importance represented by several or just a few references are Verticillium wilt (*Verticillium dahliae*), white blister rust (*Albugo tragopogonis*), charcoal rot (*Macrophomina phaseolina*), Fusarium wilt (*Fusarium* spp.), Rhizopus head rot (*Rhizopus arrhizus*), and *sunflower chlorotic mottle virus* (SuCMoV). In addition, information on sunflower diseases is also available in a Progress Report by Masirevic (2005a) based on contributions of the sub-group leaders of the Working Group Sunflower Diseases, presented at the 10th FAO European Research Network on Sunflower Consultation Meeting in Novi Sad in 2005.

Table 1. A list of references concerning sunflower diseases for the period 2004-2007 available for the author

Disease	Pathogen	No. of records
Downy Mildew	*Plasmopara halstedii*	46
Broomrape	*Orobanche cumana*	31
White rot	*Sclerotinia sclerotiorum*	17
Stem canker	*Diaporthe helianthi*	15
Alternaria blight	*Alternaria helianthi, A. helianthinficiens*	15
Rust	*Puccinia helianthi*	12
Phoma black stem	*Phoma macdonaldii*	10
Virus	*Sunflower chlorotic mottle virus*	7
Verticillium wilt	*Verticillium dahliae*	5
Charcoal rot	*Macrophomina phaseolina*	4
White blister rust	*Albugo tragopogonis*	4
Fusarium wilt	*Fusarium* spp.	3
Rhizopus head rot	*Rhizopus* spp.	1

DISCUSSION

Downy mildew. Based on a literature survey for the years 2004-2007, most of the publications for this period have dealt with this devastating disease caused by *Plasmopara halstedii* (Farl.) Berl. et de Toni. It continued to occur in almost all parts of the world where sunflower was grown, except for Australia. The biology and ecology of this organism is well-known as are many aspects of its pathogenicity, host – pathogen interaction and genetic and chemical control. Its capacity to diverse, both in virulence and fungicide, however, is very high, giving a continuous challenge to scientists.

The most detailed and up-to-date list of global distribution of *P. halstedii* pathotypes has been compiled by Gulya (2007) in a paper presented at the 2nd International Downy Mildew Symposium, Olomouc, Czech Republic. In this accurate overview he comprised as many as 35 pathotypes (races), an unbelievably high number considering the fact that in most sunflower producing countries from just a few to 12 well-distinguished virulence phenotypes exist. In Europe, France, Germany and Spain reported the highest numbers but the pathogen is rather diverse in the USA, Canada, and in South Africa as well. Furthermore, there are five *P. halstedii* pathotypes (300, 330, 710, 730, 770) that are universally distributed globally, recorded from North and South America, Europe and Africa. Apart from the quantitative aspect of virulence, it is interesting to consider the dynamics of diversity as well, i.e. the changes in a given region over time. In this respect, France leads with the highest number of new pathotypes arisen in the last 6-7 years (Vear et al., 2006). Considering population changes for virulence, a good example has recently be found in the USA by Gulya (unpublished), where 3 out of 11 pathotypes (710, 730, 770) were recorded from North and South Dakota and Minnesota in each year during the 1998 to 2007 period whereas two others (300, 772) appeared in one year and a third one (300) in two years only. Recently, Delmotte et al. (2008) analyzed the possible origin of *P. halstedii* populations existing in France using different molecular methods. Based on single nucleotide polymorphisms they assumed a multiple introduction into France of the pathogen populations exhibiting differences in virulence phenotype.

Like other biotrophic obligate parasites, *P. halstedii* has a narrow host range. In other words, though it has originally been described to occur on a number of Composites and was found to attack a few wild *Helianthus* species as well, until recently no much attention has been paid to any alternate host as potential infection sources. Recently, however, two records of the natural occurrence of this Oomycete on wild asteraceous plants appeared, on velvetleaf (*Abutilon theophrasti*) by Masirevic (2005b) in Serbia and on *Rudbeckia fulgida* by Hong (2006) in Virginia. With these records, a total of five asteraceous wild plant species (the other three being *Xanthium strumarium*, *Ambrosia artemisiifolia* and *Iva xanthifolia*) are known to be as alternative hosts of *P. halstedii*. The natural host state in each case has been proved with successful reinoculation to cultivated sunflower.

With the rapid improvement of molecular techniques and their use in plant pathology, new developments have opened new insight into research on fungal biology, detection technology, and genetics and host - pathogen interactions. For example, Hammer et al. (2007) in Germany, and Ioss et al. (2007) in France, using different approaches, were successful in detecting fungal structures from sunflower host tissues.

Furthermore, it became possible to study the genetic recombination in *P. halstedii* through parasexual events using DNA fingerprinting (Spring and Zipper, 2006). In attempts to characterize the molecular structure of this Oomycete, Thines and co-workers (2005) detected and characterized an exceptional length of ITS that was due to multiple repetitions in the ITS-2 region. Further, ITS sequence data were also used to detect possible differences between isolates differing in virulence and/or in geographic origin.

Recently, two papers dealt with the isozyme analysis of *P. halstedii* isolates in order to find out intraspecific polymorphism in sub-populations of this organism. Guchetl et al. (2007) studied eight different isoenzymes. While interracial differences on esterase were inessential, the other seven isoenzymes appeared to be monomorphous for all pathotypes studied (330, 700, 710 and 730) suggesting that downy mildew populations in the Krasnodar region had low intraspecific variability for these traits. Komjáti et al. (2008) in Hungary used cellulose-acetate gel electrophoresis to analyze sixteen isozyme systems of 10 field isolates and 35 single-spore lines representing 10 different virulence phenotypes. Apart from sunflower, isolates were also from cocklebur (*Xanthium strumarium*) and from *Helianthus x laetiflorus*. Three isozymes, isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH) and phosphoglucomutase (PGM) revealed some polymorphism among the isolates. PGM differentiated two groups among the isolates from cultivated sunflower, while the other enzymes were polymorphic between isolates from different hosts. However, polymorphisms did not relate to virulence phenotype.

To develop and release sunflower cultivars resistant to different pathotypes is of extreme importance to growers. Therefore, breeders are continuosly searching for new genes or gene clusters conferring resistance to *P. halstedii* (Sreten et al., 2007), and selecting for such genes using molecular markers. In the recent years, Radwan et al. (2004) in France and Dussle et al. (2004) in Germany achieved considerable results with PCR markers for the *Pl5*/*Pl8* locus from complete CC-NBS-LRR sequences and, with the localization of the Pl_{arg} gene using SSR markers, respectively. Furthermore, in inheritance studies of resistance to the 703 pathotype Pankovic et al. (2007) used both traditional segregation tests and PCR markers and obtained identical results related to the *Pl6* gene conferring resistance to 730.

At the Fargo conference, Felicity Vear presented an outstanding review of recent breeding work for resistance to sunflower pathogens (Vear, 2004). At that time she outlined the importance of durable resistance to combat the increasing number of new *P. halstedii* pathotypes overcoming the *Pl* gene-mediated resistance. Since then the Clermont-Ferrand group has proceeded with this work and some of their results have already been published (Tourvieille et al., 2005; Vear et al., 2006). Durable resistance was found to be independent of major gene resistance so they proposed for the future to combine both types of resistance in new cultivars for more effective and long-lasting genetic control. Partial resistance to *P. halstedii* in high oleic sunflower hybrids have been reported by Baldini et al. (2006) as well in Italy.

The hypersensitive reaction (HR), a well-known phenomenon among plant pathologists, has been the subject of investigations by Radwan et al. (2005) to characterize this mechanism in the sunflower downy mildew system. RT-PCR analysis showed that resistance was associated with the activation of a hsr203J-like gene, a molecular marker of HR in tobacco. Activation of this gene was specifically observed during the incompatible interaction and coincided with cell collapse in hypocotyls. No such HR or a significant activation of the hsr203J-like gene were observed during the compatible combination suggesting that HR failed to halt the parasite, rather it trigged a systemically-aquired resistance (SAR) taking place in the upper part of the hypocotyl and this might arrest pathogen growth.

Apart from durable resistance, induced resistance might be useful for improving downy mildew management. In the recent years, Hungarian and Spanish laboratories conducted studies to better understand this type of host defense. The plant activator benzothiadiazole (BTH) significantly depressed disease symptom appearance and pathogen growth in susceptible sunflowers treated and inoculated at the germling stage (Körösi et al., 2007). Furthermore, microscopical observations revealed a high similarity between genetic (*Pl*-gene mediated) and induced resistance responses in compatible combinations. Roldan Serrano et al. (2007) recently published a paper about chitinase and peroxidase activities in BTH-treated sunflower inoculated with *P. halstedii*. They found an increased level of activity for both enzymes in susceptible but not in resistant seedlings. In our laboratory, we compared susceptible, partially resistant and resistant interactions for various enzyme activities (unpublished) and also tested the glutathion S-transferase (GST), defensin (PDF) and catalase (CAT) gene expression. Preliminary results of this work will be presented by Körösi et al. in this conference.

Resistance or tolerance to metalaxyl has already been noted in France and the USA. Quite recent records, however, came from Spain (Molinero-Ruiz et al., 2005) and Germany (Spring et al., 2006). It is interesting to note that, as an alternative, Fernández-Ocaña et al. (2004) conducted experiments with the essential oil of *Bupluerum gibraltarium*. They found this oil acting as a host defense activator rather than directly inhibiting sporulation.

Broomrape. *Orobanche cumana* Wallr. is a parasitic plant that infects sunflower causing considerable damage. In Spain, parasitized plants exhibited lower shoot dry weight, and they were shorter (due to reduction in internode length) with smaller head diameter as compared to healthy ones (Alcántara et al., 2006). In addition, a significant decrease in the mineral composition of the leaves of affected plants could be detected.

Different pathogenic races of *O. cumana* are known to exist in various regions of Europe and in the southeastern Mediterranean where the climate is favorable for this parasite. Due to this genetic diversity a new pathogenic form, race F appeared recently in Spain (Pérez-Vich et al., 2004), in Russia (Goncharov et al., 2004), in Turkey (Kaya et al., 2004), in Israel (Eizenberg et al., 2004), and in Bulgaria (Shindrova, 2006), with the highest diversity existing in Turkey. More expanded field surveys and subsequent identification processes are required to get a better view of the incidence and distribution of pathogenic variants of this parasite. In this respect, Román et al. (2007) succeeded in developing a detection method by using cpDNA diagnostic markers and they proposed this molecular protocol for use in identification work.

In a study on the mechanism of broomrape parasitism in sunflower, Slavov et al. (2004) pointed out that seed germination of the parasite was triggered by a germination stimulant secreted by the host-plant

roots. Further, they quantified indole-3-acetic acid as early as 24 h after the seeds were exposed to the germination stimulant, suggesting the role of IAA in the germination process. When comparing different populations of this parasite for their virulence on different sunflower genotypes, Veronesi et al. (2005) found that before attachment, *Orobanche* seedlings released cell-wall-degrading enzymes such as pectin methylesterase and polygalacturonase. These enzymes' activity were very high in the most virulent, recently discovered race F. Eizenberg et al. (2005) developed a new methodology that allowed them to facilitate the in-situ study of major aspects of the host - parasite interaction.

Broomrape resistance is poorly understood and new races of the parasite evolve rapidly to overcome the resistance of newly introduced sunflowers. Labrousse et al. (2004) screened a number of recombinant inbred lines derived from interspecific crossings. A considerable variation in the characters tested showed that polygenic resistance could occur in some lines. In another experimental system Echevarría-Zomeno et al. (2006) investigated the histology of host – parasite interface. Suberization and protein cross-linking at the cell wall were seen in the resistant sunflower cells in contact with the parasite and confocal laser microscopy revealed accumulation of phenolic compounds during the incompatible reaction. Letousey et al. (2007) carried out molecular analysis of the resistance mechanism. RT-PCR and cDNA blot experiments revealed that the *Orobanche* resistant genotype exhibited a stronger overall defense response against *O. cumana* than the susceptible one. The SA-responsive gene, *def.* (defensin), appeared to be characteristic of LR1 sunflower resistance. Ha-DEF1 (a sunflower defensin) was found to induce cell death in the parasitic plants appearing as a brown symptom at the radicle apex of the parasite (de Zélicourt et al., 2007). The resistance phenomenon to broomrape in sunflower was also the subject of studies to map and characterize quantitative trait loci for resistance to race E and race F by Pérez-Vich and co-workers (2004). Their results suggested that resistance to broomrape in sunflower is controlled by a combination of qualitative, race-specific resistance affecting the presence or absence of broomrape and a quantitative, non-race specific resistance affecting their number.

In inheritance studies on the sunflower line J1 to *Orobanche* race F, Velasco et al. (2007) detected incomplete dominance of the *Or6* alleles and subsequent segregation ratios suggested the presence of a second gene, *Or7*, the expression of which was influenced by the environment. Meanwhile Spanish breeders were successful in finding sunflower germplasm resistant to race F of broomrape (Fernandez-Martinez et al., 2004) and those with quantitative resistance to the same race (Pérez-Vich et al., 2006). In addition, sunflower hybrids resistant to race F have been released in Spain (Pérez-Vich et al., 2004), in Russia (Goncharov et al., 2004), and in Turkey (Kaya et al., 2004).

For future broomrape management it might be of interest to consider to use host defense system as an alternative to genetic resistance. Buschmann et al. (2005) reported about positive results with BTH against *O. cumana* infestation, and later on Fan et al. (2007a), from the same laboratory, evaluated the efficacy of prohexadione-calcium against this parasite. Neither of these plant activators had a direct effect on the parasite, but rather induced host defense only.

An additional way of broomrape control could be by using biological antagonists. One of the candidates is *Fusarium oxysporum* f. sp. *orthoceras*, which was the subject of investigations by Dor et al. (2007). They studied the pathogenicity and toxin production of this fungus. Two main toxic metabolites caused mortality of germinating broomrape seeds and these were identified as fusaric acid and 9,10-dehydrofusaric acid. Müller-Stöver et al. (2004) also found *F. oxysporum* f. sp. *orthoceras* (Fo) as a potential of biocontrol agent and they were successful developing two granular formulations under laboratory conditions. In an other experiment Müller-Stöver and co-workers (2005) were able to increase control of *O. cumana* through integration of Fo with BTH-treatment. Under laboratory conditions no enhancing effect of BTH on virulence and growth of the fungus was observed. Fan et al. (2007b) achieved similar results when they combined the application of Fo and acibenzolar-S-methyl (ASM). The interaction between ASM and Fo was highly significant on *O. cumana* number and dry matter. ASM soil drenches combined with Fo were more effective than ASM foliar spray with Fo.

White rot. Sunflower stalk and head rot incited by *Sclerotinia sclerotiorum* (Lib.) de Bary is considered the most important disease of this crop in many parts of the world. Since cultural practices or fungicides are insufficient to control the disease, efforts are being made by breeders to develop resistant or tolerant cultivars. This may explain the dominance of publications dealing with various aspects of resistance.

Disease incidence of white rot may vary with location and season, as well as with sunflower genotype, and the symptoms appearing on sunflower stem or head are also diverse. For example, in the United States a three-year field survey (2005-2007) was made by Tom Gulya and co-workers (2008) in the main sunflower growing regions (North and South Dakota, Minnesota) regarding *Sclerotinia* stalk rot

occurrence. Both, the percentage of fields with stalk rot and the severity of affected fields varied between 16-30 %, and 4.4-6.3 %, respectively.

In the recent years, several reports have dealt with the evaluation methods of sunflower genotypes for resistance to *S. sclerotiorum*. Thus, Baldini et al. (2004) in Italy compared host reactions to basal stem and head inoculation, Pedraza et al. (2004) in Argentina examined the suitability of the length of susceptible period as a measurement of partial resistance, van Becealere (2004) in the USA described an improved screening method for assessing head rot resistance, and Castaño et al. (2005) compared the reaction of sunflower accessions to both *S. sclerotiorum* and *Albugo tragopogonis*. By looking for resistance sources among the wild *Helianthus* species, Cáceres et al. (2006) found differences in lesion length of leaves following inoculation but it was not the case with stem inoculation.

In a breeding program in France, Felicity Vear and co-workers (2007) aimed at improving the *S. sclerotiorum* head rot resistance using recurrent selection of a restorer population. In 4 cycles an 80 % reduction in diseased area was obtained and thereafter the population remained stable and homogenous for this character.

Maringolo et al. (2007) in Argentina successfully studied quantitative trait loci for sunflower capitulum resistance to head rot.

Stem canker. *Diaporthe helianthi* Muntanola-Cvetkovic, Mihaljcevic et Petrov (anamorph: *Phomopsis helianthi* Muntanola-Cvetkovic, Mihaljcevic et Petrov) has become a serious threat in sunflower production in the early 1980s in Europe and subsequently in other parts of the world, e.g. in North and South America. Relatively soon after its appearance, it became one of the most limiting factor of sunflower production in many parts of Europe, including the former Yugoslavia, Romania, Hungary, and France. However, following several years of epidemics in these countries, the disease occurrence lessened probably due to unfavorable weather conditions (dry and hot). In contrast, Huguet (2005) reported about a severe attack of this pathogen from a region of Uruguay close to the Argentinian border having an average incidence of 39%.

Walcz and Nébli (2006) investigated the persistence of this pathogen in infected stems and achenes. They found that *D. helianthi* perithecia even disposed to outdoor conditions for 3 years produced viable ascospores, as well as a few pycnidia (the latter occurring most on achenes). The fact that *D. helianthi* can be distributed with seed underlines the importance of phytosanitary measures in seed production and commerce.

Molecular studies on the intraspecific diversity of this fungus using intergenic spacer sequence analysis revealed a high homology among French/Yugoslavian and among Italian isolates (Pecchia et al. 2004). The phylogenetic tree obtained from the aligned data revealed three separate groups. The analysis also showed that all isolates originating from countries with regular and severe outbreaks of the disease (e.g. France, Yugoslavia) formed a well-defined taxon with relatively low variability compared to isolates from Italy where the disease is much seldom to occur. In another paper, Rekab et al. (2004) pointed out a polyphyletic nature of this fungus.

Besides traditional methods of resistance testing (Walser et al., 2005), Quaglia and Zazzerini (2007) reported about an *in vitro* screening for sunflower calli to *D. helianthi* fungal culture filtrate. Looking for recent publications regarding resistance breeding programs, only two reports were available. A collaborative work between Bulgaria and Germany (Encheva et al., 2004) evaluated somaclonal variation, and a study from Hungary (Csikász et al., 2006) in which selection of elite lines was described for specific resistance alleles.

Alternaria blight. The disease can be incited by two fungi *Alternaria helianthi* (Hansf.) Tubaki et Nishihara and *A. helianthinficiens* Simmons, Walcz et Roberts, but Gonorazky et al. (2005) described *A. alternata* as well as one of the seed infecting species found in Argentina. Calvet et al. (2005) determined the average decrease in the photosysnthetic rate in diseases leaves, and Leite et al. (2006) showed that disease severity could be used as an independent variable in a sunflower – *Alternaria* leaf spot management system by providing recommendations for resistance breeding or for studies on sowing date.

Madhavi et al. (2005a) compared six wild *Helianthus* species for resistance to *Alternaria* blight: *H. occidentalis* and *H. tuberosus* were found highly resistant, and *H. hirsutus* moderately resistant. Furthermore, on growth media supplemented with leaf extracts of these plant species the inhibition of fungal growth corresponded to *in vivo* responses of the particular species to inoculation. Further, the resistant *Helianthus* species possessed higher levels of constitutive as well as induced total phenols and total sugars as compared with susceptible sunflowers (Madhavi et al., 2005b).

Resistance breeding was the subject of several papers appeared in the recent years. De Oliveira et al. (2004) reported about mutation breeding from Brazil, and Murthy et al. (2005) assessed heritability of resistance using molecular markers. In India, ploidy manipulation and introgression of resistance to *A. helianthi* using wild *Helianthus* species as resistance source (Sujatha and Prabakaran, 2006), sporophytic and gametophytic recurrent selection for improving partial resistance (Rani and Ravikumar, 2006; 2007), and the description of transcripts during the necrotrophic interaction with *A. helianthi* (Anjana et al., 2007) reflected to the relative dominance of this disease in this country.

Rust. *Puccinia helianthi* Schwein. has a world-wide distribution but it has been considered as a severe pathogen causing considerable yield losses mainly in Australia and Argentina (Huguet et al. 2007). However, Zazzerini et al. (2005) reported about a considerable occurrence and spread of this disease from Mozambique as well.

The diversity of the sunflower – *P. helianthi* pathosystem has got a special attention by Sendall and co-workers (2006) describing a rapid and frequent virulence changes in the rust fungus population. Virulence data accumulated over 25 years coupled with studies on genotypic diversity and sexual reproduction permitted them to conclude that *P. helianthi* may evolve in wild sunflower populations providing a continuum of genetically heterogenous hosts on which this fungus can potentially complete its sexual cycle.

In Spain, Prats et al. (2007) carried out experiments to characterize the mechanism of resistance. Microscopical observations revealed that rust development depended on host genotype, i.e. impairment of rust spore germination and of appressorium formation associated with different excretion of coumarin on leaf surface. Mohase et al. (2006) in South Africa investigated the effect of rust infection on intercellular beta- 1,3-glucanase and chitinase activities, PAL acitivity and total salicylic acid content in relation to susceptibility vs. resistance interactions. Rust infection selectively increased the activity of pathogenesis-related proteins and other parameters studied. Treatment of susceptible plants with BTH induced intercellular glucanase activity and reduced susceptibility to rust. Induced resistance was also the subject of another paper by Amzalek and Cohen (2007) from Israel. Besides BTH, they used other inductors as DL-3-amino-n-butanoic acid (BABA), 2,6-dichloroisonicotinic acid (INA), and two enantiomers of BABA as well.

Phoma black stem. The disease is caused by *Phoma macdonaldii* Boerema (teleomorph: *Leptosphaeria lindquistii* Frezzi) appearing as black spots on stems and seldom on leaves of affected plants. Though its occurrence is quite common in several European countries, the disease is extremely severe in France where basal stem lesions often result in lodging. This could be the reason of the absolute dominance of French publications that appeared in the recent years. Darvishzadeh et al. (2007a) undertook experiments to determine the partial resistance of sunflower genotypes to seven isolates and highly significant differences were observed among genotypes, isolates and their interactions. Two genotypes exhibited specific resistance with a wide range of isolate-nonspecific partial resistance appearing as well. In addition, QTLs were also found associated with isolate specific and non-specific partial resistance (Darvishzadeh et al., 2007b). Alignan et al. (2006) developed a 1000-element cDNA microarray containing genes putatively involved in primary metabolic pathways in order to identify genes responsible for partial resistance. They were successful in identifying 38 genes differently expressed among genotypes, treatments and times. Comparative genetic analysis for the characterization of QTL involved in resistance of sunflower to *Ph. macdonaldii* has been made by Bert et al. (2004), and QTL mapping of partial resistance to stem and root necrosis in sunflower as well as inheritance studies were the subject of investigation by Abou Al Fadil et al. (2006, 2007).

Verticillium wilt (*Verticillium dahliae* Kleb.) is considered an important disease affecting sunflower in most production areas in Argentina (de la Vega et al., 2007), and is of concern also in Canada and the Unites States. Estimation of yield losses is difficult because of the absence of highly efficient chemical control (Creus et al., 2007). Therefore, host resistance is a major concern of breeders in these countries. Resistant breeding is in progress in Argentina (Maranesi and Mancuso, 2007) and in the United States (Radi and Gulya, 2007).

Charcoal rot (*Macrophomina phaseolina* [Tassi] Goidanich) may cause premature death of sunflowers grown on light, sandy soil under hot and dry climate. The disease is well-known in the Southern part of Europe, but the first occurrence in Slovakia was unexpected and probably due to the extremely warm and

dry seasons at that time (Bokor, 2007). In Hungary, Walcz and Piszker (2004) have developed an inoculation method for screening sunflower lines for resistance to this pathogen.

White blister rust (*Albugo tragopogonis*) is know to occur as a pathogen of significance only in South Africa, but it was recorded recently from Germany (Thines et al., 2006a) where the percentage of affected plants varied between 20 and 80 %. Another record is known from Belgium (Crepe et al., 2006). Thines et al. (2006b) studied the fatty acid profile, ultra structural characteristics and ITS sequencing of this fungus. Castaño et al. (2005) evaluated the reaction of a number of sunflower accessions originating from the North Central Regional Plant Introduction Office, Ames, Iowa, USA. Statistical analysis showed differential responses to white rust severity, incidence and relative incubation period among the accessions tested.

Fusarium wilt (*Fusarium* spp.) has been reported as a pathogen of concern only from Russia (Antonova, 2004) where it appeared to be harmful for sunflower production. Based on the extent of necrosis incited by the fungus on the main root and the root – hypocotyl transition zone of sunflower seedlings, some tolerance to pathogen attack could be detected among the genotypes (Antonova et al., 2005). In a breeding program a number of new breeding lines were developed exhibiting relatively good field tolerance (Goncharov et al., 2006).

Rhizopus head rot (*Rhizopus* spp.) exists under warm climatical conditions, like in North Africa, Australia and India. However, with the global warming it seems to occupy new areas, such as the Mediterraneum and southern Hungary. The most typical disease symptoms include rotting of sunflower head with a loose cover of grayish fungal spore mass. In Hungary Walcz et al. (2004) examined the effect of *Rhizopus* head rot on the oil content, oil quality and the germinability of seeds. All these parameters showed a negative tendency in the affected seeds as compared to healthy ones.

Virus (*Sunflower chlorotic mottle virus*, SuCMoV) has recently been detected in commercial hybrids and wild sunflowers in Argentina (Lenardon et al., 2005; Lenardon and Gioletti, 2007). More than two hundred lines were screened for resistance using artificial inoculations under greenhouse conditions and only three lines showed partial resistance in which virus replication was delayed and morphological traits (plant height, leaf width) were less affected. Arias et al. (2005) studied the mechanism of oxidative damage in SuCMoV infected sunflowers. Jan and Gulya (2006) in the United States reported about the registration of three virus resistant sunflower genetic stocks.

CONCLUSIONS

In this review I tried to outline the recent progress in research and development of sunflower diseases involving various aspects of each, in most cases depending on the amount of information available. Unfortunately there was no means to include all relevant experimental data, due to the page limitation for plenary papers. Similarly, I did not try to make any statistics in relation to diseases, topics or countries. Instead, I want to express my hope and feeling that the great number of references discussed in this review will give the reader an up-to-date summary of the most recent pathology research, the impact of diseases on sunflower production, and challenges remain for researchers.

ACKNOWLEDGEMENTS

I would like to thank everybody for their help in providing me with their research details. Special thanks are due to Thomas Gulya for the critical review of the manuscript and for correcting my English.

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Could a crop model be useful for improving sunflower crop management?

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ABSTRACT

In France, there is a need for improved sunflower crop management, in order to meet the greater requirement for oil by increasing both seed yields and the area of this crop. The objective of this article is to review the main characteristics of sunflower crop management in France and in other countries, in order to emphasize the need for improvement, and to evaluate if the recent advances in crop modelling could help to find solutions. In France, a better adaptation of crop management to water availability is needed, as well as a more efficient control of diseases without applying more fungicides. The results of these objectives would also trigger major improvements in other countries, but there is also a need to control insects and to adapt crop management to the goals of oil quality. The main sunflower crop models are reviewed in this article, with an emphasis on the most recent ones. Their ability to contribute to improving sunflower crop management, although they do not take into account diseases and insects, is discussed. Confidence in the decisions based on simulations, and the way to evaluate it, is also examined.

Key words: crop management – crop models – management strategies – model evaluation – sunflower.

INTRODUCTION

In France, there has been a stagnation in sunflower seed yield during the past 20 years. The annual mean seed yield has ranged from 2.1 to 2.7 t ha^{-1}. The highest value was observed in 2007, but it was mainly the result of the high rainfall during summer. During that period of time, the area cultivated in sunflower has decreased by almost 50 %. Overall, there has been a decrease in seed production, although there is a need for greater quantities because of the increased demand for biodiesel. Hence, both increases in seed yield and in the area cultivated in sunflower are necessary. Improvements in crop management would probably contribute to these objectives. However, during the past few years, sustainable agriculture has become a priority in the European Union. In France, the French government decided in 2007 that the applications of pesticides should decrease by 50% within 10 years. This has to be taken into account when improving crop management. The objective of this article is to review the main characteristics of sunflower crop management in France and in other countries, in order to emphasize the need for improvement and to evaluate if the recent advances in crop modelling could help to find solutions.

DISCUSSION

1. The main problems in sunflower crop management

Sunflower crop management in France is characterized by few applications of pesticides. Most of the time, insects are not a major problem. Hence, only 40% of the sunflower area received one insecticide in 2006. The reduction of plant population due to damage by slugs, birds or game animals has been observed more often. However, only 54% of the sunflower area was sprayed against slugs in 2006, while no treatments are allowed against birds or game animals. Moreover, the application of fungicides is rare (only 7% in 2006). Fungal diseases are mainly controlled by seed treatments, long crop rotation, destruction of volunteers and of some weeds, and by the use of resistant or tolerant varieties. However, pathological premature ripening due to phoma or macrophomina is often observed, especially in dry areas. Herbicides are applied in almost 100% of the area, but between-row cultivation also contributes to weed management. This cultural operation was observed in 41% of the area in 2006. Hence, sunflower may contribute to the decrease in the application of pesticides in France, through an increase in the area of this crop to the detriment of other crops. This would be effective as long as improvements in crop protection were focused on other ways than increasing the application of pesticides. For instance, date of sowing, seeding rate and the amount of N fertiliser have an effect on several diseases, such as Phoma black stem (Debaeke and Pérès, 2003; Seassau et al., 2008). This indicates that there are possibilities for decreasing disease incidence without applying fungicides.

In France, sunflower is mainly cultivated on clay soils. Most of the time, seeds are sown after a deep tillage (72% of the area in 2006), while there is very little direct sowing (2% in 2006). There is almost no irrigation (only 4% of the area in 2006). The range of soil depth is wide, resulting in a large range of seed yields. For instance, in south-west of France in 2006, mean yields were 2.28, 2.34 and 2.73 t ha^{-1}, respectively, on shallow (13% of the area), medium (75%) and deep soils (12%). However, there is little adaptation of crop management to the expected water availability (soil field capacity, expected rainfall and irrigation). The main adaptation to reduce the effect of water shortage is earlier sowing in south of France, because severe deficits in summer are expected. In the Aude region however, where mid-summer storms are predictable, the date of sowing is delayed. The objective is to postpone the seed filling period, so that it occurs during mid-summer. The amount of N fertiliser applied is also adapted to the target yield, which results from the expected water availability. However, there are no further adaptations of the crop management to the expected water availability. For instance, the drought-tolerance of commercial cultivars, if it exists, is unknown and thus not available for farmer's decision.

The first objective of any improvement in crop management is a better adaptation to the expected water availability. It would result in more accurate date of sowing, planting density, amount of N fertiliser (more accurate target yield) and variety maturity type. The choice of the variety should also account for the differences in leaf area and in stomatal closure, which play an important role in drought tolerance (Casadebaig, 2008). The second objective is to control diseases more efficiently without foliar-applied fungicides, especially those responsible for premature ripening in dry areas.

In other countries, there are some differences in crop management, compared to France. The following section is not an exhaustive list of the main differences which have been noticed, but it is the description of three of them which could also be improved by crop modelling. Firstly, in countries other than France, crop management is sometimes more adapted to the expected water availability, especially through the target plant population. The objective of plant number per hectare in Australia is 20-25,000 in marginal dryland, 25-35,000 in favourable dryland, 35-50,000 in limited irrigation and 50-75,000 in full irrigation (Serafin et al., 2007). In the USA High Plains, plant population for irrigated sunflower should be between 42000 and 54000 final plant per hectare, while it should be lower for lower yield potentials (Meyer et al., 1999).

Secondly, the occurrence of insect problems is more acute outside Western Europe, where insect damage on sunflower is less. In North America, there is a wide pest complex because sunflower is native to this region of the world (Charlet et al., 1997). On other continents, numerous insects also attack sunflower. For example, in Africa, sunflower has been grown for a long time as an ornamental plant. Insects attacking ornamental crops later moved to commercial ones (Charlet et al., 1997). In the countries where insects cause significant yield reductions, the planting date can play a role in controlling them. For instance, in Canada, delaying planting until late May or early June has been effective in reducing densities of stem weevil larvae (The sunflower production guide, Manitoba Agriculture, 2006). It also helps to prevent the first major emergence of the overwintering sunflower midge population. On the contrary, early planting reduces seed damage of sunflower seed weevils because early planted sunflowers complete anthesis and are no longer susceptible to egg laying at the time of peak populations (Manitoba Agriculture, 2006).

Thirdly, in some countries, crop management contributes to seed quality. In France, sunflower oil is mainly used for biodiesel or for food. Oil with a high oleic content is required for biodiesel. The quality required is obtained through the cultivation of high oleic varieties, while the rest of the crop management is similar to that for other varieties. However, planting date can affect the oil quality, because warm temperatures during anthesis and the seed-filling period increase the seed content in oleic acid (Blamey et al., 1997). Hence, in Australia, for example, planting dates are grouped into an early and a late sowing window (Serafin et al., 2007). For spring sowing, high oleic acid varieties are preferred. Hence, the high temperatures occurring during seed filling for this sowing time is not a problem. In order to produce high linoleic varieties, sowing in the late plant window (December-January) is recommended so that crops fill seeds in the cooler autumn months.

In order to adapt crop management accurately to each situation, many data are needed because environmental conditions are highly variable, between years and between locations. Hence, the optimum of one cultural operation is also highly variable from one experiment to another. For instance, Robinson (1978) stated that "disagreement on the optimum plant population is common". Moreover, there are many cultural operations which interact with each other. For example, the optimum plant population density tends to be greater with irrigation (Blamey et al., 1997; Debaeke and Nolot, 2000). It is not possible to conduct the huge number of factorial experiments needed for an accurate adaptation of crop management.

In France, the recommendation of an early sowing date is mainly based on field surveys. For instance, in the south west of France, the results of 300 fields per year from 1996 to 2006 show a decrease in yield when the sowing date was delayed after 10 April (CETIOM, 2008). The difference in yield was 0.27 t ha^{-1}, between sowings before 10 April and after 10 May. However, these results are rough estimates, because they compare different fields with possible differences in other cultural operations and in soils. In order to take into account possible interactions with other factors (variety, soil depth…), and/or to give more site-specific recommendations, many more data would be necessary. There is also a need to keep adapting crop management to keep up with technical progress (new varieties…), and with changes in objectives (quality) and in environmental conditions (the possibility to irrigate or climate change). The number of years necessary to give recommendations taking into account these changes would be too great, if results from either experiments or surveys were used. All these difficulties can be overcome by using crop models, because thousands of situations can be simulated in a few hours, once these tools are validated.

2. Sunflower crop models

Villalobos (2000) reviewed sunflower crop models at the 15th International Sunflower Conference in Toulouse. At that time, several specific models of sunflower had been developed, and a few others were applicable to several crops including sunflower (generic models). These models are mathematical representations of crops and soils which take into account dynamically and on a daily basis the effects of weather and crop management on seed yield. The QSUN model was developed in the early nineties (Chapman et al., 1993). It takes into account sowing date, irrigation and variety. The OILCROP-SUN model (Villalobos et al., 1996) also considers these factors, along with fertiliser management.

Two models which have been developed since 2000 provide further possibilities. A simple model based on published relationships calculates oil quality along with seed yield (Pereyra-Irujo and Aguirrezabal, 2007). The cultural operations taken into account are the effect of sowing date, plant density and variety.

Another sunflower crop model was developed by Casadebaig (2008) to gain new insight into the way to discriminate yield build-up between varieties. Generally, in sunflower models, varieties only differ in yield components and maturity types. In this new model, varietal parameters are required for crop development, leaf area and its ability to intercept light, response of leaf expansion and stomatal closure to soil water deficit, harvest index and the maximum percentage of kernel in achenes. These parameters are easily measurable, in order to be able to account for the dozens of new varieties appearing each year on the market (Casadebaig et al., 2008). Sowing date, plant density, irrigation and N fertiliser are also considered.

However, the sunflower crop models presented above do not include diseases, insects or weeds. There has been one attempt to connect the EPIC crop model adapted to sunflower to Phomopsis stem canker (Debaeke and Chabanis, 1999). The climatic risk of contamination by ascospores was predicted from spring and summer rainfall. Then, the disease symptoms were simulated using the relationship between infected stems and the fraction of intercepted photosynthetically active radiation (IPAR), which was simulated by the EPIC crop model. Yield loss was then correlated with the symptoms, bearing in mind the period of contamination. The relationship between symptoms of Phomopsis stem canker and the IPAR or Leaf area index (LAI) was also reported by Debaeke and Estragnat (2003). Debaeke and Pérès (2003) were also able to correlate Phoma black stem damage with IPAR or LAI at anthesis.

3. The use of sunflower crop models to adapt crop management

Sunflower crop models could be used to optimize crop management, by considering crop response to long-term historical weather records. For example, simulated seed yields were compared for a range of sowing dates, in order to select the best one (Meinke et al., 1993; Rinaldi et al., 2003; Soriano et al., 2004; Casadebaig, 2008).

In Casadebaig (2008), seed yield was simulated for 5 sowing dates, 7 locations, 3 available soil water content and 25 years. In most combinations of location x soil water capacity, seed yield decreased with delaying the sowing date until the third or the fourth date, because of a greater water deficit (Fig. 1). Then, an increase in seed yield was observed between the third or fourth date and the fifth date, due to the delaying of seed filling until the period of mid-summer storms. This pattern was less marked on deep soils with high water capacities. Hence, depending on location and on soil water capacity, the greatest yield could be obtained at the early sowing date, at both the early and the late sowing dates because of mid-summer storms, or at all sowing dates because of a little water deficit due to both deep soils and humid climates.

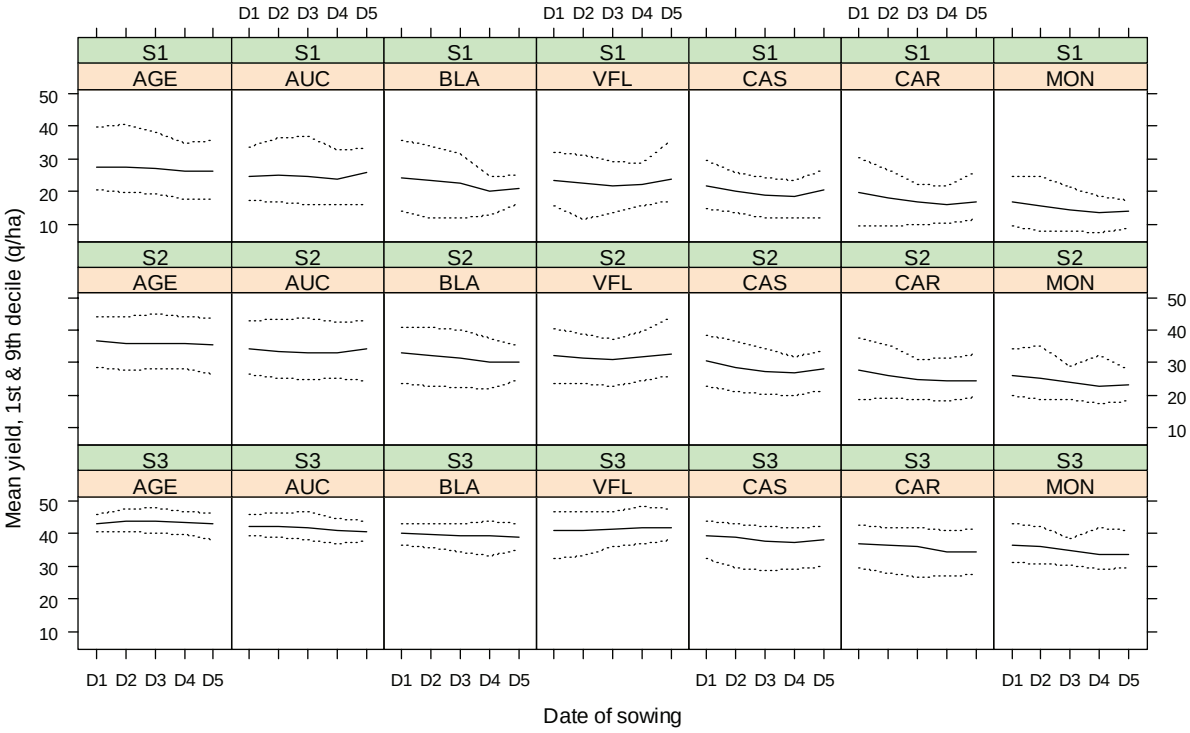

Fig. 1. Simulated seed yield versus sowing date in 7 locations and for 3 soils (Casadebaig, 2008).

Sowing dates were 1 March (D1), 25 March (D2), 15 April (D3), 10 May (D4) and 25 May (D5). Locations were representative of South of France, from the Western side (left on the Figure) to the eastern (right on the Figure): Agen (AGE), Auch (AUC), Blagnac (BLA), Villefranche de Lauragais (VFL), Castelnaudary (CAS), Carcassonne (CAR) and Montpellier (MON). Soil water capacities were 80 mm (S1), 150 mm (S2) or 250 mm (S3).

The best maturity type was similarly studied by Meinke et al. (1993). Debaeke et al. (1998) and Rinaldi et al. (2003) also compared the effect of irrigation strategies on simulated yields. Oil seed quality can also be taken into account, along with seed yield, when using a crop model to optimize crop management (Pereyra-Irujo and Aguirrezabal, 2007).

These simulations could assist farmers in making management decisions. It provides information on the effect of one or several cultural operations, in each specific soil x climate situation, which takes into account the variability between years. Experiments or surveys fail to give such precise information. However, users of crop models should be aware of 2 limits: (1) accuracy and robustness, and (2) relevance (factors not taken into account). Crop models will be powerful tools to assist farmers as long as these limits are properly managed.

Model accuracy is the ability to give simulations close to the measurements. Robustness is the capability to be accurate in other environmental conditions than those prevailing for the data set used for calibration. Both are crucial for helping farmers to make good decisions. Model accuracy is evaluated by comparing simulations and measurements not used for calibration. However, the minimum of accuracy necessary to help farmers to make the best decision is usually not discussed. This would need specific works that have never been done when using sunflower crop models. For instance, Rinaldi et al. (2003) observed a good correlation between simulated yields and independent measurements (almost perfect regression slope (0.95) and intercept (-0.07), and a fairly good R^2 value of 0.74). Observed values were obtained for several years, in locations, irrigation regimes and sowing dates similar to those prevailing for the use of the model. This evaluation of the model was encouraging. However, it was not a proof that simulations were accurate enough to make the good decision, which was to use a threshold value of 40 % of total soil water to trigger irrigation. Moreover, robustness is not usually discussed, even though the ability to give results in other situations than those prevailing in experiments is exactly the expected benefit of a crop model.

Many factors affecting seed yield or quality are not taken into account by crop models. For example, sowing date does not only have an effect on climate conditions during crop growth. Diseases and insects are also affected (Leterme, 1992; Debaeke et al., 2001; Manitoba Agriculture, 2006). Models considering these factors would be very helpful. However, this does not seem as if it will become a reality in the near future. There are numerous diseases and insects which depend on many other factors than those in the sunflower field (cropping history, spatial cropping pattern …). Moreover, their effects depend on plant tolerance or resistance, and on the application of pesticides.

However, crop models could be useful for contributing to define management strategies. Debaeke and Nolot (2000) illustrated the definition of management strategies based on a target yield (which depended on water availability), and also based on the combination of avoidance and/or tolerance of limiting factors and vegetative rationing. The limiting factors involved were both nutritional and disease ones. For each combination of soil and climate, a crop model would be useful for establishing the potential yields allowed by the water availability, solar radiation and temperature. Results of potential yields would depend on sowing date, plant number and on variety. These results could be associated with the knowledge of diseases and insects in order to define management strategies. One strategy could aim at the maximum yield. According to the hypothesis that several combinations of sowing date x plant number x variety exist, the one recommended would be that minimizing the risks of major diseases and insects. In order to minimize them further, other management strategies could aim at lower target yields. Crop models could also help to estimate the risks of diseases and insects by simulating variables correlated with them. Examples of such correlations are given in section 2 of this article (IPAR or LAI correlated with Phoma black stem or Phomopsis stem canker). Models could also be used for insect damage. For instance, they could simulate the stages of development when sunflower is more susceptible to damage from a particular insect.

CONCLUSIONS

The issue investigated in this article is the possibility of using crop models for improving sunflower crop management. In France, a better adaptation of crop management to water availability is needed, as well as a more efficient control of diseases without applying more fungicides. A huge number of experiments would be needed to reach these objectives, while surveys give only rough estimates on the effect of cultural operations. Moreover, both experiments and surveys need too many years to keep up with the continuous technical progress (new varieties…), and for the quick changes in objectives (quality) and in environmental conditions (the possibility to irrigate or climate change) that are expected in the future. Crop modelling is the only way to obtain data quickly enough. Similarly, crop models could be helpful in countries other than France, although their needs for improving crop management may be different. There have been recent advances in sunflower crop models, in simulating oil quality and in defining differences between varieties. Although diseases and insects are still not taken into account, crop models could be used to trigger management strategies. These strategies would be based on simulated potential yields and on knowledge of diseases and insects. However, the condition for using simulated results to improve crop management is the confidence in the model. Until now, models have been mainly evaluated by comparing the simulations and the measurements made in a few independent experiments, which is not enough. There is a need to evaluate the ability of models to help to make the best decision in a large range of environmental conditions.

ACKNOWLEDGEMENTS

The authors wish to thank the organizers of the 17th International Sunflower Conference for their invitation to write this article.

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Phenotypic plasticities of yield, phenological development and seed traits

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ABSTRACT

Understanding, quantifying, and exploiting the interaction between genotype and environment (G x E) is at the core of plant improvement. This paper focuses on G x E from a physiological perspective. We present a theoretical framework largely based on Bradshaw's principles of phenotypic plasticity updated to account for recent developments in physiology and genetics. Against this framework we discuss (a) associations between plasticities of different traits and (b) plasticity of seed size and composition. We show that plasticity of sunflower phenological development could be positively associated with yield plasticity under conditions when this is a desirable trait, i.e. when there is no trade-off between yield in low and high yielding environments. We propose that allometric models linking rate and duration could be useful to quantify phenotypic plasticity of agronomically important seed traits.

Key words: genetics – genotype x environment interaction – phenotypic plasticity – physiology.

INTRODUCTION

Grain and oil yield, and quality traits of sunflower depend on environmental (E), genetic (G) and G x E factors. Table 1 is a meta-analysis of 69 sunflower trials over 18 years in northern Argentina (n = 8,974), highlighting the challenge involved in breeding and selection for oil yield in sunflower where environmental and G x E sources of variation dominate. Understanding, quantifying, and exploiting G x E is at the core of plant improvement.

Table 1. Meta-analysis of 69 sunflower trials over 18 years in Argentina (n = 8,974). The partitioning of oil yield variance uses a Restricted Maximum Likelihood approach (REML) assuming all variables are random.

Random term	Variance component	s.e.
year	72692	32521
year.trial	64000	13060
year.trial.rep	4340	653
year.trial.rep.block	2623	327
genotype	2972	671
year.genotype	5704	612
residual (avg across trials)	46731	6567

Breeders are well aware of the issues involved in G x E, whereas physiologists and ecologists look at the same type of problem from the perspective of *phenotypic plasticity* or *norms of reaction* (Bradshaw, 1965; Bradshaw, 2006; De Witt et al., 1998; Pigliucci, 2001; Pigliucci et al., 1995). *Phenotypic plasticity* is "the amount by which the expressions of individual characteristics of a genotype are changed by different environments" (Bradshaw, 1965). The aim of this paper is to discuss selected aspects of phenotypic plasticity of sunflower yield and seed traits from a physiological perspective.

This article has three parts. First, we introduce some principles related to phenotypic plasticity that provide the theoretical background for the paper. Second, we explore the notion of positive associations between plasticities. Using data from sunflower trials involving a large number of hybrids and environments, we show preliminary evidence for a positive link between phenotypic plasticity of yield and phenotypic plasticity of phenological development. Third, we present a novel quantitative model to analyse seed size variation in terms of rate and duration of seed growth. For most grain species, including sunflower, we show that plasticity of seed size could be ascribed to specific allometric conditions, and that plasticity of seed size could be an important driver of yield plasticity. This allometric model could also be applied to quality related traits, e.g. oil concentration.

DISCUSSION

This paper is informed by three established principles (1-3) and a newer, less tested proposal (4):

1. "The plasticity of a character is an independent property of that character and is under its own specific genetic control" (p. 119 Bradshaw, 1965). Bradshaw (1965) insightfully formulated this proposal over forty years ago, and Reymond et al. (2003) have demonstrated unequivocally that phenotypic plasticity is a trait on its own, with its own genetic control. A corollary to this principle is that plasticity evolves (Pigliucci, 2005; Zhivotovsky et al., 1996) and therefore could be considered as a breeding aim on its own. The findings of Reymond et al. (2003) open a new, more robust opportunity to use QTLs as breeding tools, and highlight the need for appropriate quantitative models that relate traits and environmental drivers, or alternatively, establish physiologically meaningful relationships between traits.
2. Plasticity is specific for a character and is specific in relation to particular environmental influences (Bradshaw, 1965). This adds a layer of complexity to the subject, because the plasticity of a trait (e.g. kernel oil concentration) may be high or low depending on the environmental drivers.
3. There is a hierarchy of plasticities, i.e. stable traits are often associated with plastic, related traits (Bradshaw, 1965). The trade-off between seed number and size is a typical, agronomically relevant case of this principle whereby high plasticity in number is associated with low plasticity in size. Sadras (2007) has provided an evolutionary interpretation that matches the notion of a hierarchy in the plasticities of seed size and number in annual plants.
4. There are cases of *positive* associations between plasticities of certain traits. Analysis of the association between plasticity of fruit yield and plasticity of phenology in wine grape favoured the hypothesis of a positive, rather than negative (principle 3) correlation between plasticities (Sadras, Petrie, and Robinson, unpublished).

Does phenological plasticity contribute to yield plasticity?

Finlay and Wilkinson (1963) developed a method to quantify trait plasticity, that has been widely applied to the analysis of grain yield in annual crops. Calò et al. (1975) used this approach to quantify phenological plasticity of grapevine. Fig. 1 illustrates the rationale of this method applied to the analysis of plasticity of flowering time of sunflower hybrids grown in diverse environments of northern Argentina. The coefficient of phenotypic plasticity is the dimensionless slope of the linear regression between date of flowering of an individual variety in a particular environment, and the mean value of the trait across varieties in that particular environment. A variety with slope = 1 has average stability over all environments, a variety with slope > 1 has above-average plasticity, and a variety with slope < 1 has below-average plasticity.

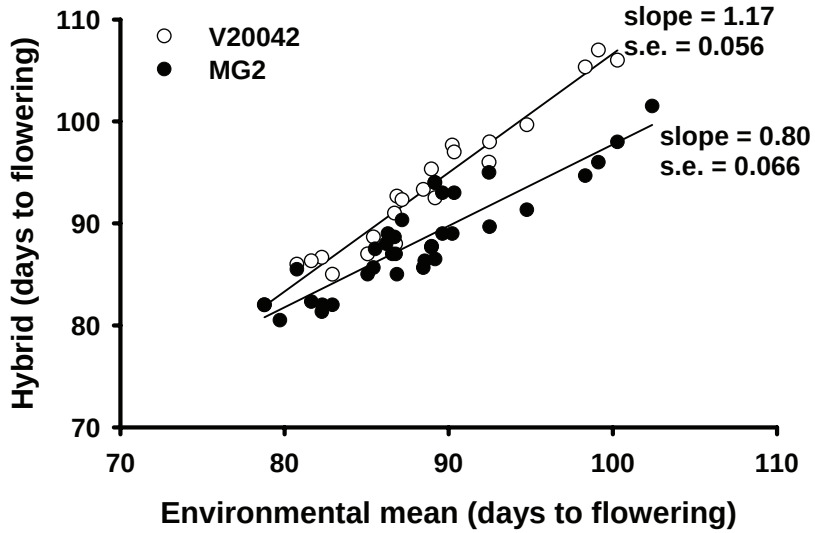

Fig. 1. Quantification of phenotypic plasticity of flowering in sunflower using the method of Finlay and Wilkinson (1963).

In the case study of this paper, environments resulted from the combination of locations and seasons, and the data set comprised 32 hybrids grown in at least 15 environments. For this data set, plasticity for yield ranged from 0.72 to 1.29 (Fig. 2). All hybrids performed similarly in the more stressful environments (i.e. the slope of the regression between minimum yield of each hybrid and its yield plasticity was not significantly different from zero, P = 0.34). Higher plasticity was associated with the ability to capture the benefits of better environments, with a rate of increase in maximum yield of 1939 kg/ha per unit increase in plasticity (P < 0.0001). A similar conclusion was reached from analysis of oil yield: oil yield plasticity ranged from 0.72 to 1.30, was correlated with plasticity of grain yield (r = 0.90, P < 0.0001) and was related to maximum (rate = 1024 kg oil/ha per unit increase in plasticity, P < 0.0001) but not with minimum oil yield (P > 0.25). High yield plasticity in this particular combination of hybrids and environments is therefore a desirable trait, as it does not involve tradeoffs between stress tolerance and yield potential.

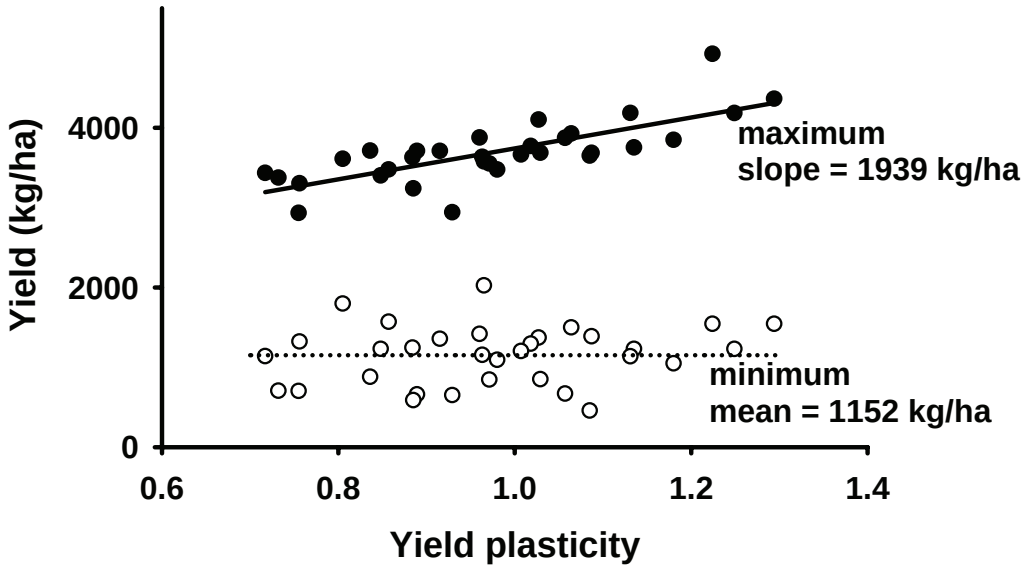

Fig. 2. Phenotypic plasticity of grain yield in sunflower hybrids was related to their ability to capture the benefits of the best environments (slope of maximum yield vs plasticity significant at P < 0.0001) and independent of their performance in the more stressful environments (slope of minimum yield vs plasticity not different from zero; P = 0.34).

In a broad sense, phenological development is recognised as the more important attribute of crop adaptation (Passioura, 1996; Passioura, 2007; Richards, 2006; Sadras and Trápani, 1999). This relates to a series of tradeoffs. Firstly, there is a trade-off between late flowering that allows for canopy and root development (Giménez and Fereres, 1986) and the decline in potential grain set generally associated with low radiation-to-temperature ratios of late flowering crops (Cantagallo et al., 1997). Secondly, in some environments, flowering date may also involve trade-offs between the risk of frost and the risk of heat stress, terminal drought, rainfall at harvest or diseases. For the combination of hybrids and environments in this analysis, we found yield plasticity was higher in late-flowering hybrids, with mean flowering date accounting for 47% of the variation in yield plasticity (Fig. 3) and 40% of the variation in oil yield plasticity (not shown). Flowering plasticity was unrelated to mean flowering date, and accounted for 20% of the variation in yield plasticity (Fig. 3) and 16% of the variation in oil yield plasticity. Maximum yield was associated with both mean flowering date (r = 0.51, P = 0.003) and flowering plasticity (r = 0.40, P = 0.02) whereas minimum yield was weakly related to mean flowering date (r = 0.34, P = 0.06) and unrelated to flowering plasticity (P = 0.34).

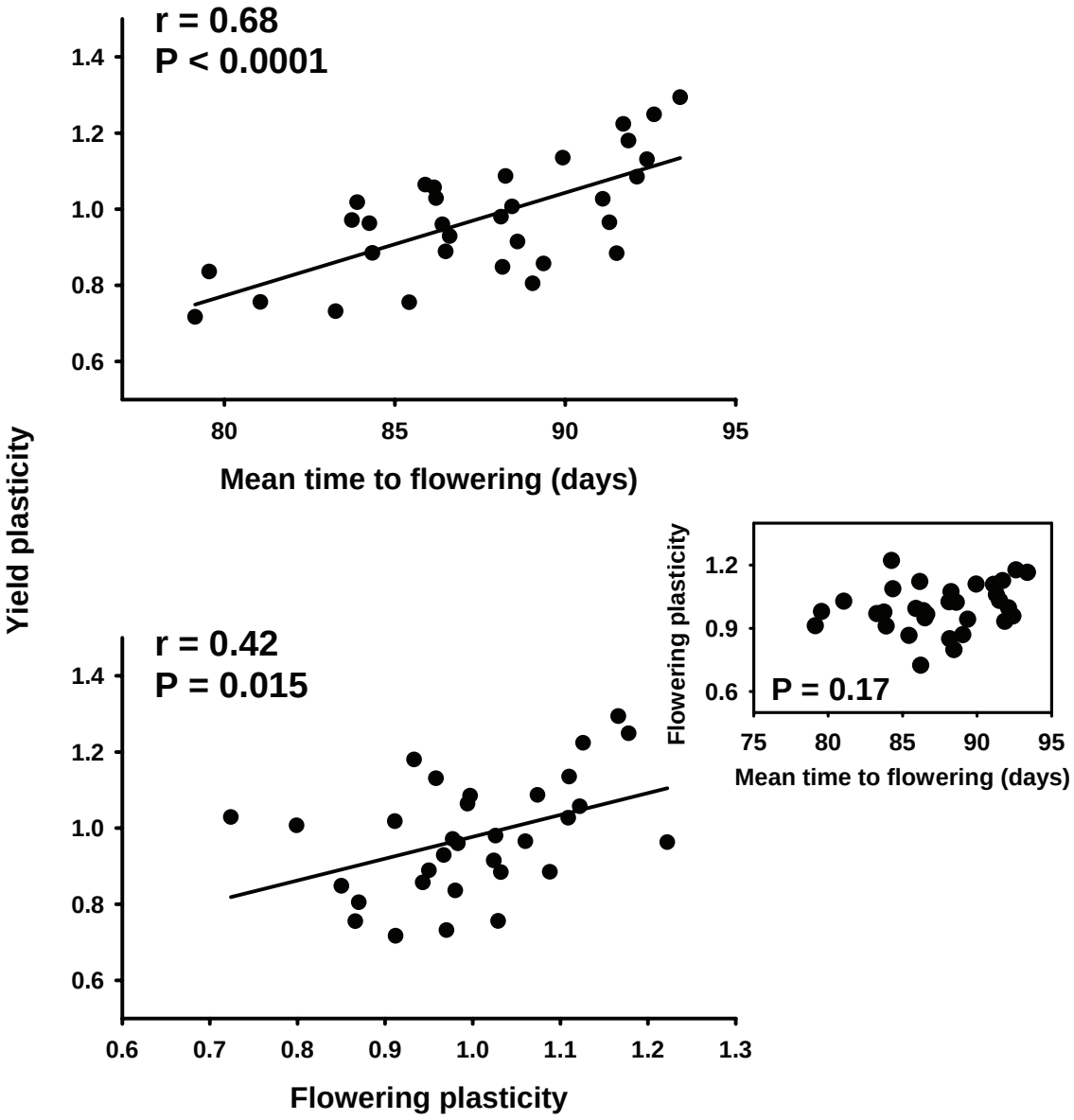

Fig. 3. Plasticity of grain yield in a collection of 32 sunflower hybrids in northern Argentina was associated with both late flowering, and flowering plasticity. Inset shows flowering plasticity was not associated with mean flowering date.

The relationships between plasticity in yield and plasticity in phenology deserve further attention. Biologically, this relationship adds a new dimension to the understanding of crop adaptation. From a breeding perspective, it would be of interest to establish the genetic basis of phenological plasticity (Principle 1), and eventually exploit this trait where plasticity in yield is a desirable trait, i.e. when performance in stressful environments does not compromise performance in better environments.

Plasticity of seed size: allometric conditions and relationship with yield plasticity

Here we explore the allometric conditions for seed size plasticity using a multi-species comparative approach, and investigate the links between seed size plasticity and yield plasticity using a limited data set of sunflower hybrids grown in contrasting environments.

Allometric conditions for plasticity of seed size and quality traits

There are many growth processes that can be approximated to sigmoidal patterns with characteristic rates and durations, including leaf expansion, seed growth, accumulation of oil in seed and accumulation of sugar and pigments in fruits. For any such process, we can express the maximum value of the trait (A) as the product of rate and duration:

$$A = \text{rate} \times \text{duration} \quad (1)$$

Sadras et al. (2007) proposed an allometric formulation of this model (Fig. 4):

$$\log \text{duration} = \log A - \alpha \log \text{rate} \quad (2)$$

The advantage of this model is that the scaling exponent α indicates three types of responses: the trait is stable as a result of full compensation between rate and duration (α = –1), the trait is variable as a result of rate (α > -1) or duration-dominated growth (α < -1). Sadras et al. (2007) used this approach to demonstrate that accumulation of anthocyanins in berries of grapevine Cabernet Sauvignon in a warm environment is highly plastic (α = -0.75 ± 0.041), in contras to sugar accumulation which is very stable (α > -1.11 ± 0.050). Fig. 5 illustrates the application of this concept to the analysis of seed size in grain crops. These particular experiments showed relatively stable seed size in soybean, with a scaling exponent correspondingly close to –1, and large variation in seed size of sunflower, with a corresponding scaling exponent significantly greater than –1 (P < 0.05), i.e. a flat line reflecting rate-dominated seed growth. These results cannot be considered general for these species, but particular for the combination of cultivars and environments (Principle 2).

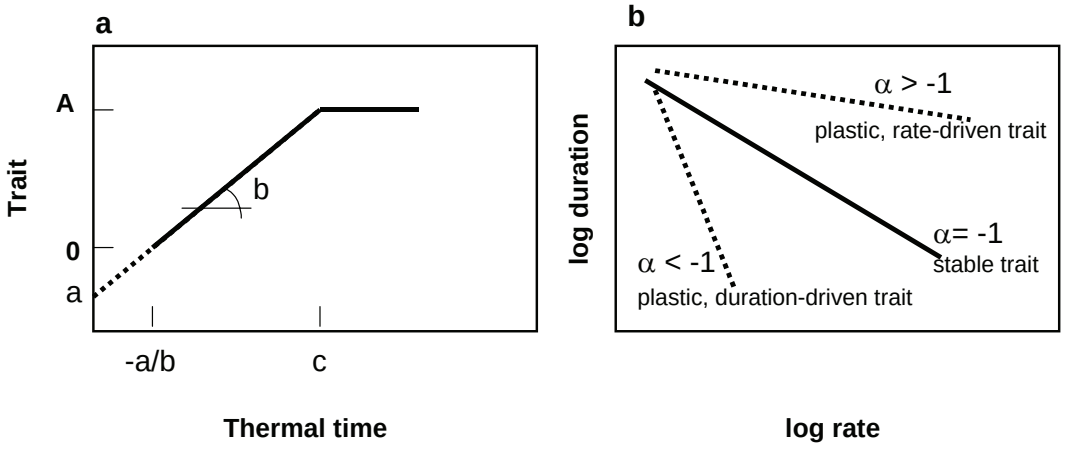

Fig. 4 (a). Many plant traits, including seed size, seed oil content and leaf area, conform to an approximate sigmoidal pattern with characteristic rates and durations. (b) The allometric relationship between duration and rate allows for a quantitative characterisation of trait plasticity. Adapted from Sadras et al. (2007).

A broader test of the concept included 45 data sets involving nine crop species, and sources of variation including genotype, environment, and their interaction (Fig 6). Relative variation in seed size ranged from 5 to 274%, and the scaling exponent was strongly concentrated in the range from 0 (large, rate-driven seed size range) to –1 (narrow seed size range due to mutually cancelled effects of rate and duration). The range of seed size declined when the scaling exponent declined from approximately 0 to –

1. An $\alpha \approx -1$ (rate and duration effects cancel each other) is necessary and sufficient for small variation in seed size, whereas $\alpha \approx 0$ is necessary but not sufficient for large seed size variation. The magnitude of seed size variation is dependent on the variation in the rate of seed growth when $\alpha \approx 0$. This double condition for seed size variability is summarised in a multiple regression model with α, and range of rate of grain filling as independent variables, which accounted for 73% of the variation in range of seed size.

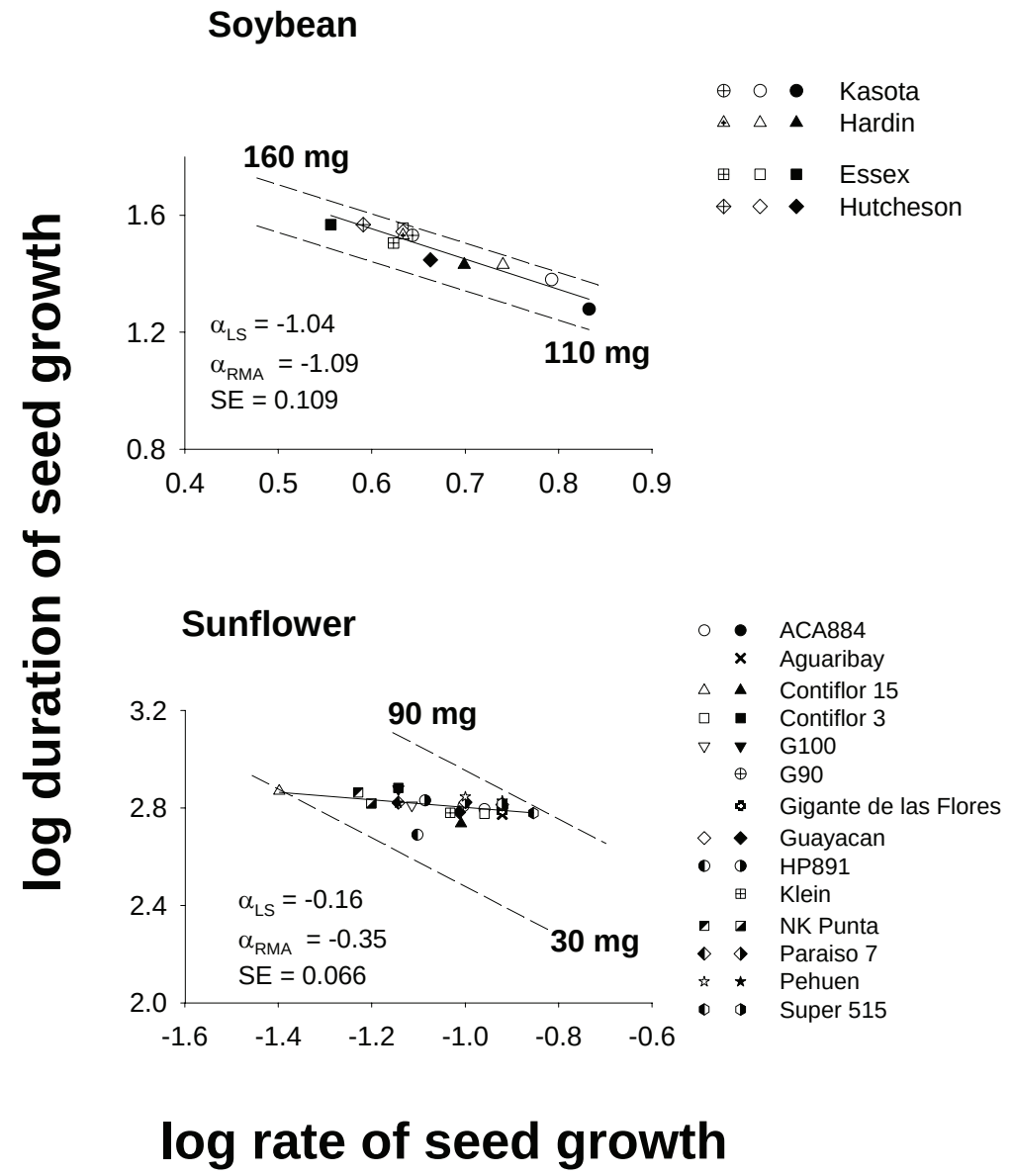

Fig. 5. Examples of intra-specific scaling relationships between rate and duration of seed growth in sunflower and soybean. Multiple symbols for a cultivar indicate different experiments or seasons. The solid line is the least squares regression, and dashed lines are isolines of seed size with $\alpha = -1$. Standard errors (SE) are common to the scaling exponents calculated with model I (α_{LS}) or model II (α_{RMA}) regression. Data sources: sunflower, López Pereira et al. (1999a); soybean (control treatment), Egli (1999). For soybean, rate is in mg seed^{-1} d^{-1} and duration in d, and for sunflower rate is in mg seed^{-1} ^{o}Cd^{-1} and duration in ^{o}Cd. Variate units do not affect the magnitude of the scaling exponent. Adapted from Sadras and Egli (2008).

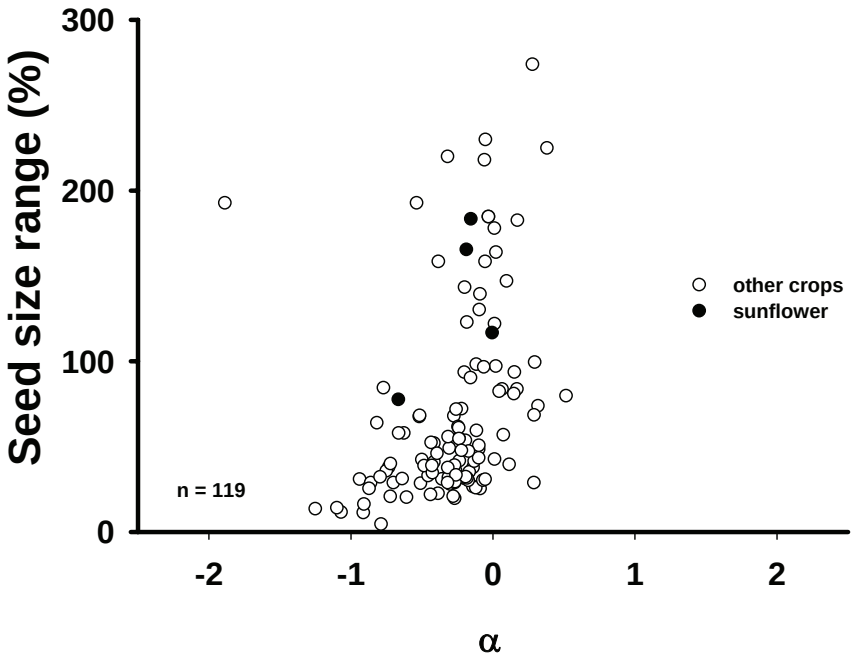

Fig. 6. Relationship between seed size range and α, the scaling exponent relating duration and rate of seed growth. Adapted from Sadras and Egli (2008).

Allometric analysis allowed for an integrated perspective on the interplay between rate and duration of seed filling, which in turn accounts for the genetic and environmental factors modulating seed size in grain crops. This allometric approach could be useful for evolutionary, agronomic and physiological analysis of seed size, and may also be used for other processes such as leaf growth or accumulation of oil or tocopherols in sunflower seed, where a framework of rates and durations is applicable. It would be of interest to consider the genetic substrate of parameter α for traits of agronomic interest (Principle 1).

Seed size plasticity and yield plasticity

The allometric relationship for sunflower in Fig. 5 was derived from crops grown under favourable conditions, i.e. hybrid grain yield ≥ 4 t/ha, oil concentration ≥ 50% (López Pereira et al., 1999a). Under these conditions, the duration of grain filling is typically around 30-35 days or about 650 ^{o}Cd (base = 4^{o}C), and differences in seed size are related to differences in rate of grain filling (de la Vega and Hall, 2002; López Pereira et al., 1999b). Relationships between rate and duration of grain filling could be different, however, in environments where excess or deficit of water supply during grain filling accelerate leaf senescence (Grassini et al., 2007; Hall et al., 1985).

Here we explore the relationships between seed size plasticity, quantified with parameter α and yield plasticity quantified with the method of Finlay and Wilkinson (1963) for a set of four sunflower hybrids grown under six environmental conditions in Argentina (for details see de la Vega and Hall 2002). The size of the data set is restricted due to the need to conciliate the time consuming sampling necessary to derive seed growth curves and α, and the relatively large number of cultivars and environments required to calculate yield plasticity. Growing conditions include a timely October sowing and a late December sowing conducive to lower yields. One of the seasons (1997/98) was "El Niño", with excessive rainfall and cloudy days detrimental to sunflower yield even for timely sown crops (Magrin et al., 1998). Yield plasticity ranked Aguará < Morgan 734 < Contiflor 15 (Fig. 7). Yield plasticity of hybrid GV25086 was similar to that of Contiflor 15 (not shown). The differences in yield stability among hybrids are partially related to their patterns of seed growth (Fig. 8). In response to late sowing, Contiflor 15 and Morgan 734 reduced both rate and duration of grain filling and Aguará slightly increased the rate of seed filing at the expense of shorter duration. Even for a set of few hybrids and growing conditions, Fig. 8 illustrates the

complex interplay of rate and duration of seed filling, and relationships between seed filing pattern and yield plasticity are not straightforward. Allometric relationships between rate and duration were loose, with large standard errors (not shown). Despite of this, the scaling coefficient α summarised the contrasting rate-duration relationships of these hybrids, and captured a substantial part of the variation in yield plasticity (Fig. 9). This reinforces the interest in the previous proposition of exploring the genetic basis of α.

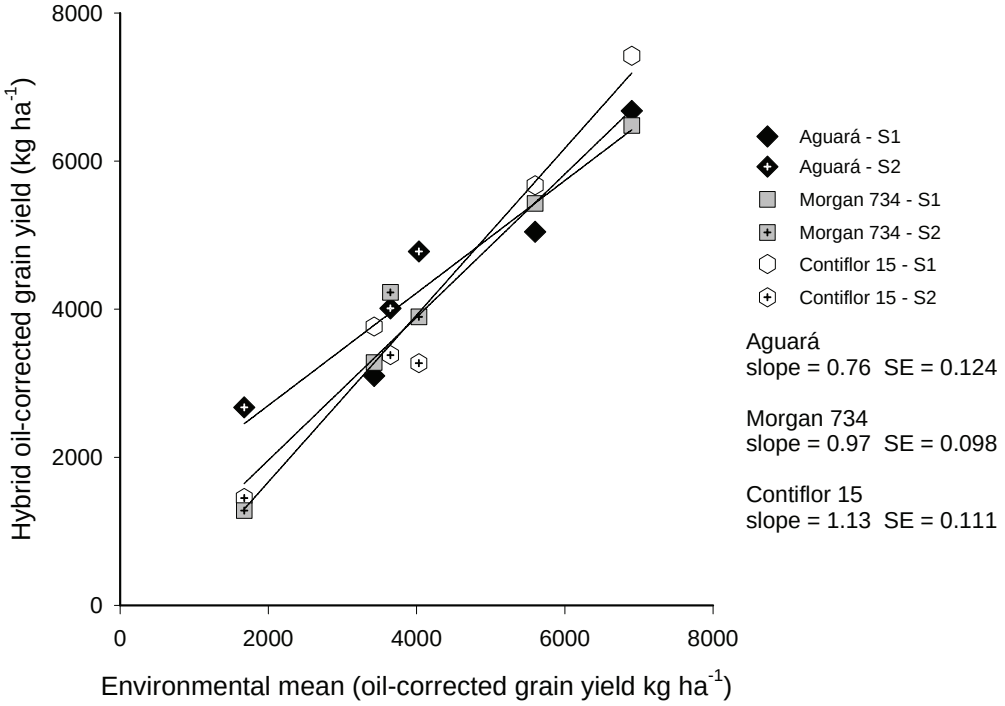

Fig. 7. Yield plasticity (slope of regressions) of three sunflower hybrids grown under six environmental conditions in Argentina. S1 is a timely October sowing, and S2 is a December sowing conducive to low yields.

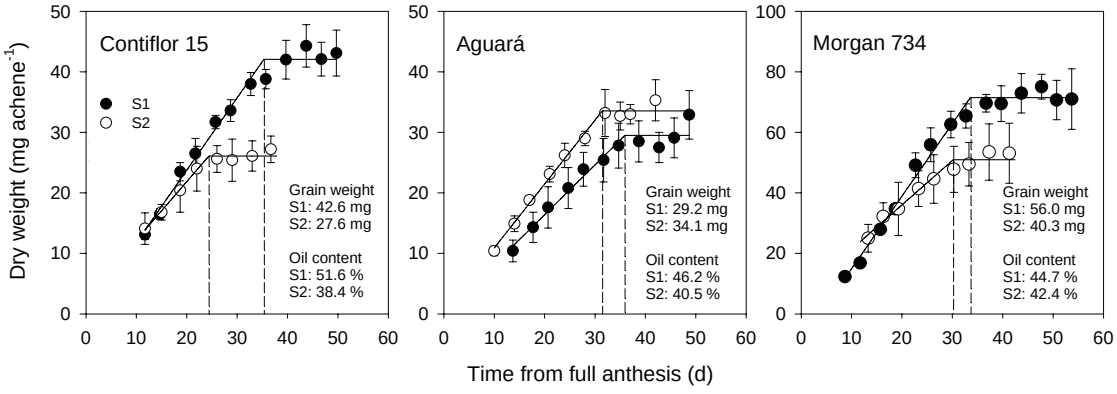

Fig. 8. Dynamics of seed growth of three sunflower hybrids sown in October (S1) or December (S2) 1996 at Venado Tuerto, Argentina. Adapted from de la Vega and Hall (2002).

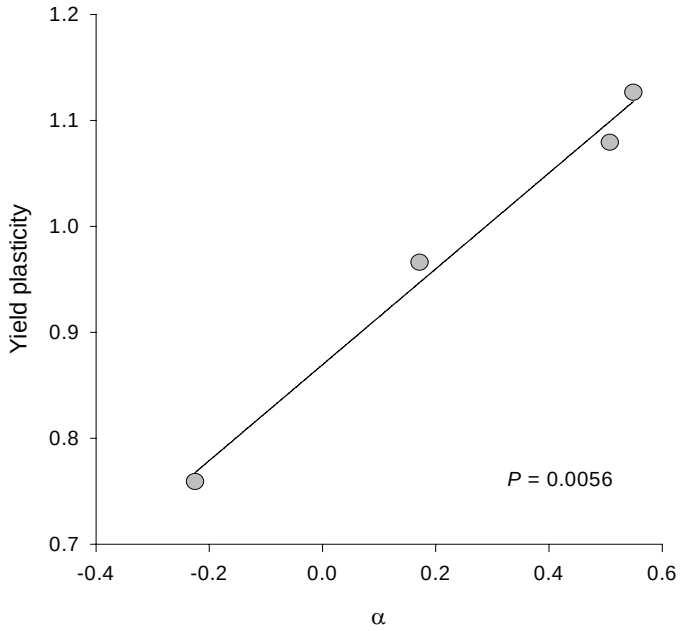

Fig. 9. Relationship between yield plasticity and α, the scaling exponent relating duration and rate of seed growth, for four sunflower hybrids grown in contrasting environmental conditions.

CONCLUDING REMARKS

The insightful vision of Bradshaw (1965), providing the contemporary definition of phenotypic plasticity and the notion that plasticity is a trait of its own, with its own genetic control acquires a new dimension when Reymond et al. (2003) demonstrate that the plasticity of certain traits could be traced back to specific QTLs. Against this conceptual framework, this paper showed that a physiological viewpoint of phenotypic plasticity can contribute to the understanding of G x E of sunflower yield. For the first time, here we showed that phenotypic plasticity of phenological development could be positively associated with yield plasticity under conditions when yield plasticity is a desirable trait, i.e. where there is no trade-off between performances in low and high yielding environments. Allometric models linking rate and duration could be useful to quantify phenotypic plasticity of agronomically important seed traits.

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Sunflower germplasm development utilizing wild *Helianthus* species

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ABSTRACT

The narrow genetic base of cultivated sunflower has been broadened by the infusion of genes from the wild species, which have provided a continued source of agronomic traits for crop improvement. The genus *Helianthus* comprises 51 species (14 annual and 37 perennial), all native to North America. The available genetic diversity from the wild species is continuing to be used to broaden the genetic background of the crop. Recent advances in culturing of otherwise abortive interspecific hybrid embryos have proved to be highly effective for making the difficult-to-cross wild perennial *Helianthus* species widely available for breeding purposes, either for specific major gene transfer or for the transfer of quantitative trait genes. These techniques are discussed and illustrations are shown of how they are being used to incorporate genes from several different ploidy levels of wild perennial species into cultivated sunflower for Sclerotinia stalk rot resistance and other diseases. Significant results have been reported on the germplasm development with regard to resistance to new races of downy mildew, rust, broomrape and other major diseases. In addition, new CMS and corresponding fertility restoration genes have been continuously identified and established, together with new genes helping to improve oil quality, herbicide resistance, and salt and drought tolerance. Thus far, only a small portion of the available genetic diversity of the wild *Helianthus* species has been used globally. As a whole, there is no doubt that wild *Helianthus* species will continue to provide new genetic variability to the sunflower breeding community, helping to maintain sunflower as a viable major global oilseed crop.

Key words: amphiploids – genetic diversity – genetic resources – *Helianthus* – interspecific hybridization.

INTRODUCTION

Sunflower production continues to face challenges from both abiotic and biotic factors as well as from today's ever-changing market needs. For the most part, the crop has been doing fairly well thus far. However, the limited genetic variability in cultivated sunflower has slowed the future improvement of the crop, and has placed the crop in a vulnerable position should any major shifts of disease races or pests occur. The uniform use of a single CMS PET1 cytoplasm and a few fertility restoration genes for worldwide sunflower production makes the crop extremely vulnerable. Diversity of resistance to various diseases is strategically needed. We have seen the rapid increase in the number of rust races being identified in Australia in recent years. The continuing race shift of broomrape in Spain, Turkey and the Black Sea areas since the mid-1990s has kept researchers busy for over 10 years searching for new resistance genes. For a while, the predominant rust and downy mildew races in the USA were limited to three or four, but many new races of these two diseases have been identified in the last 10 years. All the new races have the potential of becoming the predominant races in the future in response to our introduction of resistance genes. Sunflower is not always grown on prime land, but often on marginal land with minor salt and drought problems, presenting a challenge to be productive under less than ideal conditions. An early season sunflower crop has the potential to increase production by increasing the double crop potential for producers. In order to be competitive with other premium oils in the market, the reduction of saturated fatty acids in sunflower oil will play a key role in maintaining our continuing success. Evaluations of wild species have provided information about useful genes for future sunflower improvement. However, there are still numerous genes in wild sunflower species yet to be identified and introgressed into cultivated sunflower. Extensive collection efforts for wild *Helianthus* species and the regeneration of seeds at the USDA-ARS, Regional Plant Introduction Station at Ames, Iowa have greatly increased the availability of wild *Helianthus* seed for sunflower improvement. An overall advancement of our understanding of wild *Helianthus* species and improved methods of making interspecific crosses have

increased the number of useful genes available from wild *Helianthus* species, making it possible to transfer genes that were not possible three decades ago. This report will discuss the importance of wild *Helianthus* species and their utilization for sunflower improvement in the past and present, and show examples from our current wild species breeding program, and future prospects.

DISCUSSION

Helianthus collection. The USDA-ARS National Plant Germplasm System (NPGS) sunflower collection is maintained at the North Central Regional Plant Introduction Station (NCRPIS) in Ames, Iowa. The collection contains 37 perennial species, 14 annual species, and the cultivated species, *Helianthus annuus* (Schilling, 2006). This NPGS sunflower collection is a diverse assemblage of 3850 accessions: 1708 cultivated *Helianthus annuus* accessions, 932 wild *Helianthus annuus* accessions, 437 accessions representing 11 other wild annual *Helianthus* species, and 773 accessions representing 37 perennial *Helianthus* species. This collection is the largest and most genetically diverse sunflower collections in the world and it is vital to the conservation of *Helianthus* germplasm. From 1976 to 1996, 10,000 samples of wild sunflower were distributed to 300 researchers in 30 countries. These accessions have become the basis of wild species research programs in Argentina, France, Italy, Spain, Germany, Bulgaria, Romania, Czechoslovakia, Hungary, Russia, Yugoslavia, India, China, and Mexico.

Notable is the collection at the Institute of Field and Vegetable Crops, Novi Sad, Serbia, which contains 39 of the 51 wild species (IBPGR, 1984; Cuk and Seiler, 1985). The wild species collection of the Dobroudja Agricultural Institute (DAI) at General Toshevo, Bulgaria, is also notable, containing 428 accessions representing 37 of the 51 species of *Helianthus* (Christov et al., 2001). The wild species collection maintained at INRA, Montpellier, France has more than 600 accessions of 45 of the 51 wild sunflower species (Serieys, 1992). The Instituto de Agricultura Sostenible (CSIC) Cordoba, Spain maintains 44 annual and perennial accessions of *Helianthus* (Ruso et. al., 1996).

***Interspecific hybridization*: *The early years*.** Prior to the embryo culture method developed by Chandler and Beard (1983), nearly all the interspecific crosses were conducted in a classical fashion. All the annual *Helianthus* species, except *H. agrestis*, can be hybridized and F_1s backcrossed with cultivated lines using classical breeding methods. Direct crosses of cultivated lines with many perennial *Helianthus* species are also possible using conventional methods. Hybrids of *H. mollis* Lam. x *H. annuus* L. and *H. strumosus* L. x *H. annuus* (Heiser and Smith, 1964) and of *H. decapetalus* L. x *H. annuus* (Heiser et al., 1969; Georgieva-Todorova, 1984) have been reported. Hybrids of *H. tuberosus* L. x *H. annuus* (Heiser et al., 1969; Atlagić et al., 1993), *H. annuus* x *H. hirsutus* Raf. (Georgieva-Todorova, 1984), and *H. rigidus* (= *pauciflorus*) Nutt. x *H. annuus* (Vrânceanu and Iuoras, 1988) have also been successful. Atlagić (1990) summarized five interspecific hybrids involving crosses of perennial species *H. hirsutus*, *H. laevigatus* T. and G., *H. rigidus* (= *pauciflorus*), *H. tuberosus*, *H. maximiliani* Schrad., and *H. nuttallii* T. and G. with cultivated sunflower. Whelan (1978) used wild *H. annuus* as an "intermediate" parent or "bridge" to produce the first hybrids obtained between cultivated sunflower and *H. giganteus* L. and *H. maximiliani*.

***Interspecific hybridization*: *Utilizing embryo rescue*.** The development of a two-step embryo culture procedure by Chandler and Beard (1983) greatly facilitated interspecific hybridization. They successfully produced 53 interspecific cross combinations without the exhaustive effort of endless pollination, and 21 of these combinations had not been previously produced. Jan and Chandler (unpublished data) further modified the original procedure for culturing difficult hybrid embryos of wild perennial *Helianthus* species with cultivated *H. annuus* by adding vitamins, increasing sucrose to 20 g/kg, and the conversion from liquid to a solid medium with 0.7% agar. In addition, both growth and germination media were adjusted to pH 5.5 with 2-[N-morpholino]ethanesulfonic acid (MES) buffer. Using these modified media, 18 perennial species x *H. annuus* hybrids were established in one season, and many of them represented the first hybrid combinations ever produced (Jan, 1988).

Kräuter et al. (1991) cultured 0.2 to 1.5-mm small embryos on B5 medium with 90 g/kg sucrose, and embryos >1.5 mm on a modified MS (Murashige and Skoog, 1962) medium with 10 g/kg sucrose. When these embryos reached the size of 2 to 3 mm, they were transferred to MS medium for germination. Using this method, they obtained 33 interspecific hybrid combinations with an overall success rate of 41%. Using cultivated sunflower embryos of varying sizes, Espinasse et al. (1985) concluded that a high sucrose concentration of 90 g/kg and low nitrogen content were required for culturing small young embryos less than 2 mm in size.

As suggested by Dewey (1980), induced polyploidy could also serve as a bridge for interspecific gene transfer in sunflower. Jan and Chandler (1989) successfully doubled chromosomes of P21 x *H. bolanderi* F_1 hybrids, and increased seed set on doubled heads. Jan (1988) reported the success of a modified colchicine chromosome-doubling technique on 19 embryo-cultured wild x cultivated interspecific hybrids, and its positive effect on backcross seed set. Chromosome doubling of each head was verified by pollen grain size and stainability (Alexander, 1969). Chromosome doubling increased pollen grain size and stainability of interspecific hybrids. The increased pollen grain size directly reflected chromosome doubling and provided a reliable criterion for classifying treated plants.

Chromosome doubling restores normal fertility of amphiploids by providing an identical pairing partner for each chromosome. However, this increased fertility is likely to reduce the enforced interspecific chromosome pairing and gene exchanges during meiosis when an F_1 head is not chromosomally doubled. It would be helpful if the researchers could backcross onto both doubled and nondoubled heads, and at the same time intercross doubled heads for amphiploid production. More cytological evaluations are needed to compare the efficiency of interspecific gene transfer with or without the assistance of chromosome doubling of F_1s. Without chromosome doubling, we expect very low number of BC_1F_1 seeds and a high frequency of weak BC_1F_1 plants. With chromosome doubling, due to preferential pairing of *H. annuus* chromosomes during meiosis, we expect a reduced pairing of *H. annuus* chromosomes with chromosomes of wild *Helianthus* species.

***Interspecific hybridization*: *Introgression of genes into cultivated lines*.** In recent years, interest in interspecific hybridization has been greater for transferring useful genes from wild species into cultivated lines to develop pre-breeding germplasms for sunflower improvement. Characteristics such as disease and insect resistance, salt tolerance, drought tolerance, fatty acid variation, CMS, and fertility-restoration diversity have been emphasized.

By successful hybridization between *H. petiolaris* and *H. annuus* and backcrossing with *H. annuus*, Leclercq (1969) transferred the *H. annuus* genome into cytoplasm of *H. petiolaris* Nutt. and obtained the first cytoplasmic male sterile plants. Whelan (1980; 1981) and Whelan and Dorrell (1980) used the same technique to obtain cytoplasmic male sterility conditioned by the cytoplasm of three species, *H. petiolaris*, *H. giganteus*, and *H. maximiliani*.

Due to the use of a single male-sterile cytoplasm for worldwide hybrid sunflower production and its consequence of genetic vulnerability, a large portion of the interspecific hybridization in sunflower has focused on the identification of new CMS sources and their fertility restoration genes. Of the total 70 CMS sources resulting from interspecific hybridization, 39 were derived from wild *H. annuus* and 23 from other wild annual species, and only eight from wild perennial species. Extensive research is now focused on the identification of fertility restoration genes using both cultivated and wild species, and evaluation of their inheritance.

Rapid improvement of interspecific F_1 meiotic abnormality and low fertility was demonstrated by Whelan (1978; 1979) when he discovered CMS-PET2, G1G1, and MAX1. The differences of the parents were shown as translocations and a paracentric inversion as indicated in F_1 meiosis, which can quickly be eliminated after one or more backcrosses with cultivated lines (Whelan, 1982).

Helianthus tuberosus x *H. annuus* hybrids have been used widely in the Former Soviet Union (FSU) as a source of disease resistance. Hybrids of *H. annuus* x *H. resinosus* Small (2n=102) had stainable pollen from 0 to 50%, and meiotic diakinesis had 28 to 36 bivalents with 1 to 6 univalents (Georgieva-Todorova, 1983). The high number of bivalents suggests a high homology between the chromosomes from *H. resinosus* and those from *H. annuus*. In general, good pollen stainability is expected in the F_1s of hexaploid *Helianthus* species crossed with *H. annuus*. Atlagić (1990) reported average pollen stainability of 49.8%, 40.9%, and 64.6, respectively, for the hybrids of *H. annuus* with *H. pauciflorus*, *H. tuberosus* and *H. laevigatus.* Seiler's (1991a; 1993) release of 12 interspecific germplasm lines derived from perennial accessions of *H. hirsutus*, *H. resinosus*, and *H. tuberosus* also supports the reasonably good fertility of *H. annuus* x hexaploid accessions and some selected *H. annuus* x tetraploid accessions.

An unusual cytoplasmic-nuclear interaction causing plants with reduced vigor has been observed, and a single dominant gene was needed to restore normal plant growth (Jan, 1992). With continuous backcrossing with HA 89 as the recurrent parent into the cytoplasms of five diploid perennial species, *H. mollis*, *H. maximiliani*, *H. grosseserratus* Martens, *H. divaricatus* L., and *H. angustifolius* L. and selection for normal segregants, Jan (1992) discovered the vigor-reducing effects of these cytoplasms and a single nuclear vigor-restoration gene was needed to restore the vigor. The vigor-reducing cytoplasmic effects also have been observed in progenies when backcrossing HA 89 into cytoplasms of *H. hirsutus*, *H. occidentalis* Riddell, and *H. giganteus*. A considerable number of cultivated lines were found to possess

the same vigor restoration gene, and it was suspected to have been derived from *H. tuberosus* because of that species' popular use in early breeding programs in the FSU. Our recent discovery of a different vigor restoration gene derived from *H. giganteus* suggested the existence of different vigor restoration genes in varying perennial *Helianthus* species compensating for specific cytoplasmic effects causing reduced vigor (Jan, 2003).

Transferring genes from wild annual species into cultivated lines can be accomplished rather easily with conventional crossing and backcrossing. Seiler (1991b, c) released 15 interspecific germplasm lines having genes from wild annual species, and 13 tolerant to sunflower downy mildew, using the conventional method of crossing and backcrossing. Jan and Chandler (1985a) transferred resistance genes for powdery mildew (*Erysiphe cichoracearum* DC.) from *H. debilis* Nutt. and rust (*Puccinia helianthi* Schwein.) and downy mildew resistance genes from wild *H. annuus* into cultivated sunflower (Quresh et al., 1993; Quresh and Jan, 1993; Tan et al., 1992).

Crossing cultivated sunflower with wild perennial *Helianthus* species often results in serious problems of early hybrid embryo abortion, as well as high levels of sterility in the F_1 or BC_1F_1 generation. However, utilizing an embryo-culturing technique, 26 interspecific hybrids of wild perennials x cultivated line P21 were produced. Subsequent chromosome doubling of the F_1s of diploid and tetraploid wild accessions crossed with P21 improved backcross and sib-pollinated seed set drastically (Jan, 1988). Amphiploids of wild species utilizing *H. gracilentus* A. Gray, *H. pumilus* Nutt., *H. hirsutus*, *H. strumosus*, *H. maximiliani*, *H. nuttallii*, *H. mollis*, and *H. grosseserratus* crossed with cultivar P21 have been produced by sib-pollination of chromosomally doubled heads of each cross. These amphiploids can be maintained by sib-pollination, have improved pollen stainability and larger pollen grains, and have improved backcross seed set (Jan and Fernández-Martínez, 2002).

Interspecific gene transfer facilitated by the chromosome doubling of extremely difficult diploid perennials x *H. annuus* and tetraploid x *H. annuus* crosses has been demonstrated. Positive results of gene transfer from *H. hirsutus* into cultivated sunflower have been obtained (Jan and Zhang, 1995). By monitoring the rust resistance genes of *H. hirsutus*, which is immune to the four North American (NA) rust races, the hexaploid amphiploid was backcrossed with *H. annuus* twice. The resulting triploid BC_2F_1s had a complete set of 34 chromosomes of *H. annuus*, plus 17 chromosomes from *H. hirsutus*, and were all resistant to the four NA rust races. Several BC_3F_1 plants had 2n=36 or 37 chromosomes and were resistant to NA rust races 1 and 2, and further backcrossing resulted in many BC_4F_1 race 1- and 2-resistant plants with 2n=34. More recently, Jan et al. (2002) produced four sunflower germplasms with resistance to broomrape (*Orobanche cumana* Wallr.) race F, with resistance genes transferred from wild perennial *Helianthus* via interspecific amphiploids. In addition, interspecific amphiploids of perennial x cultivated have provided fertility restoration genes for the new CMS cytoplasms derived from *H. giganteus* (Jan, 2004) while no *Rf* genes were identified in cultivated lines. Surprisingly, *Rf* genes for this CMS were identified in four out of the seven amphiploids tested.

Chandler (1991) reviewed sunflower genomic relationships and came to the conclusion that there is little evidence of the existence of distinct genomes in *Helianthus*. The author's observation of many interspecific hybrids agrees with Chandler's statements. Even the most sterile interspecific hybrids involving diploid perennial species and cultivated *H. annuus* had satisfactory chromosome pairing (Jan and Chandler, 1985b). In order to utilize this high degree of chromosome similarity between cultivated lines and wild *Helianthus* species for interspecific gene transfer, the best approach would be to backcross without F_1 chromosome doubling. Without chromosome doubling, maximum chromosome pairing between cultivated lines and the wild species will be achieved. With chromosome doubling, preferential chromosome pairing of identical chromosomes in each parent will reduce the interspecific chromosome pairing and gene exchanges. However, the latter approach may have the advantage of having improved backcross fertility, and the reduced degree of gene exchange will enhance the quick recovery of a recurrent parent genotype carrying the specific selected gene. This was demonstrated with the rust resistance gene transfer from *H. hirsutus* into cultivated line HA 89 via amphiploidization, where chromosomes from *H. hirsutus* demonstrated their ability to challenge the perfect pairing of *H. annuus* chromosomes and to incorporate the resistance genes into the *H. annuus* genome (Jan and Zhang, 1995).

***Interspecific hybridization*: *Amphiploids*.** Colchicine treatment of interspecific F_1 hybrids resulted in high frequencies of chromosome doubling and the production of amphiploids (Jan and Fernández-Martínez, 2002). The tetraploid amphiploids produced included crosses of P21 x *H. bolanderi* (Jan and Chandler, 1989), *H. gracilentus* x P21, *H. grosseserratus* x P21, *H. cusickii* A. Gray x P21, *H. mollis* x P21, *H. maximiliani* x P21, and *H. nuttallii* x P21. These amphiploids have restored fertility, and provide easily available genetic diversity for the improvement of cultivated sunflower. The first hexaploid

amphiploids in sunflower have also been produced from crosses of *H. hirsutus* x P21 and *H. strumosus* x P21.

The interspecific amphiploids will enable the establishment of a number of chromosome addition lines for genetic studies of specific chromosomes of both cultivated and wild *Helianthus* species. With the available amphiploids and some specific interspecific crosses, the potential exists to establish additional lines with HA 89 chromosome pairs in *H. californicus*, and the chromosome pairs of *H. hirsutus*, *H. angustifolius*, *H. cusickii*, *H. gracilentus*, *H. grosseserratus*, *H. nuttallii*, *H. strumosus*, and *H. giganteus* in HA 89.

***Male sterility*.** A single male-sterile cytoplasm, PET1, derived from *H. petiolaris* subsp. *petiolaris* (Leclercq, 1969) and the identification of dominant fertility restoration genes (Enns et al., 1970; Kinman, 1970; Vrânceanu and Stoenescu, 1971) advanced sunflower production from the use of open-pollinated cultivars to hybrid production 40 years ago. This source of cytoplasmic male sterility and a few fertility restoration genes, including the widely used Rf_1 and Rf_2 genes, have been used exclusively for sunflower hybrid production worldwide (Fick and Miller, 1997).

A total of 70 CMS sources have been identified from progenies of crosses between wild *Helianthus* accessions and cultivated lines, from wild accessions grown in observation nurseries, or from induced mutation. Fertility restoration genes have been reported for 34 CMS sources, and detailed inheritance studies have been conducted for only 19 of the CMS sources (Serieys, 2002). In general, it is relatively easy to isolate stable CMS cytoplasms, but the identification of simple and completely dominant fertility restoration genes has been far less successful.

Many CMS sources from wild *H. annuus* (ANN1 through ANN9) were discovered in field-grown populations. All these CMS lines except ANN8 were completely male-sterile with degenerated anthers. Restoration genes were found for ANN2, 3, 4, and 7 using a set of 20 fertility-restoration testers, plus male-fertile plants of each respective wild species accession. Inheritance studies of fertility restoration of ANN2 and ANN3 indicated complete fertility restoration by single dominant genes (Jan, 1991). Serieys (1994) also reported complete male sterility and full fertility restoration by single dominant genes for CMS-ANO1, CMS-NEG1, and CMS-PRP1. The utilization of these CMS sources for potential hybrid production should be pursued.

***Diseases*.** Diseases limit production in a majority of sunflower producing countries. Sunflower is a host to a wide array of diseases that can cause serious economic damage in terms of yield and quality, with the fungal diseases the most numerous and economically serious. In the USA, the major diseases of concern are downy mildew, rust, Sclerotinia head and stalk rot, and Phoma black stem. Verticillium wilt, Phomopsis stem canker, Alternaria leaf spot, Septoria leaf spot, charcoal stem rot, and Rhizopus head rot occur to a lesser degree. In Europe and adjacent Mediterranean countries, downy mildew, Sclerotinia head rot, Phomopsis, Botrytis gray rot, and charcoal rot are considered the most important diseases. Some diseases are important in only a few countries, such as Verticillium wilt in Argentina and white rust (*Albugo*) in South Africa. Genetic resistance to the prevailing North American races of rust has been identified in three wild annual species, *H. annuus*, *H. petiolaris*, and *H. argophyllus* T. and G. (Jan et al., 2004a). Genes for rust resistance are frequent in the wild progenitors of the cultivated sunflower (Quresh et al., 1993). In most cases rust resistance appears to be conditioned by single dominant genes.

Downy mildew can be controlled by single, race-specific dominant resistance genes. Multi-race resistant germplasm and single-race resistant germplasms have been developed from wild sunflower species (Miller and Gulya, 1988; Tan et al., 1992; Jan et al., 2004b). Wild *Helianthus annuus*, *H. petiolaris* and *H. praecox* Engelm. and A. Gray are sources of single dominant genes for single race resistance, while *H. argophyllus* is the source of dominant genes for all known races of the fungus (Miller and Gulya, 1988; Miller et al., 2002).

Sclerotinia wilt (white mold) causes the greatest losses to sunflower on a global basis. This is in part due to the wide host range of *Sclerotinia sclerotiorum* (Lib.) de Bary being a facultative parasite that attacks 360 species of plants. It appears that Sclerotinia resistance is complex and controlled polygenically involving many genes, each with small effects. This means that the breeding strategy using wild species as a source of resistance needs to be quite different than for other diseases. A detailed approach and strategy for developing Sclerotinia stalk rot resistance will be discussed later in this section.

There are reports of identification of cultivated sunflower genotypes with low susceptibility or moderate resistance to Sclerotinia white mold. Wild species have also been identified as a potential source of genes for Sclerotinia tolerance. Interspecific hybrids with perennial *H. maximiliani* (Maximilian's sunflower) exhibited higher levels of resistance than head rot resistant inbred lines

(Cerboncini et al., 2002; Ronicke et al., 2004). Rashid and Seiler (2004) identified potential sources of Sclerotinia head and stem rot resistance in populations of perennial *H. maximiliani* and *H. nuttallii* from Canada. Perennial *H. resinosus* has been identified as a good source for resistance to Sclerotinia head rot by Mondolot-Casson and Andary (1994). The Sclerotinia disease complex appears to be very complicated. The prospect of finding a single dominant gene for resistance does not look promising, but progress is being made in the development of germplasm with increased tolerance to Sclerotinia head rot. Currently there are no commercial hybrids which possess a satisfactory level of resistance to Sclerotinia rot.

Some progress has been made in increasing the resistance to midstalk Sclerotinia rot in cultivated sunflower. Kohler and Friedt (1999) indicated that progenies of interspecific crosses involving *H. mollis* and *H. tuberosus* had increased levels of tolerance to midstalk white mold infection. Miller and Gulya (1999) developed four maintainer and four restorer oilseed lines with improved tolerance to midstalk Sclerotinia rot.

Sclerotinia sclerotiorum generates substantial quantities of oxalic acid, which has been identified as one of the key components in the infection process. One strategy for resistance is to obtain plants that are resistant to free oxalic acid by engineering them to degrade it. A wheat (*Triticum aestivum* L.) oxalate oxidase gene (OXO) has been identified and transferred into sunflower via transformation (Scelonge et al., 2000). A transgenic sunflower line, *H. annuus* cv. SMF3, constitutively expressed the wheat OXO gene (Hu et al., 2003) and exhibited enhanced resistance against the oxalic acid-generating fungus Sclerotinia. This approach to white mold resistance in sunflower awaits further testing and commercialization.

Phomopsis brown stem canker was first discovered in sunflower in Yugoslavia in 1980 and now is considered a serious problem in much of Europe (Mihaljcevic et al., 1982; Acimovic, 1984; Škorić, 1985). Cuk (1982) reported that wild *H. debilis* and *H. pauciflorus* are potential sources of resistance to *Phomopsis helianthi* Munt-Cvet. et al. Kurnik and Walcz (1985) reported resistance to stem canker in *H. argophyllus*, tolerance in two other wild species, and susceptibility in local populations of *H. tuberosus.* Dozet (1990) observed a high degree of resistance in two populations of *H. tuberosus*. Cultivated hybrids developed from *H. tuberosus* and *H. argophyllus* have high field tolerance to Phomopsis brown stem canker (Škorić, 1985). Škorić (1985) hypothesized that the resistance may be controlled by two or more complementary genes.

Alternaria leaf spot causes losses in cultivated sunflower in the USA and other parts of the world. In warm climates with high rainfalls, it causes defoliation and reduces yield significantly (Sackston, 1981). All 21 annual taxa and 18 of 21 perennial species evaluated were susceptible to *A. helianthii* (Hansf.) Tub. and Nish. spores applied in a suspension. Perennial species *H. hirsutus, H. pauciflorus* subsp. *subrhomboideus,* and *H. tuberosus* appear to resist infection by *Alternaria helianthi* (Morris et al., 1983). Lipps and Herr (1986) showed that 13 accessions of *H. tuberosus* had significantly less Alternaria leaf spot than commercial hybrids and concluded that the species is a potential source of resistance to leaf spot. Several wild annual species, *H. praecox, H.* x *laetiflorus* Pers., *H. debilis* subsp. *cucumerifolius,* and *H. debilis* subsp. *silvestris,* had high levels of resistance to Alternaria and *Septoria helianthi* Ellis and Kellerm. in field evaluations (Block, 1992). Although potential sources of resistance to *Alternaria* have been identified, resistance genes have not been transferred to cultivated lines.

Powdery mildew is a widely distributed pathogen of cultivated sunflower in warmer regions of the world (Zimmer and Hoes, 1978). This foliar disease is found mostly on senescing leaves, and is generally not of major economic concern. *Helianthus debilis* subsp. *silvestris, H. praecox* subsp. *praecox, H. bolanderi* A. Gray and 14 perennial species exhibited powdery mildew tolerance in both field and greenhouse tests (Saliman et al., 1982). Not all populations of some perennial species are resistant; populations of *H. grosseserratus* and *H. maximiliani* showed differential reactions. Jan and Chandler (1985a) characterized resistance to powdery mildew from *H. debilis* subsp. *debilis* as incompletely dominant. They incorporated genes from this species into a cultivated background and have released a germplasm pool PM1 having the resistance genes (Jan and Chandler, 1988).

Currently, cultivated sunflower does not possess resistance to Rhizopus head rot. Yang et al. (1980) reported that four out of 32 wild species and subspecies tested were resistant when inoculated with *R. arrhizus* A. Fischer and *R. oryzae* Went. The resistant sources were: *H. divaricatus, H. hirsutus, H.* x *laetiflorus,* and *H. resinosus*. Further breeding will be needed to transfer the identified sources of resistance into cultivated sunflower.

So far, most genotypes of sunflower have exhibited susceptibility to the pathogen *Phoma macdonaldii* Boerema. Under natural infection, wild sunflower species *H. maximiliani, H. argophyllus, H. tuberosus,* and *H. pauciflorus* possess excellent resistance to Phoma black spot (Škorić, 1992).

Interspecific lines based on *H. tuberosus* have resistance to charcoal rot. Wild species *H. mollis*, *H. maximiliani*, *H. resinosus*, *H. tuberosus*, and *H. pauciflorus* have also shown resistance. The number of genes and the inheritance of resistance to the pathogen have not been ascertained, although resistance appears to be dominant.

Broomrape (*Orobanche cumana*) is a parasitic weed that infects sunflower roots causing severe crop losses in Southern Europe and the Black Sea region. It has also been observed in Australia, Mongolia, and China and is generally associated with drier climates. Five resistance genes (Or_1 through Or_5) have been used successfully for broomrape control following the progression of races A through E. Since broomrape is a highly variable pathogen, the breakdown of resistance is a frequent phenomenon, and multiple sources of resistance are needed. Ruso et al. (1996) evaluated wild annual and perennial sunflower species reaction to Spanish races and found two annual species, *H. anomalus* Blake and *H. exilis* Gray that had resistance and all 26 perennial species were resistant.

Recent studies indicated the development of a new broomrape race in Spain, designated race F, which attacks all commercial sunflower hybrids, overcoming the previously effective resistance genes (Domínguez et al., 1996). High levels of resistance to race F have been observed in populations of wild perennial sunflower (Fernández-Martínez et al., 2000). Jan et al. (2002) released four race F resistant germplasms, BR1 through BR4, which were derived from wild perennial sunflowers *H. maximiliani*, *H. grossesserratus*, and *H. divaricatus*. Fernández-Martínez et al. (2004) released four sunflower germplasms, K-96, L-86, P-96 and R-96, with resistance to race F based on cultivated sunflower from Eastern Europe. Resistance to race F appears to be controlled by dominant-recessive epistasis, complicating the breeding by requiring the genes to be incorporated into both parental lines of a resistant hybrid (Akhtouch et al., 2002). Other germplasms have been released which have resistance to various races (other than race F) of broomrape including seven germplasms based on cultivated sunflower from the FSU, Romania, and Turkey (Miller and Domínguez, 2000).

Diseases*: *Current progresses in developing Sclerotinia stalk rot resistant germplasm utilizing wild perennial Helianthus. Sclerotinia stalk and head rot caused serious economic loss for more than 50 percent of the seed yield. Cultivated sunflower lacks resistance to *Sclerotinia*, although some differences in susceptibility exist. However, the over 51 species of *Helianthus*, consisting of diploid, tetraploid and hexaploid, represent a diverse potential source of Sclerotinia resistance genes. Evaluation of wild germplasm indicated that several wild perennial species possess high levels of resistance to Sclerotinia head rot and stalk rot.

Since 2005, a program focusing on the transfer of Sclerotinia stalk rot resistance from wild *Helianthus* species of different ploidy levels (2x, 4x, 6x) into adapted sunflower germplasm via interspecific hybridization was started at the Sunflower Research Unit in Fargo. In our initial experiment, hexaploid perennial *H. californicus* DC. was identified to be highly resistant to Sclerotinia stalk rot, and was crossed with the moderately tolerant line HA 410 (Miller and Gulya, 1999) followed by continuously backcrossing with HA 410 until BC_4F_1 (Feng et al., 2006).

At the same time, to expand the diversity of the resistance gene sources, interspecific amphiploids were identified that segregated for high levels of resistance to Sclerotinia stalk rot. These amphiploids had high crossability and played a critical role as bridges for interspecific gene transfer, avoiding the direct crossing of HA 410 with those wild *Helianthus* species known to cross with extreme difficulty. Thus, in 2006, amphiploids involving six wild diploid or tetraploid species were crossed with HA 410 and further backcrossed twice to transfer stalk rot resistance (Jan et al., 2006). Furthermore, based on two years of information, an additional project was started to transfer Sclerotinia stalk rot resistance from three diploid perennial species to HA 410 in 2007.

Hexaploid *H. californicus* was crossed with HA 410 in 2005 resulting in F_1 plants with 2n=68 chromosomes, which were backcrossed with HA 410 from BC_1F_1 through BC_4F_1. The chromosome numbers of the BC progeny were gradually reduced to 2n=34 (Table 1). As a result, their pollen fertility increased from 4.6, 31.3, and 38.5, to 73.9% in the BC_4F_1 generation, suggesting the continuing improvement of fertility as more *H. californicus* chromosomes were eliminated. Consistent with the improvement of pollen fertility, seed sets increased from 0.05% in BC_1F_1 up to 35.3% in BC_4F_1. It was noticed that the variation of pollen fertility was high among the BC progenies, for example, 4.6 to 62.1% in BC_2F_1, 5.0 to 95.6% in BC_3F_1, and 10 to 96.9% in BC_4F_1. This wide range of pollen fertility was expected primarily due to the variation in chromosome numbers of the individual BC progenies. Currently, of the 79 BC_4F_1 plants, 14 plants with 2n=34 have produced sufficient seed for field testing. Also, progenies derived from advanced backcross generations (BC_4) would be ideal genetic stocks for identifying chromosome segments of wild species in the cultivated background.

Table 1. Chromosome number, pollen fertility and seed set of F_1 and backcrossed progenies of *H. californicus* with HA 410 in 2005-2007.

	F_1	BC_1F_1	BC_2F_1	BC_3F_1	BC_4F_1
2n	68	50-53	40-49	35-44	34-40
Fertile pollen %	37.8	4.6 (0.9-10.2)	31.3 (4.6-62.1)	38.5 (5.0-95.6)	73.9 (10-96.9)
Seed set % (Seeds/florets)	2.71	0.05 (48/99,900)	3.35 (183/5,460)	11.9	35.3

However, the BC_1F_1 generation with 2n=51 had the most unbalanced genome relationship, obviously corresponding to the low backcross seed set. Since we only started to observe 2n=34 plants in the BC_4F_1 generation, it is obvious that deriving genes from hexaploid species often takes a long time, but the resulting germplasm will be much more of the cultivated type than that resulting from using other faster approaches. The disadvantage of this approach is less genetic variability at the 2n=34 stage for the selection of QTL as in the case for Sclerotinia resistance.

For interspecific amphiploids, a sufficient number of F_1 hybrids between the five amphiploids and HA 410 were produced in 2006 (Jan et al., 2006), which was followed with two more cycles of backcrosses with HA 410. The chromosome number, pollen fertility and seed set of crosses of interspecific amphiploids crossed with HA 410 and the backcrossed progenies are summarized in Table 2.

Table 2. Chromosome number, pollen fertility and seed set of F_1 and backcrossed progenies of interspecific amphiploids with HA 410 in 2006 and 2007.

	F_1			BC_1F_1			BC_2F_1
Parentage	2n	Fertile pollen (%)	×HA 410 seed set (%) (seeds/florets)	2n	Fertile pollen (%)	×HA 410 seed set (%) (seeds/florets)	2n
H. strumosus × P21 2n=102	68	89.4 (74.3-97.9)	19.7 (755/3,800)	49-51	26.0 (6.6-42.5)	1.9 (282/11,900)	34-41
H. grosseserratus × P21 2n=68	51	43.3 (2.4-72.8)	9.1 (165/1,818)	37-44	35.3 (6.2-84.6)	3.5 (77/4,240)	34-38
H. maximiliani × P21 2n=68	51	49.9 (2.4-66.3)	13.7 (711/5,190)	37-47	29.9 (2.4-70.9)	2.0 (97/7,920)	34-37
H. nuttallii 730 × P21 2n=68	51	29.7 (3.7-57.1)	1.1 (32/2,800)	36-43	41.0 (1.3-84.1)	8.3 (460/7,610)	34-37
H. divaricatus × P21)× (*H. grosseserratus* × P21) 2n=68	51	27.3 (1.0-48.4)	18.1 (835/4,620)	36-46	21.3 (1.4-85.1)	6.0 (434/6,020)	34-37

A total of 145 BC_2F_1 plants from five crosses between selected amphiploids and HA 410 were obtained. Because the amphiploids had a full set of 2n chromosomes from the cultivated sunflower, the elimination of the wild species chromosomes after each backcross was faster than that of the backcrosses of *H. californicus* x HA 410, and the 2n=34 progenies also had slightly higher pollen fertility and seed set (Table 1). After two backcross cycles, of the 145 BC_2F_1 plants, 47 plants had 2n=34 chromosomes and have produced sufficient seed for field testing. With continuous selection of target traits, amphiploids are expected to be extremely efficient in selecting the trait while eliminating the other undesirable wild species genes. As for the use of hexaploid wild species, the rapid elimination of wild species genes may prove amphiploids less efficient for transferring QTL.

For the diploid resistance source, *H. maximiliani*, *H. giganteus* and *H. grosseserratus* were used to pollinate NMS HA 89, and the resulting F_1 hybrids were obtained by rescuing the 5-day-old immature embryos on artificial medium as described by Feng et al. (2006). For the crosses of diploid perennials and HA 410, a total of 181 embryos were obtained from the interspecific crosses of NMS HA 89 with *H.*

maximiliani, *H. giganteus*, and *H. grosseserratus*, respectively (Table. 3). By using embryo rescue, 67 hybrid seedlings were established in the greenhouse, suggesting that the interspecific hybridization using wild species as the pollen donor was successful. Pollen fertility of the F_1 hybrids from NMS HA 89 crossed by diploid wild perennials was very low (around 1%) (Table 3). Consequently, only 155 BC_1F_1seeds were produced from 64,618 florets pollinated with HA 410. This result was consistent with the conclusion that diploid perennial species could be crossed with cultivated sunflower, but the frequency of successful crosses was low (Atlagić et al., 1995). The extremely low backcross seed set of the F_1 plants is the most limiting stage for transferring genes from diploid perennials. However, since the F_1 plants are generally perennial, sufficient BC_1F_1 seeds can be obtained by repeated pollination. The forced chromosome pairing between the cultivated and the wild diploid perennials will promote chromosome recombination and result in BC_1F_1 plants with a large number of wild species traits for the selection of QTL.

Table 3. Pollen fertility of F_1s between NMS HA 89 and wild diploid *H. maximiliani*, *H. giganteus* and *H. grosseserratus*, and backcross seed set with HA 410 in 2007.

Parentage	No. F_1 embryo/florets	No. seedlings	Fertile pollen %	BC seeds/florets
NMS HA 89 × *H. maximiliani*	10/8083	9	1.7 (0-1.7)	21/11408
NMS HA 89 × *H. giganteus*	23/5480	15	0.6 (0.3-0.8)	26/6750
NMSHA89 × *H. grosseserratus*	148/14200	43	1.0 (0-1.6)	108/46460
Total	181/ 27763	67	--	155/ 64618

In conclusion, potential interspecific pre-breeding Sclerotinia resistance lines from diploid, tetraploid and hexaploid germplasm have been produced during the past three years. Evaluation of these pre-breeding lines for their reaction to Sclerotinia stalk rot will verify the effectiveness of each approach for the selection of QTLs. The effectiveness of using each of the above approaches will also be verified by tracking of the wild species' specific molecular markers in progeny plants when they first reach the 2n=34 stage and are ready for seed increase for the field evaluation. Ultimately, we expect to identify and release germplasms with improved resistance to Sclerotinia stalk rot within the shortest time period possible.

***Insects*.** North America has the greatest problems with insect pests because the insect pests of sunflower have co-evolved with their native sunflower hosts in natural communities. In the major production area of North America, there are about 15 principal insect pests of cultivated sunflower, and of this total about six are considered of major importance as potential economic pests from year to year (Charlet and Brewer, 1997). The insects of main concern include: the sunflower beetle, the sunflower stem weevil, the red and gray seed weevils [*Smicronyx fulvus* (LeConte), and *S. sordidus* (LeConte)], the banded sunflower moth, *Cochylis hospes* Walsingham, the sunflower moth, *Homoeosoma electellum* (Hulst), and the sunflower midge, *Contarinia schulzi* Gagne.

Host-plant resistance is a pest management method that utilizes the plant's own defense mechanisms against the insect. Since wild sunflower are native to North America where their associated herbivores and entomophages co-evolved, there is an opportunity to search for insect resistance genes in the diverse wild species. Sunflower moth tolerance was observed in annual *H. petiolaris* and perennials *H. maximiliani*, *H. ciliaris* DC., *H. strumosus*, and *H. tuberosus* (Rogers et al., 1984). Stem weevil tolerance was found in perennials *H. grosseserratus*, *H. hirsutus*, *H. maximiliani*, *H. pauciflorus*, *H. salicifolius* Dietr., and *H. tuberosus* (Rogers and Seiler, 1985). Sunflower beetle tolerance was observed in annuals *H. agrestis* Pollard and *H. praecox*, and in perennials *H. grosseserratus*, *H. pauciflorus*, *H. salicifolius*, and *H. tuberosus* (Rogers and Thompson, 1978; 1980). Charlet and Seiler (1994) found indications of resistance to the red sunflower seed weevil in several native *Helianthus* species.

Interspecific germplasm using wild species as resistance sources have been created. In preliminary testing, Charlet et al. (2004) noted that germplasm derived from *H. petiolaris* had the lowest number of stem weevils. Among material tested in a banded sunflower moth evaluation nursery, germplasm derived from *H. praecox* subsp. *hirtus* had less than 2% damage. Germplasm that incorporated *H. strumosus* and *H. tuberosus* had very little red sunflower seed weevil damage in test plots. Breeding populations of promising germplasms are being developed for further testing.

Oil and oil quality. Variability for oil concentration exists in the wild species. Annual *H. anomalus* has the highest oil concentration of 460 g/kg, the highest ever observed in a wild sunflower species, followed by *H. niveus* (Benth.) Brandegee subsp. *canescens* with 402 g/kg, *H. petiolaris* with 377 g/kg, and *H. deserticola* Heiser with 343 g/kg (Seiler, 2007). Perennial *H. salicifolius* had a concentration of 370 g/kg (Seiler, 1985; Seiler and Brothers, 2003). Cultivated sunflower generally contains 450 to 470 g/kg. Reduced concentrations of saturated palmitic and stearic fatty acids have been observed in a population of wild *H. annuus* that had a combined palmitic and stearic acid concentration of 58 g/kg (Seiler, 1998). This is 50% lower than in oil of cultivated sunflower. A combined palmitic and stearic acid concentration of 65 g/kg was observed in a wild perennial species, *H. giganteus* L. (Seiler, 1998).

Salt and drought tolerance. Several species of *Helianthus* are native to salt-impacted habitats. Interspecific germplasm derived from *H. paradoxus* Heiser has been identified with high salt tolerance, withstanding salt concentrations up to EC 24.7 d/Sm. It appears that one major gene controls salt tolerance, although a modifier gene may also be present, possibly recessive in control (Miller, 1995). Two salt-tolerant parental oilseed maintainer lines, HA 429 and HA 430, have been released (Miller and Seiler, 2003). Blanchet and Gelfi (1980) evaluated stomatal resistance, leaf-water potential, photosynthetic activity, leaf structure, and number of stomata. They concluded that *H. argophyllus* is the best candidate source for drought tolerance genes because its pubescent leaves reflect sunlight, reduce water loss, and exhibit low transpiration rates. *Helianthus niveus* subsp. *canescens* was their second choice.

Herbicide tolerance. A wild population of annual *H. annuus* from a soybean field in Kansas that had been repeatedly treated with imazethapyr for seven consecutive years developed resistance to the imidazolinone and sulfonylurea herbicides (Al-Khatib et al., 1998). Resistance to imazethapyr and imazamox herbicides has great potential for producers in all regions of the world for controlling several broadleaf weeds. Several populations of wild sunflower (*H. annuus* and *H. petiolaris*) from the USA and Canada have been screened for resistance to these two herbicides. Eight percent of 50 wild sunflower populations had some resistance to imazamox and 57% had some resistance to tribenuron in the central U.S (Olson et. al., 2004). In Canada, 52% of 23 wild *H. annuus* populations had some resistance to tribenuron (Miller and Seiler, 2005). Genetic stocks IMISUN-1 (oil maintainer), IMISUN-2 (oil restorer), and IMISUN-3 (confection maintainer) have been developed and released (Al-Khatib and Miller, 2000). Miller and Al-Khatib (2002) also released one oilseed maintainer and two fertility restorer breeding lines with imidazolinone herbicide resistance. Genetic stocks SURES-1 and SURES-2 with resistance to the sulfonylurea herbicide tribenuron have been developed and released by Miller and Al-Khatib (2004). Additionally, two oilseed germplasm lines, HA 442 and RHA 443 have been released with imidazolinone resistance (Miller et al., 2006). The imidazolinone and sulfonylurea herbicides may control broomrape in areas of the world where this parasitic weed attacks sunflower (Alonso et al., 1998).

CONCLUSIONS AND PROSPECTS

Significant progress has been made in increasing the number of accessions in the wild sunflower species collection to preserve the wild species and increase the available genetic diversity for improvement of the crop. Interspecific gene transfer for sunflower improvement has been practiced since the very early years by breeders in the FSU and it has continued to play a key role as the crop developed into a major global oilseed crop. Recent advances in culturing of otherwise abortive interspecific hybrid embryos have proved to be highly effective for making the difficult-to-cross wild perennial *Helianthus* species crosses widely available for breeding purposes, either for specific major gene transfer or for the transfer of quantitative trait genes. Significant results have been reported on the germplasm development with regard to resistance to new races of downy mildew, rust, broomrape and other major diseases. In addition, new CMS and corresponding fertility restoration genes have been continuously identified and established, together with new genes helping to improve oil quality, herbicide resistance, and salt and drought tolerance. Thus far, only a small portion of the available genetic diversity of the wild *Helianthus* species

has been used globally. As a whole, there is no doubt that wild *Helianthus* species will continue to provide new genetic variability to the sunflower breeding community, helping to maintain sunflower as a viable major global oilseed crop.

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Current advances in sunflower oil applications

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ABSTRACT

The fatty acid and triglyceride molecular species of a given oil determine its physical, chemical, and nutritional properties. Thus, applications for a specific oil depend mainly on its fatty acid composition and the way in which the fatty acids are arranged in the glycerol backbone. Minor components, such as tocopherols, could also modify oil properties, such as thermo-oxidative resistance. Sunflower seed commodity oils predominantly contain linoleic and oleic fatty acids, with lower contents of palmitic and stearic acids. High-oleic sunflower oil, which could actually be considered as a commodity oil, contains oleic acid of up to around 90%. New sunflower varieties with different fatty acids and tocopherols compositions have been selected. Due to these modifications, they possess new properties and are much better adapted for direct home consumption, food industry and other applications such as biolubricants and biodiesel production.

Key words: biodiesel – fatty acids – oil quality – oxidative stability – tocopherols – triglycerides.

INTRODUCTION

Oils are mainly constituted by triglycerides, but also contain small quantities of diglycerides, polar lipids, tocopherols, free fatty acids, etc. Triglycerides, which account for more than 95% of total oil, consist of a glycerol molecule with three fatty acids esterified in the hydroxyl residues, one in the central position of the glycerol molecule and the other two at the terminal positions. The most common fatty acids forming these triglycerides in sunflower are: saturated palmitic and stearic acids, monounsaturated oleic acid and polyunsaturated linoleic acid. The final use of each type of oil is defined by both its physical and chemical characteristics, which depend on its fatty acids and triglyceride composition. For instance, the difference between oils and fats is due to the amount of their saturated fatty acids. Their thermo-oxidative stability depends mainly on the amount of polyunsaturated fatty acids they contain (oils with a high content of these unsaturated fatty acids are more unstable), and their content and type of tocopherols. Therefore, the performance of an oil for a specific use will depend on these characteristics. Considerable research efforts are being put into the following aspects. On the one hand, more stable sunflower oils are being obtained by increasing their content in monounsaturated fatty acids (oleic acid) and decreasing their content in polyunsaturated fatty acids (linoleic acid). These oils are also suitable for biolubricants. Their stability could also be increased by modifying their tocopherol content. On the other hand, healthy substitutes for animal, tropical or hydrogenated fats required by the food industry are being obtained by increasing their content in saturated fatty acids, mostly stearic, which does not modify the plasma cholesterol content.

DISCUSSION

Sunflower oils

Depending on their particular use, oils or fats must have a specific composition to fulfill the requirements of each application. Deep-frying, and other industrial processes for food preparation, require fats and oils with a high thermo-oxidative stability. In these applications, due to easy storage and pouring, oils are better than fats. For margarine, spreads, confectionery, and related products, fats with a certain degree of plasticity are required. For biolubricant production, oil liquid at temperatures below 0ºC with a good thermo-oxidative stability is required. Biodiesel production only requires a minimal stability and standard sunflower oils are equally as good as canola or other vegetable oils, but, probably for this application, palm oil is even better.

By lowering the content of unsaturated fatty acids or modifying minor components, such as tocopherols, the stability of oils could be enhanced, making them suitable for deep frying and biolubricant uses. Increasing the saturated fatty acids content will increase the proportion of solid fat and, therefore, its melting temperature. With the exception of animal fats, palm oil fractions and lauric oils, natural fats hardly fulfill the requirements of most industrial processes. Nevertheless, the above mentioned fats are considered unhealthy by many authors and by the World Health Organization (WHO, 2003) because of

their high content in palmitic, myristic and lauric fatty acids, so they have been substituted by hydrogenated vegetable oils. However, the hydrogenation process generates *trans* isomers of unsaturated fatty acids, which are also considered to be nutritionally undesirable. In general, dietary recommendations encourage the intake of unsaturated fatty acids, such as oleic and linoleic, and stearic as a saturated fatty acid (Kelly et al., 2001; Mensink, 2005; WHO, 2003).

Different sunflower lines with modifications in the fatty acid composition of their oils have been obtained (Table 1). Since the selection of the high-oleic mutant by Soldatov (1976), several new fatty acid mutants have been obtained by ionization, radiation or chemical mutagenesis, among them three independent high-palmitic lines, with around 30% of palmitic acid in their oils, two in standard high-linoleic background and another in high-oleic background (Ivanov et al., 1988; Osorio et al., 1995; Fernández-Martínez et al., 1997), and some high-stearic acid in high-linoleic background (Osorio et al., 1995; Fernández-Moya et al., 2005) have been obtained. Lines with high-stearic in high-oleic background were obtained later by recombination (Fernández-Moya et al., 2005). In spite of their higher saturated acid content, these sunflower oils have a low content of saturated fatty acid in the middle position of the triglyceride (Alvarez-Ortega et al., 1997), differentiating them completely from animal, palm and hydrogenated fats.

Table 1. Fatty acid composition of several sunflower oil mutant lines with modifications in their oils, compared to the standard sunflower oil.

Sunflower	Oil	Fatty acid composition (%)				
line	phenotype	16:0	16:1	18:0	18:1	18:2
Standard	Normal	7		6	29	58
HA-OL9[a]	High oleic	5		3	**90**	2
CAS-4[b]	Medium stearic	6		**12**	28	53
CAS-3[b]	High stearic	5		**26**	15	53
CAS-30[c]	High stearic	6		**30**	10	50
CAS-15[c]	High stearic-oleic	6		**24**	**62**	5
CAS-5[b, d]	High palmitic	**31**	5	3	12	48
CAS-12[e]	High palmitic-oleic	**32**	6	4	**54**	3

[a]Soldatov, 1976; Fernández-Martinez et al. 1993.
[b]Osorio et al. 1997.
[c]Fernández-Moya et al. 2005.
[d]Ivanov et al. 1988.
[e]Fernández-Martínez et al. 1997.

New research has been carried out to obtain fractions with improved properties from these oils. Thus, high stearic and oleic sunflower oils have been cold-fractionated to obtain stearin and olein fractions (Table 2). In this case, because of the unimpaired distribution of triglycerides species between the fractions, fatty acid composition analysis is not a satisfactory method to characterize them, and, instead, the triglyceride composition has to be determined. Table 2 shows the triglyceride subclasses of these fractions; the liquid olein fraction has mostly triunsaturated triglycerides, mainly OOO, (see Table 2 for abbreviations) and OOL, and monosaturated triglycerides, mainly EOO, and in a lesser amount POO and EOL, whereas the stearin has a higher content of disaturated triglycerides than the olein fraction, EOE and POE being the principal triglycerides. The melting properties of the stearin, measured as the solid content at different temperatures by differential scanning calorimetry are similar to cocoa butter.

Table 2. Triglyceride subclasses composition of the liquid fraction (olein) and solid fraction (stearin) obtained from high stearic and oleic sunflower oil[1].

Sunflower	Oil Fraction	TAG Types (%)		
		SUS	SUU	UUU
HEHO	Olein	3.1	56.7	40.2
HEHO	Stearin	73.3	17.9	8.8

[1]SUS, disaturated triglycerides; SUU, monosaturated triglycerides; and UUU, triunsaturated triglycerides. S, saturated; U, unsaturated; HE, high stearic acid; HO, high oleic acid.

Tocopherols, good antioxidant molecules, are one of the minor components of sunflower oil, with α-tocopherol, or vitamin E being the standard in commodity sunflower oil, new lines with modified profiles of tocopherols have been obtained (Table 3). These new lines have been obtained from germplasm of

wild and cultivated sunflower (Demurin, 1993; Velasco et al., 2004). The tocopherols accumulated in these lines mainly depend on modifications on the genes which control the biosynthetic pathway. The oils containing γ-tocopherol and δ-tocopherol have the advantage of a higher oxidative stability, but a reduced vitamin E content.

Table 3. Tocopherol composition of oils extracted from modified sunflower lines.

Oil Type	Tocopherol composition (%)			
	α-T	β-T	γ-T	δ-T
Standard α-T	95	4	1	0
Medium β-T[a]	50	50	0	0
High β-T[b]	75	25	0	0
High γ-T[a]	5	0	95	0
High δ-T[b]	5	0	30	65

[a]Demurin, 1993
[b]Velasco et al., 2004

Sunflower oil applications

Standard sunflower oil possesses good properties for low temperature and general food applications (salad dressings, emulsions, etc), but for high temperature applications and deep frying, oils with a lower content of polyunsaturated fatty acids are required, and these are the high-oleic oils. The oil properties at a high temperature also depend on the tocopherols, oils with a higher content of γ and δ-tocopherols being more stable than oils with α and β-tocopherols. Margarine and plastic fat production demands oils with high contents of saturated fatty acids such as palmitic or stearic acids, preferably stearic because of the unhealthy effect of palmitic acid, as stated in Kelly et al. (2001): "The food industry might wish to consider the enrichment of foods with stearic acid in place of palmitic acid and trans fatty acids".

Thermo-oxidative treatments to test oil stability are usually carried out at 180 °C for 10 h monitorizing the formation of polar and polymer compounds. TAG polymerization in the different oils increased with time (Fig. 1). In this regard, oils could be classified into three groups; standard oils, with a high content of polymerised TAGs, up to around 17% after 10 h treatment; high-oleic sunflower and palm olein oils with around 10% of polymerised TAGs after the same treatment; and the high-palmitic and high-oleic sunflower oil with only 6% of polymerised TAGs at the same time. This indicates that oils with the higher content of oleic and palmitic acids are the best for high temperature applications. Rejection levels of 12% of polymers have been recommended in current regulations for discarding used frying fats for human consumption (GFSR, 2000). As a result, commodity oils, soybean, canola and standard sunflower oils must be rejected after 8 h at 180ºC, while high-oleic sunflower could still be used after 10 h and the high-palmitic and high-oleic oil would be even further from rejection.

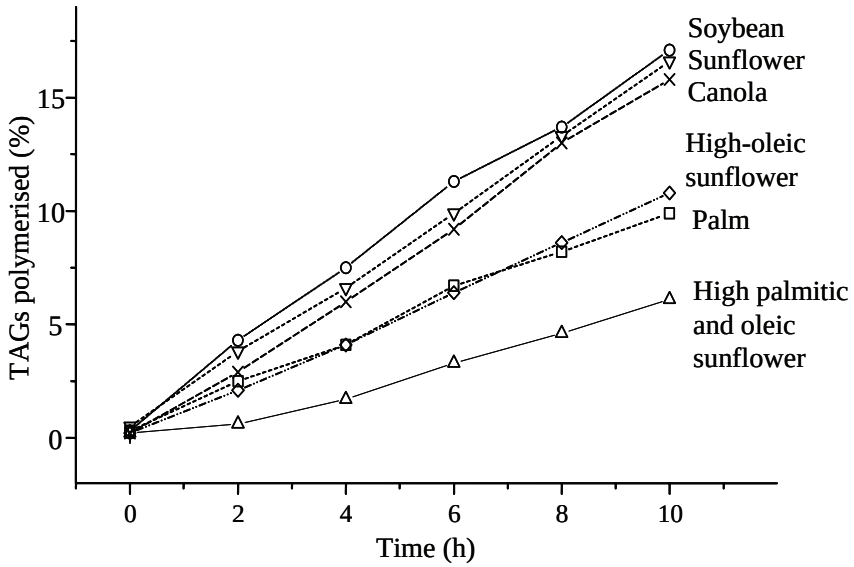

Fig. 1. TAG polymerization at 180ºC of oils of vegetal origin. Soybean, canola and sunflower are the standard commodity oils, palm is a commercial palm olein, and high-oleic and high-palmitic high-oleic sunflower oils are genetically modified sunflower oils (Marquez et al., 1999).

As stated above, tocopherols could also modify the thermo-oxidative stability of the oils. Fig. 2 shows the polymerised TAGs at 180ºC of genetically modify sunflower oils. Oils tested in this experiment were standard, high-oleic containing α-tocopherol, high-oleic and high-palmitic containing α-tocopherol and high-oleic and high-palmitic containing γ-tocopherol.

After 10 h at 180ºC, standard and high-oleic sunflower oils have 17.4% and 8.2% of polymerised TAGs, while the high-palmitic and oleic oils have only 2.3% and 1.4%. Furthermore, after 25 h of experiment, the polymerised TAGs were only 8.7 and 4% of polymerized TAGs and had less than 12% of polymers and were therefore still suitable for human consumption (Marmesat et al., 2008). These two high-palmitic oils have a very high oxidative stability and the oil with γ-tocopherol is the best as it always has less than half of the polymerised TAGs than the same oil with α-tocopherol and, even more, after 25 h it was less polymerised than the standard sunflower after 2 h, making this oil extremely stable.

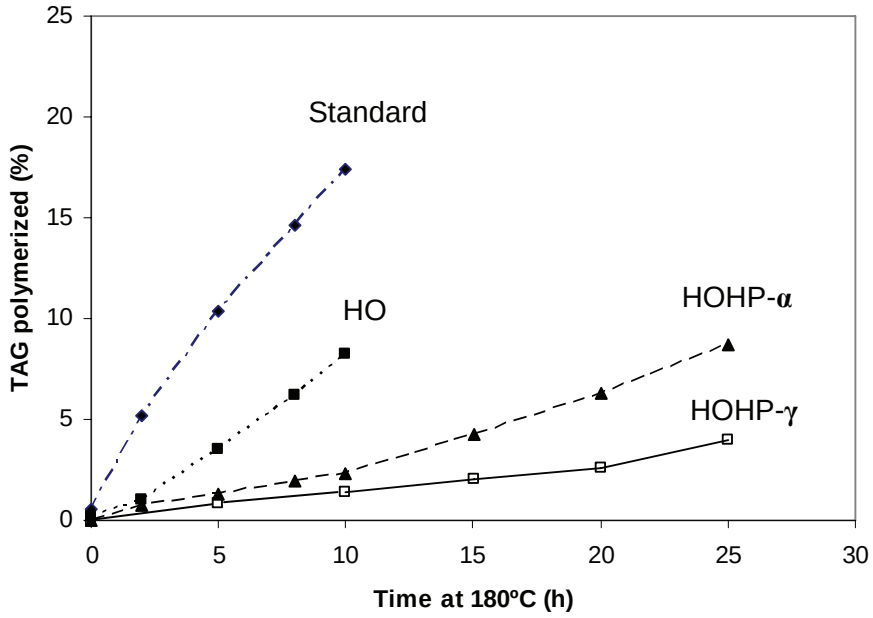

Fig. 2. TAG polymerization at 180ºC of standard sunflower oils compared with high-oleic oil (HO), high-oleic and high-palmitic oil containing α-tocopherol (HOHP-α), and high-oleic and high-palmitic oil containing γ-tocopherol (HOHP-γ).

High-stearic high-oleic sunflower oils, and also the liquid fraction obtained from them by cold fractionation, have good thermo-oxidative stabilities. These oils have a reduced content of polyunsaturated fatty acids, high content of oleic and some stearic acid. Experiments made to determine their oxidative stability have shown the total modified TAGs after 10 h at 180ºC of these oils in comparison with high-oleic and high-oleic and palmitic with different tocopherol contents (Table 4). Due probably to the different origin of oils and authors, differences were found in the data for standardising the results. The data presented here were corrected according to the results obtained with respect to the high-oleic oil present in all experiments, 1 being the value assigned to the high-oleic oil.

For oils with a high saturated content, which could be solid at relatively high temperatures, a new parameter must be defined, i.e. the cloud point which is the temperature at which the liquid became turbid. Oils with a cloud point of above 0ºC are difficult to transport and need special factory requirements. In winter during transport storage oil could become solid, and so good deep frying oils must have a good oxidative stability and be liquid, at least up to 0ºC. The high-stearic and high-oleic oils are very stable, but they are solid at room temperature, and the high-palmitic oils are also quite solid at temperatures of between 0 and 10ºC. High-oleic sunflower oil and olein fractions from HSHO sunflower are probably the best oils for deep frying, mainly if they contain γ-tocopherol instead of α-tocopherol.

Table 4. Total modified TAGs and cloud point of sunflower oils with different fatty acid and tocopherol compositions.

Fatty acids Phenotype	Total modified TAG High oleic = 1	Cloud point (ºC)
Standard	1.29	-8
High oleic	1.00	-8
High oleic and palmitic α tocopherol	0.61	6
High oleic and palmitic γ tocopherol	0.48	
High oleic and stearic	0.59	24
Oleine from HSHO[1]	0.76	-4

[1]High saturated, high oleic acid

For the elaboration of some food products the industry needs solid or semisolid fats, whose traditional sources have been animal and some tropical fats, such as palm and lauric oils (palm kernel and coconut). Studies in human health have demonstrated that these fats are unhealthy due to their elevated contents of medium and long chain saturated fatty acids (mainly myristic and palmitic acids). Their intake increases the plasma levels of LDL-cholesterol (bad cholesterol), which generates an increment in the risk of suffering cardiovascular diseases. The effect of fats on cholesterol levels depends on their fatty acid composition (Mensink et al., 2003). The relationship between plasma cholesterol levels and cardiovascular diseases is well-known. The ingested fatty acids modulate the lipoprotein levels (and therefore the type of cholesterol). In general, unsaturated fatty acids (oleic, linoleic, and linolenic acids) increase the HDL and diminish the LDL, and for that reason they are considered as being healthy. On the other hand, saturated fatty acids (lauric, myristic and palmitic) increase both the LDL and the HDL and therefore the ratio LDL/HDL. But stearic acid, in spite of being saturated, does not have any effect on the cholesterol content (Kelly et al., 2001; WHO, 2003; Mensink, 2005). In conclusion, the ingestion of stearic, oleic or linoleic acid does not modify the profile of lipoproteins (Thijssen and Mensink, 2005). The main reason for this is that stearic acid is transformed very quickly into oleic acid in the liver (Pearson, 1994).

To solve the problem regarding the use of hydrogenated vegetable fat, animal fat or tropical fats, a research project has been carried out with the aim of obtaining natural sunflower oils that could be used directly in the food industry for the production of margarine and similar products without the need of any chemical manipulation. New lines have been selected by classic methods, without the application of genetic engineering techniques, just the same as the high-oleic sunflower mutant. Sunflower lines with a high-stearic acid content together with oleic or linoleic acids are a healthy alternative to these unhealthy fats. In Table 4, the clouding point of some sunflower oils is shown. Among these, setting a good example, the high-stearic and oleic fat from sunflower has a clouding point of 24ºC, making it suitable for the manufacture of margarine, spreads, bakery and other products where a plastic fat is needed.

The triglyceride composition of these new oils is different to those of the standard sunflower oil, making them appropriate for industry demands (Table 5; Fig. 3). High-stearic lines contain a considerable percentage of triglycerides with two saturated fatty acid molecules, EOE and POE being the most abundant species in high-stearic high-oleic oils, and ELE and PLE those in the lines with a high-linoleic background. These triglycerides have linoleic or oleic acids in the central position of the triglyceride, which makes them appropriate for the production of margarines. With fats constituted by these types of triglycerides and keeping in mind the effect on the levels of cholesterol of these fatty acids, besides the fact that they do not contain saturated acids in the central position of the triglyceride, we can guarantee that a fully vegetable and healthy margarine can be manufactured for the first time with the healthy stearic acid (WHO, 2003) and no saturated fatty acids in the middle position of triglycerides (Renaud et al., 1995).

Table 5. Triglyceride subclasses composition of standard sunflower (RHA-274), high-oleic (CAS-9), high-stearic and high-linoleic (CAS-30) and high-stearic and high-oleic (CAS-15) oils. SUS, disaturated triglycerides; SUU, monosaturated triglycerides; and UUU, triunsaturated triglycerides. S, saturated; U, unsaturated fatty acids.

TAG type	RHA-274	CAS-30	CAS-9	CAS-15
SUS	1.8	29.0	0.9	18.4
SUU	30.7	57.0	21.5	61.9
UUU	67.5	13.8	77.5	19.1

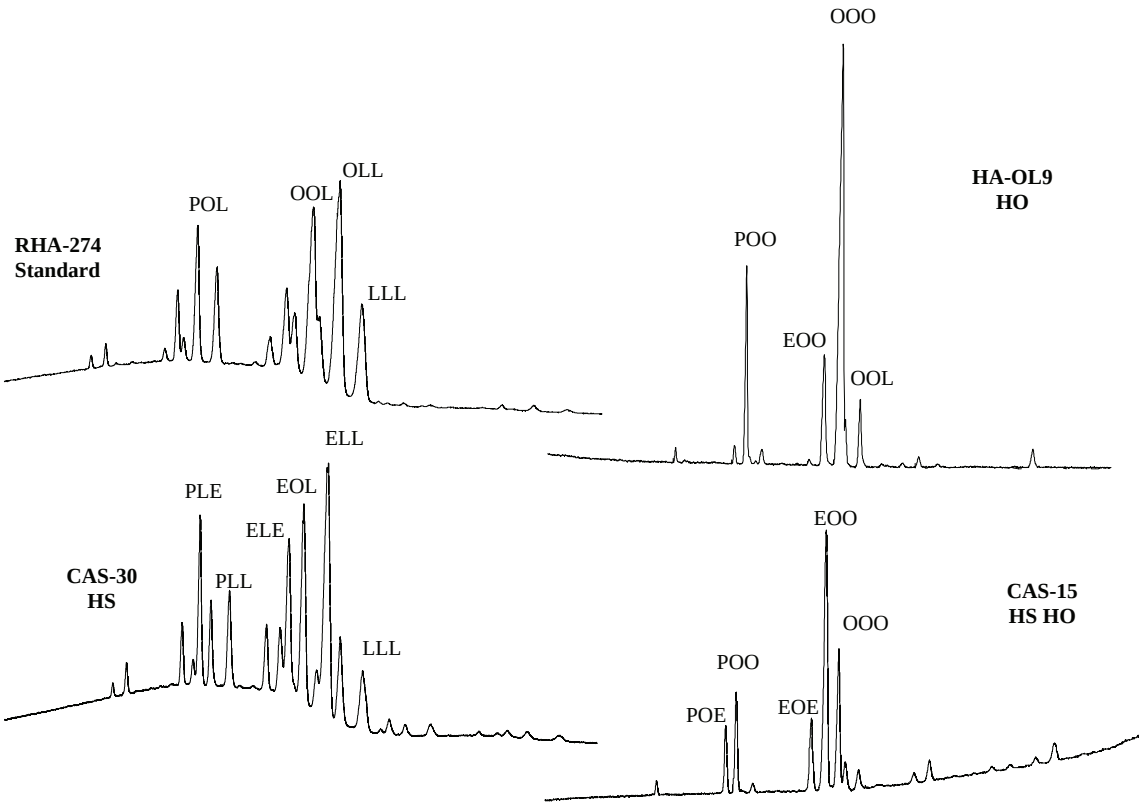

Fig. 3. Triglyceride chromatographs of standard RHA-274, high-oleic HA-OL9, high-stearic CAS-3 and high-stearic and oleic CAS-15 sunflower oils, showing the main triglycerides molecular species of each oil. P, palmitic; E, stearic;, O, oleic; and L, linoleic fatty acids.

To sum up, these new sunflower oils, with modified tocopherols and fatty acid composition, which were developed as a feedback for the food industry requirements to offer healthier products, together with the two others available nowadays (normal and high-oleic) could cover the requirements of the food industry without any chemical manipulation, with the aim of increasing the consumers' quality of life.

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Sunflower in Spain: Past and present trends in an international context

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ABSTRACT

Despite sunflowers having been brought to Spain at the beginning of the 16th century, and in-shell sunflower seed production having been traditionally grown in this country, the oilseed type was not introduced into Spanish agriculture until the end of the 1960's. The development of the oilseed sunflower in Spain has been through several stages marked in many cases by national and EU political decisions. The present trend points towards a recovery of the sunflower area lost in Spain during the last decade and the demand of vegetable oils for biodiesel production or for specific food uses may also serve to secure a plateau price and keep the oilseed oil demand higher than ever. A binding target of 10% for biofuels has been set in the EU for 2020. One output and one input trait are segmenting the sunflower market. The high oleic (HO) sunflowers, including mid oleic (NuSun), have continued to grow, NuSun representing over 96% of the total high oil sunflowers in the US, while in Europe in some countries such as France, Spain and Hungary, the HO area is expanding very quickly. The recent development of herbicide-tolerant sunflowers solved one of the historical deficits in sunflower crop management: i.e., post emergence weed control and may also serve for the chemical control of broomrape and contribute to increase seed yield. The combination of high oil value and potential yield increase makes sunflowers a competitive choice option for farmers.

Key words: herbicide-tolerant – high oleic – market trends – mid oleic – Spain

INTRODUCTION

Sunflowers were introduced into Europe via Spain at the beginning of the 16th century (Putt, 1978). After this, it moved in an eastward direction in Europe, in the beginning as an ornamental plant, and, in a second phase, becoming a food. The earliest record of using sunflower seeds as a source of oil was found in an English patent of 1716. Although this patent refers to the use of sunflower oil for wool, paint, leather, etc. manufacture, most of the crop was used for food. The commercial manufacture of sunflower oil started in Russia between 1830 and 1840.

Although "in shell" sunflower seeds have been traditionally produced in Spain, the oilseed type was not introduced until the second half of the last century. There are different stages in the history of oilseed sunflower production in Spain (Fig. 1). These have been influenced in many cases by political decisions, which have had a great impact on the area planted. These influences and other factors having an effect on sunflower evolution and present situation in Spain are analyzed in this review.

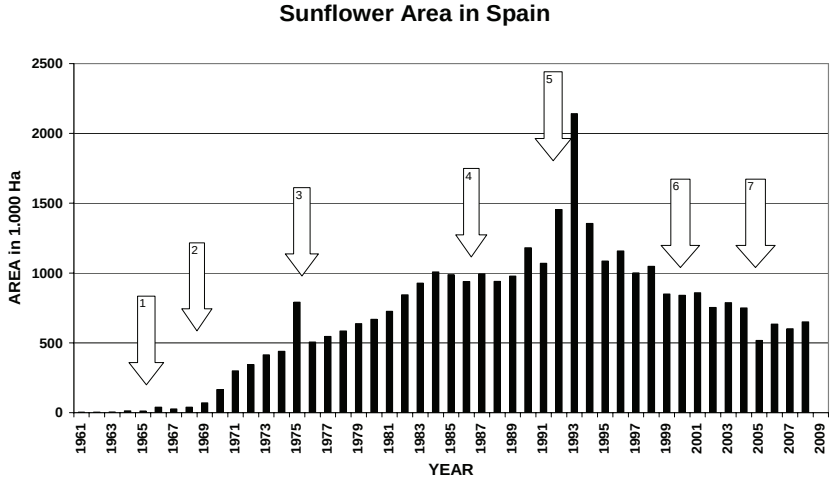

Fig. 1. Historical Oilseed sunflower planning area in Spain Source (MAPA, 2007)

DISCUSSION

Stages in the history of sunflower development in Spain

First Stage: 1965 to 1969. During this period, Spain was deficient in edible oil production and had to rely on imports for consumption. The first sowing of oilseed sunflowers with a black shell opened up a unique opportunity for Spanish farmers and industry. For farmers, to plant the traditional fallow land after cereals´ with sunflower, and for the industry to develop a crushing industry that pushed the crop area up.

Second Stage: 1969 to 1975. This period corresponds to the rapid expansion of the sunflower crop in Spain. At the end of this period the sunflower area planted in Spain was of 781,800 ha. The crushing industry played a key role in the development of sunflower crop as it provided:

- Certified seed for planting.
- Technical staff and mechanical planters to spread crop management techniques.
- Local agents who contracted and collected the crop.
- Financing of the crop, anticipating in many cases payments before harvesting

Also, by the year 1971, the World Bank had launched a program to assist in the development of sunflower in Spain, establishing a research centre for oilseed research in Córdoba in CRIDA 10 within the National Institute for Agricultural Research (INIA). Some of the young researchers from that time have made their scientific careers in working in sunflowers in Spain and are now relevant scientists in sunflower research.

Third Stage: 1975 to 1986. During this period, Spain's vegetable oil imports were restricted to state commerce. Only the Government could import both oilseed for crushing, and oil. Imports were made in the event of any shortage of oil. Additionally, the Government fixed a contract price for the crop that the industry had to pay the farmer. The Ministry of Agriculture assured itself the first bid for the oil. With this intervention, the period was marked by a notable increase in sunflower in Spain. The sunflower-planted area grew in over a million hectares in 1984. The period ends with the entry of Spain into the European Economic Community (EEC) in 1986.

Fourth Stage: 1986 to 1992. The EEC protection system was based on a *price support mechanism*, as a means of increasing agricultural output and productivity and ensuring agricultural income. The most important feature of this mechanism was to ensure that Community farmer prices *were higher* than the world average. The EEC set reference prices for a guaranteed maximum production (GMP) of oilseeds. The farmers received these prices. The crushing industry received aid for the oil extraction, which was fixed by the difference between the reference price and the international market price at the time of extraction. During the transitional period of ten years set for Spain in 1986, the Spanish Ministry of Agriculture established an Intervention price for the farmer at the intervention centres. This price would rise 1/10, to reach the annual Community price. It also established a target price for the local industries to calculate the aid for oil extraction. There was also an aid scheme for the export of surplus oil. The sunflower-planted area in Spain rose to 1,454,500 ha in 1992.

Fifth Stage: 1993 to 2000. The early development of the Common Agricultural Policy (CAP) allowed the EEC to move quite rapidly from a complete deficit to a surplus of production in the main products, and, therefore, to transform the EU from being a net importer to a net exporter on the world market. In 1992, the MacSharry reforms (named after the European Commissioner for Agriculture, Ray MacSharry) were proposed to pacify the EU's external trade partners at the Uruguay round of the GATT trade talks with regard to agricultural subsidies and to cope with EU budgetary difficulties. The 1992 reform, implemented in 1994, took a decisive step towards market orientation by gradually changing the basic mechanisms of the CAP from a *price support system* towards *direct income support.*

In 1993, Spain renounced the last years of the transitional period to become fully integrated into the new CAP. In the absence of any limitation of surface and with sunflowers taking advantage of a particularly high per hectare grant, its cultivation grew in over 2,000,000 ha. Then, the Spanish government imposed a series of restrictions on sunflower planting to limit the undesirable presence of "Premium hunters." These measures stabilized sunflower area to around 1,100,000 hectares. Two systems limited the oilseed area planted. One reduced the aid in proportion to the exceeding of the Maximum Guaranteed Area imposed by the EU/USA Blair-House agreement. The other was a reduction in the aid per hectare if the local oilseed prices exceeded the international reference prices fixed for the period to calculate the amount of aid per hectare. This last calculation could have also led to bonuses in the case of a fall in international reference prices for oilseed by fifteen percent below the reference prices.

The free import/export of oilseeds and oil in Spain, coupled with the no tariff barriers in the EU, created a complex situation for many local crushing industries and collectors. Losses were frequent in those taking long sunflower positions during the harvest due to fluctuations in international prices. Meanwhile, cereals continued to enjoy tariff protection and an intervention price in the EU.

Sixth Stage: From 2000 to 2005. The EU's total budget on agricultural spending fell substantially from 1992 to 1999. However, almost 50% of this budget was still being spent on agriculture in a declining economic sector, which did not create new jobs. Therefore, criticism has focused more and more on the fact that agriculture absorbs huge amounts of money, depriving other policies and tasks of the EU of their potential to create new jobs due to a lack of appropriate financial resources necessary for their development. Agenda 2000 seems therefore to be the Commission's attempt to define new, or, rather, additional, objectives of the CAP, by extending it to function as a rural development strategy. The 'Agenda 2000' reforms divided the CAP into two pillars: production support and rural development. Several rural development measures were introduced including diversification, setting up producer groups and support for young farmers. Agri-environment schemes became compulsory for every Member State.

In Spain, as in other EU member states, there was a progressive lowering of the sunflower planting area as per hectare aid was fixed as being the same for all crops as well as fallow. Fallow is an attractive cereal rotation in countries with periodic droughts. The intervention price for cereals was also lowered during this period. The sunflower area fall was inevitable in Spain despite the environmental fixed aid of 60€/Ha allowed by the EU to Spain for sunflowers. This aid, in many cases, was not implemented as there was no extra budget for it. Many Regional Governments in Spain were devoting all their rural development funds to other five year programs thus limiting their access to it.

In 1997, I reviewed what was then the draft of "Agenda 2000" (Alonso, 1997). Its optimistic estimate indicated that the reduction in Spain's sunflower sowing area could reach from 30 to 40%. The sunflower area planted in 2002 was 753,893 ha. i.e., a 32% reduction in the average area planted during the 1994-1998 period. The sunflower area fell further to 517,125 Ha in 2005 due to the EU policy and to the local drought conditions.

Seventh Stage: After 2005. On 26th June 2003, the EU farming ministers adopted a fundamental reform of the CAP, through the Council Regulation (EC) No 1782/2003, (Official Journal of the European Union, 2003a) based on "decoupling" subsidies from particular crops (though, as Spain did, the Member States could choose to maintain a limited amount of a specific subsidy). The new "single farm payment" is subject to 'cross-compliance' conditions relating to environmental, food safety and animal welfare standards. Many of these were already either good practice recommendations or separate legal requirements regulating farm activities. The aim was to make more money available for environment, quality, or animal welfare programmes.

The purpose of the single farm payment is to ensure income stability for farmers who are able to decide what they produce according to supply and demand. Thus, the EU has opted for less government influence and more of a market place drive for farmer's crop choice. These reforms came into force in 2004-2005. The Member States had the choice of applying for a transitional period delaying the reform in their country to 2007 and phasing the reforms up to 2012.

In 2004, after the expansion of the EU, the new EU member states had immediate access to price support measures (export refunds and intervention buying). However, direct payments will be phased over 10 years (2004-2013), starting at 25% of the rate paid to existing countries in 2004. The EU provided the 2004 entrants with access to a rural development fund for early retirement, environmental issues, poorer areas, technical assistance. The EU states agreed in 2002 that agricultural expenditure up to 2013 should not increase in real terms. This will require a cut in subsidies to the original states of around 5% to finance payments to the new members. With Romania and Bulgaria joining in 2007, the required cut will increase to 8%.

The sunflower area planted in Spain recovered in 2006 and 2007 from the 2005 lower yield. The 2007 recovery was moderated by the international increase in cereal prices at the end of 2006, when the oilseeds had relatively low prices. However, the price increase boost for vegetable oils in 2007 placed sunflowers as a very competitive alternative to cereals. This favourable situation is becoming better for sunflowers as the input prices for farming are also increasing very quickly, particularly fertilizers, and diesel. Thus, it bodes well for sunflower planting area recovery in Spain and the EU in the coming years.

Outlook on the international sunflower and oilseed situation.
During the second half of 2007, and up to the beginning of the new marketing year, prices in the oilseed complex have continued their pronounced rise, and vegetable oil prices have reached record high values (See Fig. 2).

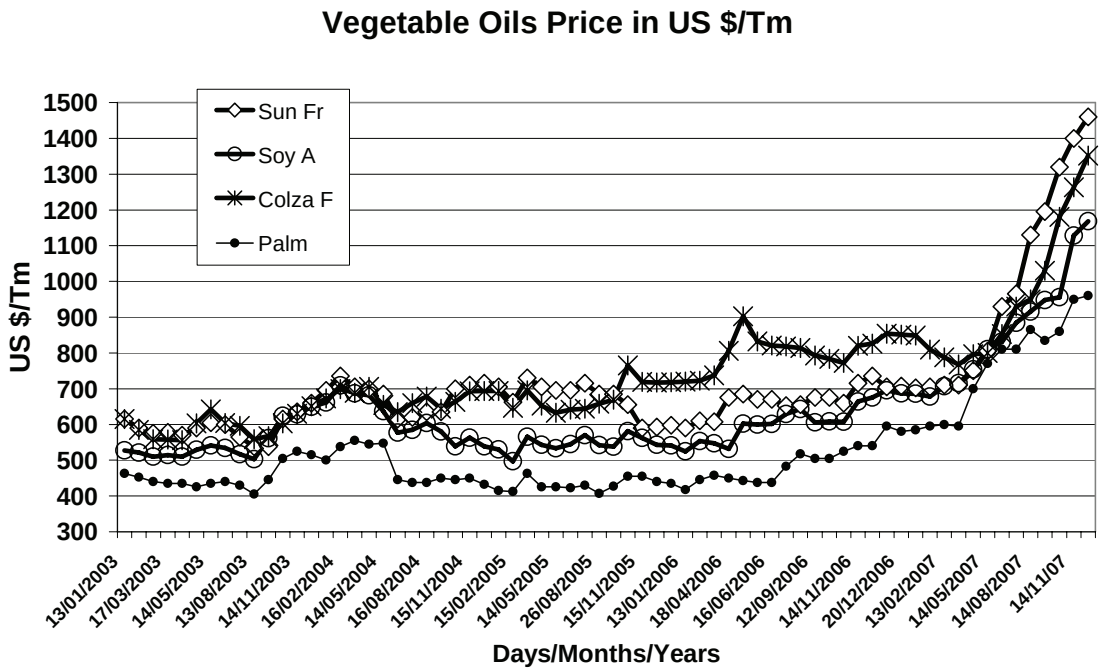

Fig. 2. Vegetable oil prices since 2003. Source: SOS. Elaborated from Oil World (2007)

According to the FAO outlook global market analysis (FAO, 2007), a key factor behind this price rise is that oil-crop markets have come under the direct influence of developments in the related feed grain market. The unprecedented rise in international maize prices has spilled over to the oilseeds and meal market, and, in particular, to the soybean complex.

World stocks and stock-to-use ratios of both oil and meals are falling to critical levels, because of a significant drop in oilseed production in 2007 (see Table 1), coinciding with a steady expansion in global demand for food, feed and energy use, thus calling for a steep reduction in inventories (See Table 2). The two main factors behind the drop in total oilseed output are: first, increased competition from grains, notably in the United States but also in China and CIS (Commonwealth of Independent States) countries, which has interrupted the steady expansion in the world oilseed area. Second, unfavourable weather conditions which have affected oilseed production in several key growing areas or countries, including the European Union, CIS, Australia, Canada, China, Turkey and the United States. The decline in production of soybeans and sunflowers has been responsible for the drop in oilseed production in 2007. Sunflower production in 2007/08 was 8.2% lower than in 2006/07. Sunflower production went down by 26.3% in the EU, 20.7% in Ukraine and 16.2% in Russia (see Table 3).

The present forecasts for global supply/demand in 2008 point towards a continued firmness in international prices for oilseeds and oilseed products. Furthermore, the growing biodiesel requirements have led to an increased demand for vegetable oils. This trend, combined with a constant rise in the consumption of vegetable oil as a food, has led to a gradual tightening in global supplies, thus explaining the recent rise in vegetable oil prices, which may continue during 2008.

Table 1. World production of major oilseeds

	2005/06	**2006/07 Prel.**	**2007/08 Proj**
	Million Tm		
Soybeans	220.59	237.27	219.85
Cottonseed	43.95	45.82	45.37
Rapeseed	48.74	46.80	47.62
Groundnuts (unshelled)	33.04	32.41	33.11
Sunflower	30.02	30.15	27.67
Palm kernels	9.98	10.27	11.11
Copra	5.50	5.28	5.37
Total	**391.82**	**408.00**	**390.10**

Source: USDA Marc 2008 (USDA;2008)

Note: The split years bring together northern hemisphere annual crops harvested in the latter part of the first year shown, with southern hemisphere annual crops harvested in the early part of the second year shown, For tree crops, which are produced throughout the year, calendar year production for the second year shown is used

In USA, the USDA January 2008 report (USDA, 2008) showed an unexpected sharp decline in corn ending supplies. In order to compensate for the corn shortage, 2.4 million acres of corn would be needed in the new plantings. On the other hand, soybean needs to gain between 8 and 10 million acres in 2008 to prevent ending supplies from dropping to dangerously low levels. But it was not just the USDA reports that gave markets a lift to new highs. There was also a continuous flow of fund money into all commodities, and this will continue during 2008. Poor weather conditions in South America's soybean growing areas, such as the warm, dry summer in Argentina, also had an effect on oilseed prices during February and March 2008.

Table 2. World oilseeds and product markets at a glance

	2005/06	**2006/07 *estim.***	**2007/08 *f'cast***
	Million Tm		
Total oilseeds			
Production	404	417	403
Vegetable Oils and Animal Fats			
Production	149	151	154
Supply (Production + Opening Stocks)	168	172	174
Utilization[1]	146	152	157
Trade[2]	72	76	79
Stock-to-utilization ratio (%)	14	13	11
Oil meals and cakes[3]			
Production	101	106	102
Supply (Production + Opening Stocks)	113	121	119
Utilization *	98	102	108
Trade *	55	59	62
Stock-to-utilization ratio (%)	15	17	11

Source: (FAO, 2007)

[1]Residual of the balance.

[2]Trade data refer to exports based on a common October/September marketing season.

[3]All meal figures are expressed in protein equivalent; meals include all meals and cakes derived from oilcrops as well as fish meal and other meals from animal origin.

Table 3. Sunflower seed; World supply and disappearance in 1000 t

	2002/03	2003/04	2004/05	2005/06	2006/07	2007/08F
Seed Production						
Argentina	3,700	3,240	3,600	3,800	3,500	4,500
Other Europe	749	876	867	830	850	490
European Union 27	5,183	6,155	6,463	5,958	6,483	4,772
Peoples' Republic of China	1,946	1,743	1,552	1,927	1,900	1,800
Russian Federation	3,685	4,850	4,800	6,450	6,750	5,650
Ukraine	3,270	4,252	3,050	4,700	5,300	4,200
United States	1,112	1,209	930	1,823	972	1,310
India	1,625	1,700	1,224	1,550	1,280	1,650
Turkey	820	600	650	750	850	700
Other	1,897	2,266	2,270	2,290	2,294	2,599
TOTAL	**23,987**	**26,891**	**25,406**	**30,078**	**30,179**	**27,671**
Seed Import						
Turkey	229	660	529	345	408	380
European Union 27	705	1,066	413	627	572	400
Other	451	442	192	316	663	294
TOTAL	**1,385**	**2,168**	**1,134**	**1,288**	**1,643**	**1,074**
Seed Export						
Argentina	213	46	107	45	70	200
United States	166	170	141	178	181	166
Russia/Ukraine	517	1239	57	616	484	365
Other	571	852	918	783	1,219	752
TOTAL	**1,467**	**2,307**	**1,223**	**1,622**	**1,954**	**1,483**
Area Harvest (1000 Ha)	**20,202**	**23,287**	**21,369**	**23,117**	**23,841**	**22,790**
Yield Tm/Ha	**1.19**	**1.15**	**1.19**	**1.30**	**1.27**	**1.21**

The US has passed a new energy bill adding a more bullish sentiment to the corn and vegetable oil markets. The new energy bill also raises the corn-based ethanol mandate.

Reduced growth in global oils/fats supplies and an unprecedented fall in meal supplies, because of a significant drop in oilseeds production in 2007, are expected to coincide with a steady expansion in global demand for food, feed and energy use, thus calling for a severe reduction in inventories. World stocks and stock-to-use ratios of both oil and meals have fallen to critical levels.

The biofuel impact

Another favourable situation for oilseeds in general, was the impact when the European Commission brought forward the legislative proposals that were adopted in 2003 in the form of the bio fuels directive (Directive 2003/30/EC of the European Parliament and of the council of 8 May 2003) (Official Journal of the European Union, 2003b) and article 16 of the energy taxation directive of the Council Directive 2003/96/EC of 27 October 2003, (Official Journal of the European Union, 2003c).

The biofuels directive expressed the clear intention of *"promoting the use of biofuels… in each Member State, with a view to contributing to objectives such as meeting climate change commitments, environmentally friendly security of supply and promoting renewable energy sources"*. It included an interim target for 2005 and a target for 2010 of a 2% and 5.75%, respectively, share of the market for petrol and diesel. These indicative targets, once adopted, were not mandatory, but they constituted a moral commitment on behalf of Member States.

During the 2007 Spring EU Council, Europe's Heads of State agreed on the 3 targets for 2020: A binding target of 20% renewable energy (RES) by 2020 and a separate binding target of 10% for biofuels; A 20% energy efficiency target (EE) and 30% greenhouse gas (GHG) reduction target. These three targets have laid the foundations for renewable energy sources to become a major pillar of the EU future energy supply.

Table 4. European Biodiesel production capacity growth 2003-2007.

Country	Production Capacity in 1000 t				
	2003 EU15	**2004 EU 15**	**2005 EU 15**	**2006 EU 25**	**2007 EU 27**
Germany	1,025	1,088	1,903	2,681	4,361
Italy	420	419	827	857	1,366
France	500	502	532	775	780
UK	5	15	129	445	657
Spain		70	100	224	508
Greece			35	75	440
Belgium			55	85	335
Austria	50	100	125	134	326
Poland			100	150	250
Portugal			6	146	246
Sweden	8	8	12	52	212
Czech Republic			188	203	203
The Netherlands			0	0	115
Slovakia			89	89	99
Denmark	41	44	81	81	90
Romania					81
Bulgaria					65
Lithuania			10	10	42
Estonia			10	20	35
Hungary			0	12	21
Latvia			5	8	20
Slovenia			17	17	17
Malta			2	3	8
Ireland			0	0	6
Cyprus			2	2	6
Total EU	**2,049**	**2,246**	**4,228**	**6,069**	**10,289**

Source EBB, Situation at 01/07/2007 (EBB,2007)

The new 10% minimum target in 2020 (The impact of a minimum 10% obligation for biofuel use in the EU-27 in 2020 on agricultural markets) has also been seen to be relative to the existing legislation which put the target at 5.75% in 2010. The current directive may fail to produce the incorporation of 5.75% in 2010 due to the market and technologies having little time to react.

Since 2003, biodiesel has proved to be a significant demand shifter in the overall vegetable oil industry. The confluence of environmental concerns, high energy prices and government incentives has led to a significant increase in the biodiesel production capacity in the EU and worldwide. In 2007, there were 185 fully operational biodiesel plants, in Europe and another 58 plants were under construction. Thus, the EU biodiesel production capacity reached 10.2 million tonnes (see Table 4). In Spain in 2007 there were 23 fully operational biodiesel plants and 26 plants under construction, with a joint production capacity of 921,000 and 2,961,200 Tm/year, respectively. Furthermore, there were another 24 projects for a joint production capacity of 2,692,000 Tm/year (See Table 5).

This huge EU biodiesel production capacity risks remaining idle and the production stagnating or declining, the same as during the 2nd half of 2007. The record high vegetable oil prices at the end of 2007 and beginning of 2008 is making many operations unviable. To make things worse, the US Federal subsidies since 2004 (up to \$264/m^3 i.e., \$ USA 300/Tm ~ €200/tm) biodiesel blends tend towards increasing biodiesel imports in the EU. The US "B99" blend (a blend of a small amount of mineral diesel with biodiesel) exported to the EU, benefits the blender's credit as this is not restricted to biodiesel produced and consumed on the US territory. Thus, "B99" exports to the EU were boosted in 2007 as in most cases B99 blends are sold in the European market as "pure biodiesel" and at a substantial discount

(over €120-180/tonne), in some cases at a lower price than that of the raw materials purchased by the EU industry for producing biodiesel.

Table 5. Biodiesel number of plants in Spain in 2007 and their production capacity in 1000 Tm

	Operational		**In Construction**		**In Project**	
Province	**Number**	**Capacity**	**Number**	**Capacity**	**Number**	**Capacity**
A Coruña			1	200	1	103
Alava	1	30				
Alicante	1	20			1	200
Almeria	1	6	1	6		
Asturias	1	4			2	270
Badajoz			2	360		
Baleares	1	16				
Barcelona	1	31				
Burgos	1	8	1	49		
Cadiz			1	200	3	330
Cantabria			1	155		
Ceuta					1	250
Ciudad Real	1	32	1	100	1	110
Cordoba			2	7		
Cuenca	2	122	1	50		
Girona	1	5				
Granada					1	80
Huelva			2	400		
Huesca	1	50	3	102		
Jaen	1	100	1	200		
La Rioja			1	250		
Las Palmas			1			
León					3	310
Lleida					1	110
Lugo					1	20
Madrid	1		1	45		
Murcia					1	140
Navarra	1	70			2	124
Pontevedra			1	300		
Sevilla	2	86	1	60	1	300
Tarragona	1	50			2	80
Teruel					2	115
Toledo	3	156				
Valencia	1	110				
Valladolid			1	70		
Vizcaya			2	400	1	150
Zamora	1	20	1	7		
Total Spain	**23**	**921**	**26**	**2,961**	**24**	**2,692**

Source (Biodieselspain,2007): Situation 31/12/2007

The new US biodiesel mandate could eventually require as much as one-third of total US vegetable oil production, assuming no vegetable oil imports to the US. Eventually, all the biodiesel produced in the States may remain there, leaving more room for the EU biodiesel factories to produce EU biodesel needs.

The Latin American biofuel industry is headed by Brazil's mature ethanol industry. Brazil will also have included mandatory B2 and B5 by 2008 and 2013, respectively, including tax breaks. Argentina also has a B5 project for 2010.

In Asia, in several southeastern countries, indicative B2 or B5 targets are gradually moving to mandatory targets. Malaysia has a B5 mandatory by 2010; Indonesia B5 by 2025; Thailand B10 by 2012 and Philippines B2 by 2009. China has no concrete biodiesel policy but is targeting 15% use of biofuels by 2020; South Korea has a mandatory B5 blending implemented in 2006 and India is preparing legislation on biodiesel to support cultivation and commercial activities of *Jatropha*-based biodiesel.

According to Rabobank reports on biodiesel (Hansen, 2006; Tan, 2007), the biodiesel production could have a considerable impact on global vegetable oil demand in 2010. Considering the 2005 vegetable oil production of 96 million tonnes, the vegetable oil demand for biodiesel production and the extra food demand may require another 18 million tonnes and 13 million tonnes, respectively, in 2010.

As was recently affirmed by Miriann Fischer Boel, the European Commissioner responsible for Agriculture and Rural development in a speech during the 2008 World Biofuels Market Congress in Brussels (Fischer Boel, 2008) the EU policy on this subject, despite being controversial, has a solid justification and everyone in the sector can be confident that no policy u-turns lie ahead. Biofuels must be a part of the future of sustainable energy production in the EU for two reasons: the fight against climate change, where biofuels are an important weapon, and energy security against future supply problems. In this speech, Ms. Fischer Boel, gave the answer to various objections raised recently against biofuels and in these answers we can find the clue to the next direction of EU policy. The first objection is that using first-generation biofuels in many cases supposedly does not cut down greenhouse gas emissions. It is true that some biofuels do not show clear benefits, but biodiesel made from European-grown rapeseed makes a greenhouse gas saving of 44 per cent compared to fossil fuels. The typical figure for ethanol made from sugar beet is 48 per cent. Under the rules proposed by the Commission, a given biofuel would count towards a Member State's target only if it made a greenhouse gas saving of at least 35 per cent compared to fossil fuels, which is a very healthy difference. And the standard applies both to domestic production and to imports. When calculating this saving, the EU proposes to take into account the value of by-products such as animal feed. The second objection to the 10 per cent target is that it will mean destructive land conversion. The Commission recognises these dangers and has proposed the following: no biofuel would count towards a Member State's usage target if it does not meet strict sustainability criteria. For example, this would exclude biofuel coming from either land with a high biodiversity value or land with high carbon stocks.

As regards the basic argument, that more biofuel means painfully high prices, and, therefore, less food for the poor, Ms Fischer Boel says, that it is not fair to make biofuel a scapegoat for the extreme market movements of recent times. According to the OECD, cereal use for ethanol in Europe, North America and Asia increased by 17 million tonnes in 2006. But, in the same year, the combined cereals supply shortage in these countries was 60 million tonnes – nearly four times as much. Clearly, this was not just a “biofuel story”.

In a 2006 study on agricultural market growth impacts on the production of biofuel, two scenarios were considered. In one of them, referred to as “High oil price scenario” with sustained crude oil prices at US $ 60/barrel, the summary conclusion was:

With sustained higher crude oil prices, there are two main forces at play that affect world markets for agricultural commodities. First, due to higher agricultural production costs that lead to lower quantities of production, commodity prices increase. At the same time, higher oil prices – and higher oil-based fuel pump prices – increase incentives to produce more biofuels (even though partially dampened by higher feedstock prices), which creates an additional demand for feedstock products. Again, this causes prices of agricultural commodities to increase.

Whatever the reasons behind the current very high vegetable oil prices, it is clear that these are limiting the potential use of biodiesel for economic reasons. On the other hand, the present crude oil prices above US $100/barrel, and the equally high values for protein meals are improving their profitability, in particular for integrated industrial facilities that can produce either vegetable oils for food or for biodiesel.

With the large demand for vegetable oils for biodiesel production and keeping in mind the mandatory uses of biofuels in many countries, we can expect that this industry will serve to prevent the oilseed market prices falling below the threshold prices at which the production cost of biodiesel is equal to the domestic tax-free diesel prices.

Sunflower oil quality traits and food and non-food market trends

High oleic sunflowers have been developed from the sunflower variety Pervenets obtained by Soldatov in 1976 (Soldatov, 1976). High oleic sunflower oil is specialty oil with high oleic acid (monounsaturated)

levels. It must be at least 77% monounsaturated to meet product descriptions but often a level of above 80% is required by the industry.

Most of the world's vegetable oils and fats show a mixed fatty acid composition and their physical-chemical properties, as well as their physiological-medical benefits, are fixed in the fatty acid composition (Table 6). In triglycerides, the hydroxyl groups of a glycerol molecule are chemically bound to different fatty acids, varying in chain length and/or in number and position of C-C double-bonds, which cause bends in the C-C chain. In high-oleic oils, such as high-oleic sunflower oil, all properties are strongly dominated by its high content of oleic acid (C18:1) and low polyunsaturated fatty acid (PUFA).

Table 6. Differences in physicochemical characteristics between the two types of sunflower oil

Characteristic	**High oleic sunflower**	**Conventional Sunflower**
Fluidity	Liquid +	Liquid +
Resistance to heat	Good +	Average -
Resistance to oxidation	Good +	Average -
Effect on Cholesterol; LDL (Bad)	Reduces +	Reduces +
Effect on Cholesterol; HDL (Good)	No Change +	Reduces -

LDL is atherogenic; HDL is antiatherogenic

This combination of fatty acid content offers many advantages:

- For nutritional purposes: no *trans* fatty acids; low saturated fatty acid content; reduction in "bad cholesterol"; GMO free.
- For all uses: high oxidative stability, reduced rancidity; extended shelf life
- In processing: It is liquid ant thus easy to transport and to store.

High oleic sunflower oil offers advantages not only for the food industry but also for the biodiesel industry. The European Standard requirements set out in regulation EN 14214 (European Standard prEN 14214, 2002) defined 25 parameters for fatty acid methyl esters for bio-diesel. Among them, the iodine value is set at 120. While conventional sunflower oil has an iodine value of about 137, high oleic sunflower has about 87. This allows to reduce the iodine values in mixtures with other vegetable oils such as soybean, whose iodine value is around 133.

The development of high oleic sunflowers was limited in the US during the 1980's and 1990's as a consequence of two patents (Fick, 1984; Fick, 1985), which ended in 2005. In Europe and South America, there was only a limited interest in some industries, which triggered a special production of this type of sunflower in Spain, France, Italy and Argentina. Thus, farmers received a premium price of between 10 and 30 percent depending on market demand with wave fluctuations, and high oleic sunflower oil had a from 20 to 40 percent higher price than conventional sunflower oil.

The NuSun or mid-range oleic sunflower, whose oleic acid levels vary from 50 to 70 percent, was introduced in 1999 into the U.S. as a response to U.S. food processors' desire for a vegetable oil low in saturated fatty acids and suitable for fast-food frying applications without hydrogenation. In 1999, the U.S Food and Drug Administration (FDA) proposed regulations that changed the Nutrition Facts label to allow consumers to identify the amount of *trans* fat in a food by listing the grams of *trans* fat. Later, the U.S.A. FDA published a final rule that amended its regulations on food labeling requiring *trans* fatty acids to be declared on the nutrition label of conventional foods and dietary supplements (68FR41434). This was effective January 1, 2006. The NuSun sunflower planting area grew very quickly in the US, and by 2004 it was already the dominant oil sunflower grown in the States, and in 2007 it reached about 90% of the oil type sunflower grown in this country. The high oleic type was grown in about 6%. Thus, high and mid oleic sunflowers constitute about 96% of US oilseed sunflowers (Kleingartner, 2004; Kleingartner, personal communication).

In the EU, the *trans* fatty acid concern is also causing an impact on the food industry. In 2002, the Health Council of the Netherlands recommended that *trans* fat should be limited to 1% of calories. In a report on dietary reference intakes, the Health Council of the Netherlands recommended, among other things, that *trans* fat should be limited to one percent of calories. In 2003, the Danish government announced that, as from January 2004, the amount of *trans* fat from partially hydrogenated oil would be limited to 2% of the total amount of fat or oil in the food. Despite the initiatives being taken for an EU regulation on *trans* fats, different industries have positioned their products in line with the concept of "free of *trans* fatty acid". Some industries have chosen the use of natural saturated oils, such as palm oil, or total hydrogenation of unsaturated vegetable oils, which does not cause *trans* fatty acids, aiming to

separate the concepts: “trans fatty acids” and “hydrogenation” (FEDIOL, 2006). In some cases, high oleic sunflowers has been chosen either pure or in blends with other vegetable oils. i.e., in 2003, SOS Cuetara, in Spain changed the formulation of fats in their cookies to reduce *trans* fatty acid content to below 0.5% of the oil fraction. They developed a special spread blend branded as Oleosan containing a large proportion of high oleic sunflower oil. This was achieved by blending high oleic sunflower oil with other vegetable oils. The Spanish fish canning industry also announces in TV the use of “high oleic” oils as premium quality.

As a consequence of the continuous demand increase of high oleic oils, the high oleic area planted in some European countries has grown rapidly during the last four years, while in others their presence is only testimonial. In 2007, the West European countries grew high oleic sunflowers in about 26% of the 1,349,400 sunflower-planted hectares. Two countries, France and Spain, were responsible for most of this (Fig. 3). In Eastern Europe, the high oleic area represents less than 3% of the sunflower area planted and only Hungary grew a significant 17% of its sunflower area with high oleic varieties.

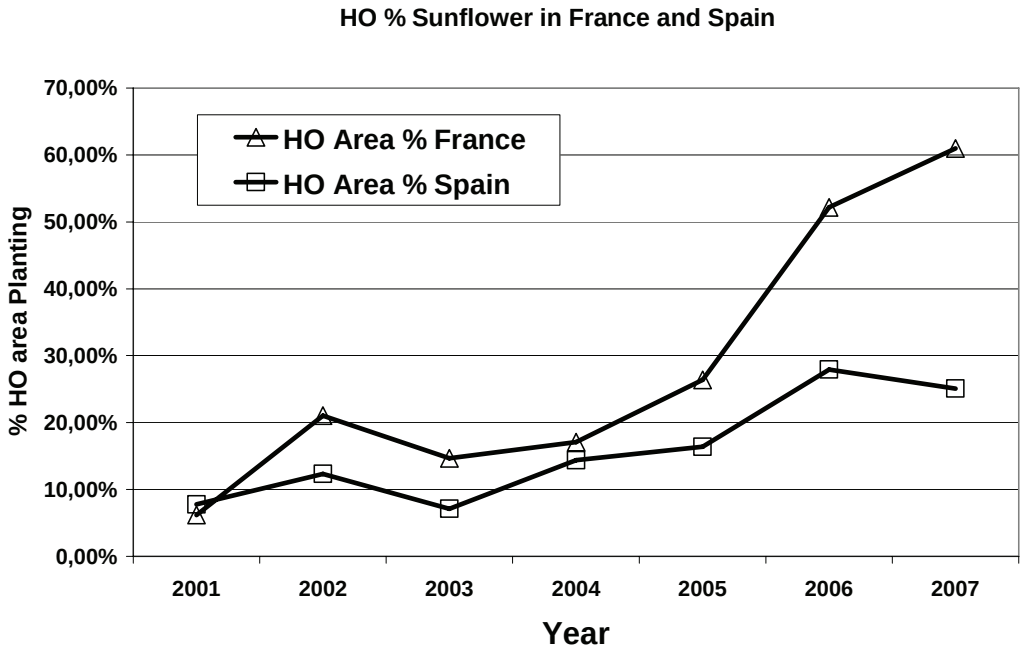

Fig. 3. High Oleic sunflower area planting growth in Spain and France in percentage of area planted.

The cosmetics and lubricant industries may also increase their use of high oleic sunflowers in the future. The EU uses more than 5 million tons per year of lubricants. This sector is dominated by mineral oils and only 2% is of vegetal origin. Besides its technical aspects as driving forces, the regulatory framework of the EU is also increasingly favouring the environment- friendly, readily biodegradable oil. Due to its high oxidative stability, high oleic sunflower oil is favourably compared to other natural oils as base oils or additives in lubricants. Compared to mineral oil-based lubricants, bio-lubricants still face disadvantages in product costs. Great perspectives are in store for very high oleic sunflower oil as it performs like synthetic esters (e.g. TMP-trioleates), but is somewhat lower in price (Vannozzi, 2006).

The continuous growth of high oleic sunflowers may be limited by two factors: the present sunflower oil prices and the lack of sunflower hybrids with the newest trait demands, such as herbicide tolerance, and/or different disease resistances.

Regarding the present very high sunflower oil prices, in order to make a contract with farmers and to preserve the identity of the seed in the whole factory supply chain, a premium has to be paid both to farmers and collectors. The amount of this premium can be either a fixed amount or a percentage of the commodity sunflower seed price. A fixed premium of 24 €/Tm of seed considered to be highly attractive by farmers two years ago, is not very encouraging in the present market situation.

The recent development in Europe, and worldwide, of herbicide-tolerant (HT) sunflowers, which allow the use of broad spectrum post-emergence herbicides is also segmenting the market very fast. The

development of performing HT and high oleic sunflower hybrids is also limiting the expansion of high oleic sunflowers.

In a similar way, the expansion of new races of well known diseases, such as Broomrape race F, caused by *Orobanche cumana*, also limits the high oleic sunflower potential area until performing high oleic and *Orobanche* resistant sunflower hybrids are developed.

Herbicide tolerant sunflowers: The new market development

Recently, two herbicide-tolerant sunflower types have been introduced into the market for weed control with post-emergence specific herbicide treatments. These sunflower hybrids provide growers with a post-emergent control option, without killing the crop. Broadleaf weeds, both annual and perennial, are the leading weed spectrums affecting sunflower yields but grasses and volunteer cereals may also be important. In the past, the lack of choice in weed control forced many sunflower farmers to rely on more expensive, less effective pre-emergent options that often did little to eliminate weed competition losses in their sunflower fields. The recent ban in the EU of the pre-sowing herbicide Trifluralin is making the use of herbicide-tolerant sunflowers and post-emergence herbicide weed control more attractive. Thus, both systems are spreading very quickly.

Clearfield Sunflower production system

The CLEARFIELD Production System for sunflowers is a unique production system comprised of non-GMO herbicide-tolerant sunflower hybrids and a herbicide of the Imidazolinone chemical family. At present, the herbicide used in sunflowers is imazamox, which is a member of the herbicide family of AHAS or ALS inhibitors. Some members of this family control susceptible weeds by inhibiting the acetohydroxyacid synthase (AHAS) enzyme also called acetolactate synthase (ALS). Several variant AHAS genes conferring imidazolinone tolerance were discovered in plants through mutagenesis and selection, and were used to create imidazolinone-tolerant maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.), oilseed rape (*Brassica napus* L.), and sunflower (*Helianthus annuus* L.). These crops were developed using conventional breeding methods and commercialized as CLEARFIELD® crops from 1992 to the present. While the Clearfield system is available for several different field crops, including corn, wheat, rice, and canola, the "IMI" chemical formulations are not interchangeable. Clearfield sunflower is not cross-tolerant to the sulfonylurea (SU) family of herbicides.

In 1996, a wild sunflower population of *Helianthus annuus*, highly tolerant to the herbicide imazethapyr, was found by Al-Khatib in a soybean field in Kansas, U.S.A (Lilleboe, 1997). The Agricultural Research Service plant geneticist Jerry Miller learned of Al-Khatib's wild sunflower collection from John Nalawaja, a colleague at North Dakota State University-Fargo, and requested seed specimens. Their goal was to transfer resistance to cultivated sunflower. This prospect was very exciting because the list of broadleaf weeds and grasses controlled by imazethapyr herbicide and other herbicides of the imidazolinone chemical family (IMI) was extensive. Also, for sunflower to spread into no-till acreage, planting herbicide tolerant hybrids was the only alternative for postemergence weed control. In 2002, Miller and colleagues released two germplasm lines (USDA lines HA 425 and RHA 426) of imazamox (the new BASF IMI herbicide) -tolerant sunflower for commercial seed companies to use in developing their own hybrids (Miller and Al-Khatib, 2002). In 1997, I collected from the Kansas farm owned by Doug French the same wild sunflower population collected earlier by Al-Khatib and, back in Spain, Koipesol's technical team proved that this herbicide tolerance offered an excellent opportunity for the chemical control of the parasitic weed *Orobanche cumana* Wallr. (Alonso et al., 1998). *Orobanche* chemical control is not race specific and may serve both to prevent the parasitic plant to spread to new areas and to control it in already infected areas.

Clearfield sunflowers, which are registered for use only with imazamox herbicide (Beyond™ in U.S.A., Pulsar® 40 in Europe) manufactured by BASF Corporation represented the first real post-emergence option for controlling key problem weeds. Imazamox herbicide provides contact and residual activity on a number of grasses and broadleaf weeds as well, including nightshade, pigweed, foxtail species, wild oats, volunteer cereals, puncturevine, non-Clearfield wild or volunteer sunflower and broomrape.

The Clearfield Production System for sunflowers started in 2003, in the U.S., when the Environmental Protection Agency registered Beyond™ herbicide. In Europe it has been introduced into different countries since 2002 and Syngenta Seeds was the first seed company marketing Clearfield sunflower hybrids.

Express Sunflowers production system

Express Sunflowers was introduced by Pioneer Hi-Bred International, Inc., and DuPont Crop Protection in 2006 in some European countries, and in 2007 in the U.S. This system facilitates a broad spectrum weed control option for sunflower growers: Sunflower hybrids with the DuPont™ ExpressSun™ trait which provides tolerance to Express® herbicide. This herbicide is a sulfonylurea. Sulfonylureas are a powerful family of herbicides discovered by DuPont in 1975 and first commercialized for wheat and barley crops in 1982. Sulfonylureas comprise a family of compounds which kill broadleaf plants by blocking the plant enzyme acetolactate synthase (ALS), an enzyme important to the plant for the synthesis of some amino acids (leucine, isoleucine and valine)

The Express Sunflowers production system only offers broad leaf postemergence weed control while the Clearfield sunflowers production system offers postemergence control of many weeds such as; broad leaf, grasses and volunteer cereals as well as broomrape control.

The expansion of herbicide tolerant sunflower and future development

Herbicide-tolerant (HT) sunflowers gain a market share very quickly once the HT traits are incorporated into performing hybrids. Often their expansion has been limited by the lack of planting seed. In some cases the sunflower area planted with HT hybrids is above 25% in only three or four years after the introduction (Table 7). This tendency is going to continue as more performing hybrids will be available and it will not be long before most of the sunflower grown will be HT.

It is more difficult to forecast which of the two methods will dominate in the market and most probably both will be present. Currently, in some countries the Clearfield method represents nearly 100% of the HT planted area. This is the case of Turkey, Spain and Serbia. In other cases, Clearfield and Express methods are both spreading but keeping to about 50% each of the total HT market in the country. Finally, in some cases, the Express method represents 75% of the HT market, for instance in Bulgaria. These differences may be caused by the different dates of introduction of each method into the different countries. Thus, we shall still have to wait a few years before we see the real share of each in the market. However, it is reasonable to think that in the areas where broomrape (*Orobanche cumana*) is a problem, the Clearfield solution will probably dominate. Also, the Clearfield method could be a better option in those cases where sunflower is rotated with cereals in areas where cereals are repeated one to three years before planting sunflower, so that the grasses could create a weed problem.

Table 7. Percentage of sunflower area planted with herbicide-tolerant production systems (Clearfield and Express) in Argentina and Europe

Argentina	**Year of introduction**	**2002/3**	**2003/4**	**2004/5**	**2005/6**	**2006/7**	**2007/8**
Bs. As. (SO, SE y Oeste)	2002/3	2%	3%	9%	15%	27%	25%
S. Córdoba	2002/4	1%	2%	7%	21%	14%	21%
E. La Pampa	2002/5	1%	7%	12%	23%	22%	13%
Europe	**Year of introduction**	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**
Turkey	2003	3%	10%	23%	25%	21%	24%
Bulgaria	2006	0%	0%	0%	9%	18%	33%
Romania	2005	0%	0%	1%	4%	9%	16%
Hungary	2006	0%	0%	6%	7%	14%	17%
Serbia	2006	0%	0%	4%	15%	5%	11%
Slovakia	2006	0%	0%	0%	0%	0%	6%
Russia	2006	0%	0%	0%	0%	1%	1%
Ukraine	2006	0%	0%	0%	1%	2%	3%
Spain	2003	0%	0,2%	0,2%	0,2%	1%	2%
France	2009	0%	0%	0%	0%	0%	0%
Croatia	2009	0%	0%	0%	0%	0%	0%
Czech	2009	0%	0%	0%	0%	0%	0%
TOTAL EUROPE		0%	0%	1%	2%	3%	6%

Source. Syngenta Seeds Marketing department

Both methods offer a tremendous potential for weed control and thus favour sunflower yield, particularly for the spreading of sunflower into no-till acreage as well as in very early planting in Mediterranean countries.

In several sunflower planting date studies made in Cordoba, Spain, during the 1980's, by the CIDA research group it was shown that sunflower planted during the winter (December and January) yielded about 30% more than when planted in March.

For some years, the Andalusian government sunflower trial network RAEA (Red Andaluza de Experimentación Agraria) planted, in the same location, sunflower hybrids at the end of January and in March in three years, 1994, 1998 and 2000. A number of common hybrids, (i.e., 32, 13 and 12 hybrids) were used both in winter and spring planting trials in 1994, 1998 and 2000, respectively (RAEA, 1994, 1998, 2000). The average yield for the three years in the nine trials (Fig. 4) was 2,069 kg/ha for the winter planting date and 1,694 kg/ha for the spring planting date. i.e., 375 kg/ha average difference. While in some locations the difference was minimal (near the coast and in very low yielding areas) the difference reached a peak of 911 kg/ha in the central part of Andalusia. Thus, the potential increase of yield due to early planting seems to be related to the flowering escape of these plantings from the extremely hot days that often occur in early June.

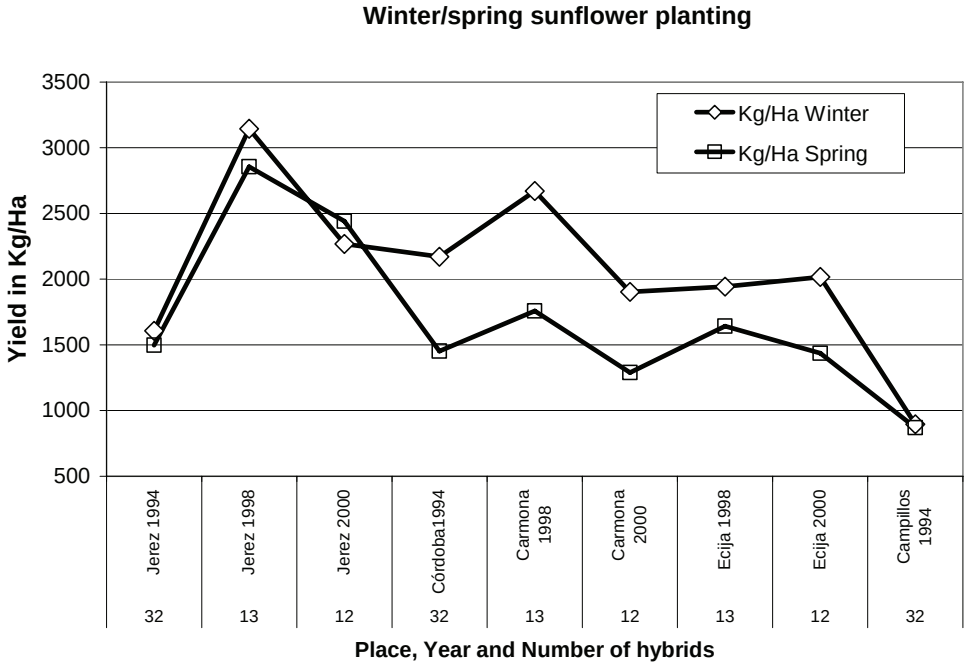

Fig. 4. Average sunflower yields of the same set (number) of sunflower hybrids planted in winter and spring in nine trials during three years. Source: RAEA (1994, 1998, 2000).

This potential yield increase was threatened by bird damage and herbicide damage from hormonal treatments in neighbouring cereal fields and weeds. The slow growth of sunflowers during January and February favoured the weed infection. There was no effective postemergence herbicide treatment for sunflowers at that time. The introduction of Clearfield sunflowers is allowing early planting in Andalusia with an effective control of *Orobanche* and weeds

CONCLUDING REMARKS

The sunflower planting area in Spain and other EU member states has been influenced in the past by political decisions and the CAP. This influence became less and less important through the successive CAP modifications. Yet, the recent political decisions at global levels on biodiesel production and use are again impacting on the prices of all oilseed oils.

The present high oilseed oil prices may be more related to production deficits than to biodiesel consumption. Sunflower oil use for biodiesel production is not significant. However, the present potential production capacity and the short term projected increase certainly may have an impact on preventing the

oilseed oil prices from going down. Even if sunflower oil remains mostly as food oil in the near future, the biodiesel demand may impact on all oilseed oil prices including sunflower oil.

A high oilseed oil price is an incentive to increasing production, but cereal prices are also very high causing a competition for land among crops. High transport costs as a consequence of high crude oil prices and high fertilizer prices will also play a role in the farmer's choice of what crops to plant. Sunflowers may be favoured in this complex equation, as it is an easy-to- grow crop which uses deep soil fertilizers which every year escape from cereals. Additionally, the low test weight of sunflower seed will encourage more and more local crushing. i.e., few seed exports/imports.

The niche high oleic sunflower market may become dominant in the next years at least in Western Europe as has happened in the U.S with the NuSun high oleic sunflower oil may also be of interest to the growing bio-lubricant industry and for some blending in bio-diesel production.

In the present oilseed oil price situation, and with high input costs, it is reasonable to think that sunflower area may grow again in Western Europe as well as in several ex-URSS countries. Weed and *Orobanche* control with HT-tolerant sunflowers offers an excellent opportunity to increase sunflower yield in different countries and lead this regrowth. This is particularly important for early planting dates in hot Mediterranean countries giving North African countries an excellent chance to increase their sunflower production.

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Phomopsis control in sunflower using products of biogenic origin

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ABSTRACT

The regulation of growth and development in sunflower plants was studied. Resistance to Phomopsis and productiveness of sunflower plants were induced by treating seeds with a disease resistance inducer of elicitor nature based on chitosan together with plant growth regulators.

Key words: albite – chitosan – disease resistance inducer – growth regulator – Phomopsis – zircon.

INTRODUCTION

As the world literature indicates and also our data show, the fungus *Diaporte (Phomopsis) helianthi* (Munt.-Cvet et.al.), which affects both sunflower and wild plants, possesses a high infection potential that contributes to its expansion and injuriousness in Russia and other countries in which sunflower is cultivated. The available domestic and international sets of commercial and promising newly developed sunflower cultivars and hybrids are susceptible to this disease. Therefore, Phomopsis remains a problematic disease both for Russia and for other countries.

Within the current arsenal of pest control agents in crops, including those against Phomopsis in sunflower, chemical fungicides continue to be of priority. Research conducted in Yugoslavia, Romania, France, Russia and other countries demonstrated that the available range of chemical fungicides does not reduce the infection and, therefore, the effectiveness of protective measures remains low (Piven et al., 1997; Chaban, 1990).

In this connection, research on inducing the resistance based on strengthening the natural defense mechanisms of plants has very good prospects for developing environmentally safe and high-yielding crop production technologies. In fact, the concept of induced resistance appeared together with the purpose of using induced immunity in practice. The most important prerequisite to considering induced resistance as an actual phenomenon includes the fact that the defense reactions are triggered as a response to infection both in resistant and susceptible plants (Tyuterev, 1999).

Both synthetic and natural biologically active compounds may be used as disease resistance inducers. Among the latter, most attention is currently being paid to chitin and its derivative chitosan (Reunov, 2001; Reddy et al., 1999). For the first time, high activity of chitosan in protecting plants was demonstrated by L.A. Hadwiger in 1986. He determined that the seed treatment with chitosan protects the plants from fungal and bacterial diseases and increases their yields by an average of 20% (Hadwiger, 1989). Even in the recent past, the mechanism of the chitosan action on plants was practically unknown. Nowadays, convincing data have been obtained showing high effectiveness and revealing mechanisms of the chitosan action on biological objects. Some publications (Begunov et al., 2004). reported an effect of the induced biosynthesis of chitinases and chitisanases, that resulted from treating plant cells with chitosan; they showed very high resistance against plant-pathogenic fungi and bacteria. It was determined that not only the cells, but also the plants of rice, pea, carrot and other crops sprayed with chitosan synthesized pectins along with chitinolytic enzymes and their joint action led to the complex cell wall destruction of plant pathogens that prevented their invasion of plants. Long-term tests of chitosan under laboratory and field conditions conducted by Russian research institutes (All-Russian Research Institute of Biological Plant Protection, Krasnodar and All-Russian Research Institute of Plant Protection, St. Petersburg) showed positive results by using the models including root rot pathogens in cereals, vegetables, and rice and mildew in cucumber and other crops. The highest plant protection effect of using chitosan was produced due to seed treatments (Begunov et al., 2002).

Before our work started, no references to a complex application of disease resistance inducers and plant growth regulators against Phomopsis in sunflower were available in literature. Therefore, the work

proposed by us that includes studying growth and development regulation, Phomopsis resistance induction and increase in sunflower yields by using the sunflower seed treatment with a disease resistance inducer of an elicitor nature based on chitosan, is especially important.

MATERIALS AND METHODS

In 2006 research was conducted using two sunflower cultivars: a susceptible cultivar Rodnik - growing season duration: 80 days, potential yield: 3.2 t/ha, oil content: 55%; and a tolerant cultivar Master - growing season duration: 94 days, potential yield: 4.0 t/ha, oil content: 54%. The experiment layout, as well as phenological, phytopathological and biometric studies were designed according to conventional methods (Begunov et al., 2004). The plot area was 100 m^2, three replicates and randomized block design were used. The experiment plots were planted on May 15. The soil of the plots included deep low-humic leached chernozem. The humus level in the arable soil layer was 3.4-4.1 %, pH_{salt} was 6.5, pH_{water} was7.5. Winter wheat was a predecessor.

The experiment included the use of a biogenic resistance inducer based on chitosan – chitosanium glutaminium succinate - in the mixture with phytohormonal plant growth regulators based on hydroxycinnamic (zircon), polybetabutyric (albite), heteroauxinic (IAA) acids and trace elements of chelate form (hydromics). Hydromics, zircon, chitosanium glutaminium succinate are recommended in the Russian Federation to be used for sunflower seed treatments, while albite and IAA are new experimental plant growth regulators for sunflower plants.

The tested disease resistance inducer and plant growth regulators were applied as different combinations for the seed treatments of both sunflower cultivars a day before planting. The product Maxim (active ingredient: fludioxonyl) was used as a standard. A treatment without the application of the above products served as control. Experiment design, applied compositions and application rates are shown in Table 1.

The progress of Phomopsis in sunflower plants was evaluated using the VNIIMK scale.

Table 1. Experiment design

#	Experimental treatment	Rate of application, kg,l/t of seeds
1	Chitosanium-glutaminium succinate + zircon	0.2 + 0.2
2	Chitosanium-glutaminium succinate + phloroxan + IAA + zircon	0.2+0.0005 + 0.006+0.2
3	Chitosanium-glutaminium succinate + albite	0.2 + 0.1
4	Chitosanium-glutaminium succinate + phloroxan + IAA + albite	0.2 + 0.0005 + 0.006 + 0.1
5	Chitosanium-glutaminium succinate + hydromics	0.2 + 0.15
6	Chitosanium-glutaminium succinate + phloroxan + IAA + hydromics	0.2 + 0.0005 + 0.006 + 0.15
7	Maxim (standard)	5.0
8	Control	Untreated

Biological effectiveness was calculated with the formula:

$$B = \frac{Pc-Pt}{Pc} \times 100\ \%$$

where B – biological effectiveness, %;
Pc – Phomopsis progress in control;
Pt – Phomopsis progress in an experimental treatment.

The sunflower was harvested with a Sampo combine.

The agrometeorological conditions during the sunflower growing season were rather favorable both for sunflower growth and Phomopsis progress. The hydrothermic coefficient for the growing season was 1. The mathematical data processing was done using the variance analysis method.

RESULTS

Research on determining biological parameters of the chitosanium-glutaminium succinate application against Phomopsis showed that the highest disease resistance induction of 56-60 % was caused in sunflower plants as a result of treating their seed, *i.e.* when the defense mechanisms were launched at the

early plant ontogeny stages. The optimum rate of application was 0.2 kg/t of seeds (Begunov et al., 2004). In addition, it was shown that chitosan has good hydrophylic property, complex formation ability, film-forming capacity, absence of toxicity and broad-spectrum biological activity.

All these characteristics of chitosan provided a stimulus to search for the possibilities to enhance its biological activity, add new useful properties to this polymer and create its novel compositions with non-phytotoxic biologically active compounds.

The sunflower seed treatments using chitosanium-glutaminium succinate with zircon, IAA, albite and hydromics stimulated germination and activated initial growth and development of plants. For the Rodnik cultivar, the root growth stimulation reached 170% (Treatment 6) and the stem growth stimulation reached 140% (Treatment 1). For the cultivar Master, the maximum root and stem stimulation values were recorded in the Treatments 2 and 1 and they reached 150 and 119%, respectively. On the whole, the growth-stimulating process at the first ontogeny stages was more active in the early-ripening cultivar Rodnik than in the late-ripening cultivar Master (Table 2).

Table 2. Evaluation of the growth-stimulating action on sunflower plants produced by the tested formulations under field conditions

Treatment #	Cultivar Rodnik Germination on the 14th day				Cultivar Master Germination on the 14th day			
	Root (cm)	Percentage of control	Stem (cm)	Percentage of control	Root (cm)	Percentage of control	Stem (cm)	Percentage of control
1	9.3	137	22.0	140	9.4	130	17.5	119
2	8.7	128	19.7	125	10.7	150	16.4	111
3	9.6	140	19.8	125	9.0	120	17.0	116
4	8.5	125	19.3	120	10.5	140	15.4	105
5	9.4	138	19.3	120	10.5	140	16.3	110
6	11.6	170	18.2	115	10.5	140	16.3	110
7	8.5	125	17.7	110	10.5	140	16.0	110
8	6.8		15.8		7.3		14.7	
Control								
HCP_{05}	0.55		1.34		0.50		0.68	

Our further phenological observations showed that the sunflower plants of both cultivars formed the second pair of true leaves in the treatments where the tested compositions had been applied two or three days earlier than in the standard (Maxim) and control treatments. Also, the accelerated budding and flowering stages were recorded for the plants whose seeds had been treated with the tested compositions.

Effects of the compositions of the tested disease resistance inducer and plant growth regulators were tested under natural conditions at the field. It should be noted that the weather conditions were especially favorable for the progress of Phomopsis at all the developmental stages of the plants, from germination to flowering. For that period, 21 rainy days were recorded with 250 mm of total rainfall. The disease incidence and severity were evaluated at the budding, flowering and physiological ripeness stages.

The first symptoms of the Phomopsis leaf form were detected in the control plot at the budding stage on June 20 for the cultivar Rodnik and on June 29 for the cultivar Master. By July 29, 6% of the sunflower plants of Rodnik and 4% of Master had been affected by Phomopsis. The stem form of Phomopsis appeared in the plants of Rodnik on July 10 and in the plants of Master on July 24.

Table 3 shows the results of the evaluation of Phomopsis stem symptoms at the physiological ripeness stage. The cultivar Rodnik showed 42.3% of diseased plants, with 23.6% of disease severity; for the cultivar Master these values were 26.7 % and 12.2 %, respectively.

The data analysis showed that the combined application of chitosanium-glutaminium succinate togeteher with IAA, phloroxan, zircon, and albite produced the most effective protective action on plants. Induced biological effectiveness in these experimental treatments (2 and 4) was 71-73 % for Rodnik and 81-88 % for Master. Therefore, it was shown that plant growth regulators such as albite and zircon in combination with chitosanium-glutaminium succinate strengthened the defense mechanism of sunflower plants against Phomopsis.

Among the tested plant growth regulators, the seed treatments with the combination of disease resistance inducer and zircon (Treatments 1 and 2) and albite (Treatments 3 and 4) contributed to obtaining higher yields from both cultivars.

Table 3. Biological and economic effectiveness of the tested formulations

Treatment	Rodnik				Master			
	Incidence (%)	Severity (%)	Biological effectivenes s (%)	Yield (t/ha)	Incidence (%)	Severity (%)	Biological effectiveness (%)	Yield (t/ha)
1	16.2	12.1	49	2.19	12.9	5.9	52	2.21
2	8.7	6.3	73	2.15	8.5	2.3	81	2.11
3	12.0	8.0	73	2.20	5.2	4.9	52	2.10
4	10.3	6.9	71	2.04	5.2	1.5	88	2.11
5	9.3	6.1	74	1.95	7.9	3.5	71	2.02
6	12.1	8.4	64	2.10	14.7	5.3	57	2.06
7	14.9	16.2	31	1.90	26.0	11.2	8	1.85
8 (Control)	42.3	23.6	-	1.89	26.7	12.2	-	1.76
HCP_{05}				0.083				0.085

DISCUSSION

The combined application of the tested biogenic disease resistance inducer and phytohormonal plant growth regulators albite and zircon enhances the Phomopsis resistance of sunflower plants and increases their yields.

Thus, this area of research on improving disease resistance in plants without changing their genome is a connecting link between fundamental immunology and practical crop protection. In this connection, using the products based on chitosan is very promising, and the induced resistance caused by them may be considered as one of the biological methods for disease control.

ACKNOWLEDGEMENTS

This research was supported by the ISTC Grant number 3034.

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Verticilosis en germoplasma de girasol

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RESUMEN

La verticilosis, causada por el agente patógeno *Verticillium dahliae* (Kleb), es una de las enfermedades más importantes que afecta al cultivo de girasol en Argentina. Se evaluó el comportamiento frente a *V. dahliae* de líneas de girasol del programa de mejoramiento de la EEA. Pergamino de INTA. La evaluación se realizó en 689 líneas aplicándose el método de inoculación artificial del patógeno en plántula. En la escala de severidad empleada se consideró: R: resistente, MR: moderadamente resistente, MS: moderadamente susceptible, S: susceptible y AS: altamente susceptible. De acuerdo al origen genético, se formaron 33 grupos, calculándose el porcentaje de líneas en cada una de las reacciones. La mayor amplitud de comportamiento se detectó en el compuesto P4. Se obtuvo mayor porcentaje de lecturas R en líneas provenientes de endocría de poblaciones mejoradas que en la descendencia de cruzas de líneas de diferentes orígenes. La combinación de las cruzas locales x exóticas, produjo mayor proporción de líneas de buen comportamiento que ambos grupos por separado. La resistencia a *Verticillium* puede ser obtenida a partir de diferentes fuentes de germoplasma.

Palabras clave: girasol - recursos genéticos - resistencia a enfermedades - *Verticillium* - Verticilosis.

ABSTRACT

Verticillium wilt , caused by pathogen *Verticillium dahliae* (Kleb), is one of the principal diseases of sunflower (*Helianthus annuus* L.) in Argentine. The objective of this research was to evaluate lines of the sunflower breeding program of E.E.A. Pergamino to *Verticillium* reaction. Seedling inoculation method was applied to evaluate the performance of 689 lines. The scale was R (resistant), MR (moderately resistant), MS (moderately susceptible), AS (very susceptible). According to their genetic background, 33 groups were formed and the reaction percentage in each group was calculated. The largest variability was detected in P4. A larger percentage of resistance lines was obtained from inbred lines derived from improved populations than from populations derived from crossing inbred lines of different origins. Lines derived from crosses of the type local x exotic performed better than both groups separately. Resistance to *Verticillium* can be obtained from different sources of germplasm

Key words: disease resistance – genetic resources – sunflower – *Verticillium* – Verticillium wilt.

INTRODUCCIÓN

La verticilosis, causada por el hongo *Verticillium dahliae* Kleb, se manifiesta inicialmente por un marchitamiento foliar ocasionado por el taponamiento de los tejidos de conducción que progresa desde la raíz. Se visualiza inicialmente en las hojas inferiores y luego en las superiores, posteriormente se puede observar áreas cloróticas internervales y necrosis (Zimmer and Hoes, 1978). Se afecta el rendimiento por reducción del peso de granos y el contenido de aceite (Bertero de Romano et al., 1994; Pereyra et al., 1999). Es una enfermedad de gran incidencia económica por lo cual la obtención de cultivares de buen comportamiento es una meta prioritaria en los programas de mejoramiento de la especie en Argentina (González et al., 2003).

El objetivo del presente trabajo fue evaluar el comportamiento frente a este patógeno de líneas del programa de mejoramiento de girasol de la EEA Pergamino INTA, provenientes de distinto origen genético.

MATERIALES Y MÉTODOS

En la E.E.A. Pergamino INTA se analizó la reacción de 689 líneas estabilizadas frente a *Verticillium dahliae* para evaluar la incidencia del patógeno. Se empleó el método de inoculación artificial en plántula en invernáculo (Bugbee y Presley, 1967) de alta correlación con la incidencia a campo. Se inocularon 20 plántulas de cada línea al estado de 3 hojas verdaderas (aproximadamente 20 días después de la siembra), Las lecturas se realizaron a las tres semanas de la inoculación con la siguiente escala: R (Resistente) sin

síntomas foliares, sana; MR (Moderadamente resistente) con áreas cloróticas; MS (Moderadamente susceptible) áreas cloróticas y necróticas; S (Susceptible) con predominio de manchas necróticas; AS (Altamente susceptible) con necrosis y deformaciones foliares. Los genotipos se agruparon por origen en 33 grupos en los cuales se calculó el porcentaje de líneas que tenia cada uno de los grupos en la escala de severidad descrita.

RESULTADOS Y DISCUSIÓN

En la Tabla 1 se presentan los resultados de la reacción frente al patógeno de las líneas derivadas de poblaciones y cruzamientos de líneas locales, exóticas, y locales por exóticas, en los 33 grupos (Anexo I: Descripción del germoplasma interviniente en la evaluación de *Verticillium*).

Tabla 1. Reacción frente al patógeno *V. dahliae* de las líneas derivadas de poblaciones y cruzamientos de líneas locales, exóticas y locales por exóticas

Origen	Reacción a *V. dahliae* (%)					
	Número de líneas	**R:** Resistente	**MR**: Moderada-mente resistente	**MS**: Moderada-mente susceptible	**S**: Susceptible	**AS**: Altamente susceptible
LINEAS LOCALES						
RK 489/AXB 3479	9	0	0	22	11	67
RK 456/BXC 3496	18	0	0	6	33	61
LXN 621/BXC3496	20	0	0	15	30	55
KLM 280/RK 489	17	0	6	24	11	59
KLM 280/GP 762	8	0	0	37	50	13
KLM 214/GP 762	3	0	0	0	67	33
GP 762/BXC 3496	39	0	0	10	18	72
GP 762/AXB 3479	15	0	6	47	27	20
DXT 3331/AXB 3479	5	0	0	0	40	60
BXC 97/01/KLM 214	10	0	10	20	50	20
BXC 97/01/DXT 3331	13	0	0	31	23	46
BXC 97/01/AXB 3479	28	0	7	11	46	36
RF 00/16	10	0	0	20	60	20
RF 00/01	10	0	0	30	70	0
RF 97/01	18	0	22	56	22	0
LINEAS EXÓTICAS						
ND 01	23	0	0	20	55	25
HA 89 x HAR 4	16	0	25	31	38	6
HA 301xCHERNY-66/	30	0	3	37	53	7
HA 337 / HA 335	21	0	0	48	47	5
HA 338/373 1x CHERNY-66	18	0	6	66	28	0
HA 343 x NOVINKA	10	0	0	10	70	20
LINEAS LOCALES POR EXÓTICAS						
LXN 621/HA 89	42	0	0	10	26	64
KLM 280/HA 822	28	0	0	22	39	39
HA 89/DXT 3330	4	0	0	100	0	0
AxB 3479-2-2-1/ DxT 3331-3-1-2/HA 300	11	9	36	32	23	0
LxN 621 / KLM 280/HA 300	48	0	8	23	67	2
RK 426-11 /KLM-280/HA 300	31	3	42	45	10	0
POBLACIONES						
Compuesto P2	12	0	0	17	58	25
Compuesto P3	9	0	0	44	44	12
Compuesto P4	15	7	13	20	33	27
Compuesto P6	5	0	0	20	20	60
VNIIMK 6540	66	0	18	58	24	0
VNIIMK 1646	77	22	51	19	8	0

Las mayores diferencias de comportamiento se dieron en las líneas derivadas del Compuesto P4 (de origen rumano, mezcla de Record, Sintética OS2 y Sintética Horizonte), y las menores en las líneas derivadas de la cruza de HA 89 / DXT 3330. Las líneas derivadas de las cruzas en que intervienen AXB 3479-2-2-1; DXT 3331-3-1-2 y HA 300 (derivada de Peredovik 301), tuvieron también alto porcentaje de

lecturas R y MR. Un comportamiento similar presentaron los genotipos originados en la cruza HA89 x HAR 4 (esta última línea originada a partir de Saenz Peña 74-1-2 de buena sanidad). Se obtuvieron también genotipos de buen comportamiento derivados de los cruzamientos entre RK 426-11, originada en el Compuesto RK y KLM 280, originada en el Compuesto KLM. Comparando las líneas obtenidas a partir de selección y endocría de poblaciones con los obtenidas a partir de cruzas entre líneas de distinto origen; se obtuvo mayor porcentaje de lecturas R explorando la variabilidad de las primeras (Tabla 1).

Comparando los grupos originados de líneas derivadas de locales con las de líneas exóticas y con la combinación de ambas; se obtuvo mayor porcentaje de líneas con resistencia en la combinación de ambas que en cada grupo por separado, destacándose AXB 3479/DXT 3331/HA 300 y RK 426-11/KLM 280/ HA 300. Se destaca la importancia de la variedad rusa VNIIMK 1646 como una fuente de resistencia a verticilosis, teniendo en cuenta el alto porcentaje de lecturas R y MR observado en líneas derivadas de la misma, siguiéndole en aptitud el Compuesto P4.Los resultados indicaron una amplia capacidad de respuesta en el fondo genético analizado. En consecuencia, sería posible obtener resistencia genética al patógeno a partir de fuentes de diverso origen.

Anexo I. Descripción del germoplasma interviniente en la evaluación de *Verticillium*

Designación	Origen genético / derivada de:
Locales (1)	
AxB 00/01	71/538, LC 206020
BxC 00/01	LC 206020, MP 555 (Rusa, silvestres)
BxC 97/01	LC 206020, MP 555 (Rusa, silvestres)
DxT 00/01	MP 557, Negro Bellocq
DxT 00/02	MP 557, Negro Bellocq
GP 762	Primeras líneas del programa de EEA Pergamino
KLM 214	Compuesto KLM (Klein, Local, Manfredi)
KLM 280	Compuesto KLM (Klein, Local, Manfredi)
LxN 621	Compuesto LxN (Local x Ruso)
Rf 00/16	A 871
Rf 00/01	M 731-243
Rf 97/01	S 3107
RK 426-11	Compuesto RK (Ruso, Klein)
RK 456	Compuesto RK (Ruso, Klein)
RK 489	Compuesto RK (Ruso, Klein)
Exóticas (2)	
CHERNY-66	Chernianka
HA 300	Peredovick 301, North Dakota, 1976
HA 301	North Dakota, 1976
HA 335	North Dakota, 1986
HA 337	North Dakota, 1986
HA 338	North Dakota, 1986
HA 343	North Dakota, 1986
HA 89	North Dakota, 1971
HA R 4	North Dakota, 1984 Saenz Peña 74-1-2
ND 01	North Dakota, 1984 Alto oleico
Novinka	Variedad rusa
Poblaciones	
Compuesto P2	6 B; Ienissei
Compuesto P3	Comangir (silvestre, cultivado)
Compuesto P4	Compuesto rumano: Record, Sintética OS2, Sintética Horizonte
Compuesto P6	Precoz, alto, aceite, Americano
VNIIMK 1646	Variedad rusa
VNIIMK 6540	Variedad rusa

(1) Locales: Obtenidas en INTA-EEA Pergamino; (2) Exóticas: No obtenidas en la EEA Pergamino

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Towards *Sclerotinia* resistance – *In vitro* screening of wild sunflower species

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ABSTRACT

This paper presents the work on testing the possibility of the use of *in vitro* screening for determination of wild *Helianthus* species resistance to *Sclerotinia*. For this purpose, micropropagated plants of different accessions of *H. maximiliani*, *H. mollis*, *H. rigidus* and *H. tuberosus* were grown on MS medium supplemented with 0, 0.5, 1 and 2 mM of oxalic acid. Fresh and dry weight of above-ground part, and dry weight of root could be considered as the potential parameters of wild species resistance/susceptibility to *Sclerotinia* in *in vitro* tests, as they were not affected by treatment in resistant (100%) accessions and were significantly decreased in susceptible (25%) ones in the presence of 2 mM of oxalic acid.

Key words: oxalic acid – *in vitro* screening – resistance – *Sclerotinia sclerotiorum* – wild sunflower.

INTRODUCTION

White rot caused by the fungus *Sclerotinia sclerotiorum* Lib. (de Bary) is the major disease of sunflower (*Helianthus annuus* L.) in countries with a humid climate, while in countries with moderate climate, it causes yield losses in rainy years (Škorić and Rajcan, 1992). Wild sunflowers (*Helianthus* spp.) constitute an important source of resistance against several major sunflower diseases including *Sclerotinia* (Georgieva-Todorova, 1993). Populations of several wild sunflower species were found to be tolerant to white rot (Škorić and Rajcan, 1992; Henn et al., 1997; Tavoljanski et al., 2002; Cerboncini et al., 2002; Vasic et al., 2004). Resistance screening was done either by observing naturally occurring infection (Tavoljanski et al., 2002) or by using different artificial inoculation methods (Henn et al., 1997; Cerboncini et al., 2002; Vasic et al., 2002; 2004).

De Bary was the first researcher to associate oxalic acid with *Sclerotinia* infection (Lumsden, 1979). Later, Noyes and Hankock (1981) demonstrated its importance as a factor in the pathogenicity of this fungus, while Hartman et al. (1988) found a correlation between oxalic acid production and virulence of different *Sclerotinia* isolates. There have been several attempts to create a bioassay in which resistance to oxalic acid would be used as an indicator of resistance to *Sclerotinia* (Hartman et al., 1988; Noyes and Hancock, 1981; Raducanu and Soare, 1992; Tu, 1985; Vasic et al., 1999; 2002). Whole plants or their parts were used, and correlation was found between field susceptibility/resistance of tested genotypes to *Sclerotinia* and reaction of the explants of the same genotypes when grown on a medium into which oxalic acid was added.

As maintenance of wild species collection and field screening are costly and labour-intensive, we have tested the possibility of the use of *in vitro* screening for determination of wild sunflower species resistance to *Sclerotinia*.

MATERIALS AND METHODS

Accessions of *H. maximiliani* Schrader (max), *Helianthus mollis* Lam. (mol), *H. rigidus* (Cass.) Desf. (rig) and *H. tuberosus* L. (tub) were obtained from wild *Helianthus* species collection of Institute of Fields and Vegetable Crops in Novi Sad, Serbia (Table 1). The accessions were pre-screened for *Sclerotinia* resistance by measuring sclerotia infection on stem (Vasic et al., 2004). Their resistance was determined as the percentage of healthy plants (Table 1).

The plants were propagated *in vitro* using culture of apical shoots (Vasic et al., 2001). Prior to transfer to a propagation medium, shoots were dipped into 0.1% indolebuteric acid (IBA) solution for 4 min. For the resistance screening, apical shoots of *in vitro* grown plants were placed in 250 ml Erlenmeyer flasks with 80 ml of MS medium (Murashige and Skoog, 1962), pH 5.7, supplemented with 5 g l^{-1} of sucrose, 6 g l^{-1} of agar, and different concentrations of oxalic acid (Table 1). Control plants were

grown on MS medium without oxalic acid. There were four Erlenmeyer flasks with four shoots per accession for each oxalic acid concentration. One Erlenmeyer flask was treated as one replication in the data analysis. The shoots were grown at 24ºC with a photoperiod of 16 h (light)/8 h (dark).

After six weeks of culture, the following parameters were measured: plant height, fresh and dry weight of above-ground part, root length, fresh and dry weight of root. The data were analysed using ANOVA and LSD test.

RESULTS AND DISCUSSION

Analysis of variance showed that both genotype and treatment had significant effect on the measured parameters.

Table 1. Reaction of tested wild sunflower accessions on treatment with different concentrations of oxalic acid[1,2].

Genotype	Resistance (%)	Concentration mM	h	rl	fm	dm	rfm	rdm
mol x	100	Control	10.875a	3.925a	0.476a	0.049a	0.478a	0.039a
		0.5	4.625b	5.225a	0.208b	0.0185b	0.132b	0.011c
		1	3.550b	3.650a	0.183b	0.019b	0.340ab	0.011c
		2	4.075b	5.075a	0.395a	0.040a	0.296ab	0.025b
		$LSD_{0.05}$	2.750	2.022	0.151	0.014	0.342	0.009
		$LSD_{0.01}$	3.855	2.835	0.212	0.020	0.479	0.014
mol 1298	100	control	15.200a	11.200a	0.293ab	0.030b	0.088ab	0.006b
		0.5	9.325c	2.750c	0.153c	0.022b	0.059b	0.004b
		1	13.675ab	7.525b	0.253bc	0.026b	0.117ab	0.007ab
		2	10.950bc	8.525ab	0.387a	0.042a	0.146a	0.010a
		$LSD_{0.05}$	3.328	2.732	0.113	0.011	0.062	0.003
		$LSD_{0.01}$	4.666	3.829	0.159	0.015	0.087	0.005
max 34	75	control	14.700a	11.875a	0.492a	0.048a	0.183a	0.013a
		0.5	12.425ab	3.550c	0.299b	0.025b	0.048b	0.004b
		1	11.775b	3.575c	0.277b	0.030b	0.074b	0.006b
		2	4.800c	6.550b	0.338b	0.048a	0.245a	0.017a
		$LSD_{0.05}$	2.862	2.812	0.139	0.016	0.067	0.004
		$LSD_{0.01}$	4.012	3.942	0.195	0.023	0.094	0.006
max 1631	50	control	15.400a	18.775ab	1.493a	0.105a	0.749a	0.048a
		0.5	16.225ab	14.650b	0.945a	0.066a	0.274b	0.016b
		1	14.100ab	22.325a	1.503a	0.104a	0.884a	0.051a
		2	11.825b	18.675ab	1.058a	0.077a	0.416ab	0.025ab
		$LSD_{0.05}$	3.836	4.436	0.737	0.047	0.473	0.028
		$LSD_{0.01}$	5.377	6.219	1.034	0.066	0.664	0.039
tub 675	50	control	12.600a	11.325a	0.690b	0.025c	0.257b	0.016a
		0.5	10.425a	11.175a	1.018a	0.054b	0.364a	0.021a
		1	8.600a	10.700a	0.512b	0.078a	0.201bc	0.029a
		2	2.600b	4.450b	0.134c	0.043bc	0.154c	0.048a
		$LSD_{0.05}$	4.535	4.291	0.259	0.021	0.101	0.048
		$LSD_{0.01}$	6.358	6.016	0.363	0.030	0.142	0.067
rig 1692	50	control	13.625a	12.850a	0.312b	0.030b	0.249b	0.020b
		0.5	14.750a	12.025a	0.486a	0.053a	0.380a	0.034a
		1	14.375a	10.700a	0.257b	0.025b	0.197b	0.014b
		2	11.500a	11.675a	0.259b	0.026b	0.173b	0.015b
		$LSD_{0.05}$	5.003	5.082	0.108	0.009	0.056	0.006
		$LSD_{0.01}$	6.697	5.880	0.159	0.015	0.085	0.008
mol 1530	25	control	12.175a	4.150a	0.678b	0.048b	0.215b	0.016b
		0.5	12.350a	3.475a	0.728b	0.070ab	0.497ab	0.042ab
		1	13.525a	4.050a	1.359a	0.115a	0.926a	0.067a
		2	5.350b	3.925a	0.626b	0.055b	0.314b	0.031ab
		$LSD_{0.05}$	2.239	1.290	0.623	0.050	0.483	0.038
		$LSD_{0.01}$	3.140	1.809	0.873	0.070	0.677	0.053
rig 1843	25	control	11.125a	6.575ab	0.529b	0.062b	0.473b	0.047b
		0.5	8.975a	6.350ab	0.448b	0.059b	0.372b	0.041b
		1	7.950ab	7.500a	0.781a	0.089a	0.835a	0.083a
		2	4.800b	4.825b	0.530b	0.063b	0.346b	0.038b
		$LSD_{0.05}$	3.466	$LSD_{0.05}$	0.180	0.022	0.258	0.025
		$LSD_{0.01}$	4.860	$LSD_{0.01}$	0.252	0.031	0.362	0.035

[1]Within each column, genotype means followed by different letter differ significantly at the level p=0.05.
[2]Legends for traits: h - plant height, rl - root length, fm - fresh weight of above-ground part, dm – dry weight of above-ground part, rfm - fresh weight of root, rdm - dry weight of root.

The choice of oxalic acid concentrations was made based on the research done on cultivated sunflower protoplasts (Vasic et al., 1999) and intact plants grown *in vitro* (Vasic et al., 2002). Results obtained with 0.5 and 1 mM concentrations of oxalic acid were not conclusive as there was neither any difference between resistant and susceptible accessions nor a regular pattern in measured parameter variation (Table 1). This is in accordance with the results obtained on the sunflower plants grown in the presence of oxalic acid (Vasic et al., 2002). The same applies in the reaction of tolerant accessions (*H. maximiliani* 1631, *H. tuberosus* 675 and *H. rigidus* 1692) to oxalic acid treatment, and is probably the consequence of differences in morphology and biochemistry between wild sunflower species (Heiser et al., 1969).

Concentration of 1 mM of oxalic acid had a stimulant effect on the most susceptible accessions – *H. mollis* 1530 and H. r*igidus* 1843 (Table 1). Stimulant effect of non-selective concentrations of stress agents in *in vitro* culture was also observed in the experiments with herbicides, and is thought to be a consequence of the phenomenon that stress agents when present in small concentrations act as nutrients (Olofsdotter et al., 1994).

Similarly to the work of Vasic et al. (2002), concentration of 2 mM of oxalic acid discriminated between resistant and susceptible genotypes. This oxalic acid concentration only affected plant height in the resistant accessions (*H. mollis* x and 1298) and almost all the traits in susceptible ones, except for the root length in *H. mollis* 1530 (Table 1). This is in disagreement with the results of Mouly (1989) who found that concentrations of oxalic acid lower than 4.44 mM were not selective in a bioassay with sunflower leaves. The same author recommended a concentration of 8.88 mM as optimal.

Fresh and dry weight of above-ground parts and dry weight of root could be considered the potential parameters of wild sunflower resistance/susceptibility to *Sclerotinia* in *in vitro* tests, as they were not affected by treatment in resistant accessions and they were significantly decreased in susceptible ones in the presence of 2 mM of oxalic acid (Table 1). In contrast to the results obtained in cultivated sunflower (Vasic et al., 2002), plant height and root length were not good indicators of wild sunflower resistance/susceptibility to *Sclerotinia*. This may be due to structural differences between cultivated and wild sunflowers and their biochemical reaction to *Sclerotinia*, as previously observed in *H. resinosus* Small (Mondolot-Cosson and Andary, 1994).

The results obtained in our study showed that there is potential for the use of oxalic acid bioassays for screening wild sunflower species for resistance to *Sclerotinia*. Fresh and dry weight of above-ground parts and dry weight of root were found to be good morphological parameters for discrimination between resistant and susceptible accessions, in combination with an oxalic acid concentration of 2 mM. However, more work should be done in determining the optimal oxalic acid concentration. Also, the morphological and biochemical differences between different sunflower species should be taken into account in further studies.

ACKNOWLEDGEMENTS

This work was supported by Ministry of Science, Republic of Serbia.

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Correlation between macronutrient content and sunflower resistance to *Sclerotinia sclerotiorum* measured by sclerotia infection of stem

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ABSTRACT

Nutrition of plants has a substantial impact on the predisposition of plants to be attacked or affected by pests and diseases. Since availability and nutrient quantity in the host plants is a limiting factor for *Sclerotinia sclerotiorum* infection development in sunflower, the aim of this work was to determine macronutrient content in photosynthetic tissue (leaves) of sunflower plants before and after development of fungus infection, and to find the correlation between macronutrient content and resistance to sclerotium infection. The study was carried out on eight sunflower inbred lines. Macronutrient (N, P, K, Ca and Mg) content was determined in dry plant material of control and sclerotium infected plants. There was a high positive correlation between N content in infected plants and resistance, which points to the important role of this nutrient in sunflower defence from *Sclerotinia* attack. Moderate negative correlation between K and Ca content in control plants showed that the content of these nutrients in healthy plants could be used as an indicator of cultivated sunflower resistance/susceptibility to *Sclerotinia* infection.

Key words: macronutrients – resistance – *Sclerotinia sclerotiorum* – sunflower.

INTRODUCTION

Nutrition of plants has a substantial impact on the predisposition of plants to be attacked or affected by pests and diseases. By affecting the growth pattern, the anatomy and morphology and particularly the chemical composition, the nutrition of plants may contribute either to an increase or decrease of the resistance and/or tolerance to pests and diseases (Krauss, 2001).

White rot, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary and *S. minor* Jager, is the most important sunflower (*Helianthus annuus* L.) disease in the regions with moderate climate (Masirevic and Gulya, 1992; Vasic et al., 2004). The nutrition of *Sclerotinia* during all stages of disease development is probably the most important factor in determining the extent of infection development (Lumsden, 1979). Smirnova (1967) determined that, for normal development of *Sclerotinia*, all nutrients are needed (N, S, K, Mg, and Fe), while Purdy and Grogan (1954) observed that growth and sclerotium formation only occur when the inorganic macronutrients P, K, Mg and S are present in the medium. Availability and nutrient quantity in the host plants is a limiting factor for *Sclerotinia* infection development in sunflower (Acimovic, 1998).

Because of the importance of nutrients for *S. sclerotiorum* infection development, the aim of this work was to determine the nutrient content in photosynthetic tissue (leaves) of sunflower plants before and after development of fungus infection, and to find the correlation between nutrient content and resistance to sclerotium infection.

MATERIALS AND METHODS

Six cms inbred lines (PR-ST-3A, CMS_3-8A, PH-BC_1-40A, Ha-48A, Ha-74A, and CMS_1-50A) and two restorer lines (RUS-RF-OL-168 and RHA-583), all selected in Institute of Field and Vegetable Crops, Novi Sad, Serbia, were used in the study.

The experiment was set in two variants - SC_1 – non-infected control, and SC_2 – stem infection with sclerotium. In each variant, four rows with 12 plants per genotype were sown. Plants in SC_2 plot were inoculated by incorporation of sclerotia into the middle part of the stem, at the stage of bud appearance (E4). Wounds with sclerotia were covered with wet cotton and aluminium foil as described by Vasic et al. (2002). After the infection, plants in both variants were irrigated three times a week, for three hours.

Screening was done at the stage of physiological maturity (M0), and resistance was determined as percentage of healthy plants.

Leaf samples for physiological analyses were taken 28 days after the inoculation in both SC_1 and SC_2 variants. In both cases, three healthy leaves from the upper part of the plant were taken from 10 plants from the inner rows. In SC_2 variant, leaves were taken only from the plants showing symptoms of the white rot. Macronutrient (N, P, K, Ca and Mg) content was determined in dry plant material.

Nitrogen content was determined by the Kjehdal method (Nelson and Sommers, 1973) and phosphorus content spectrophotometrically by the ammonium molybdate-vanadate method (Gericke and Kurmies, 1952). Leaf samples were dry-ashed and dissolved in 25% HCl and analyzed for potassium content by flame photometry and for calcium and magnesium content by atomic absorption spectroscopy.

Resistance of genotypes to sclerotia infection was correlated with macronutrient content in infected and non-infected plants in order to estimate their relationship.

RESULTS AND DISCUSSION

Results obtained showed that there was a difference between tested genotypes in resistance to *Sclerotinia* sclerotium infection. The most resistant genotype was Ha-48A (>90%), and the most susceptible ones were RUS-RF-OL-168 (60.0%) and Ha-74A (60.0%). However, all tested genotypes could be considered tolerant since they all had resistance over 50% (Table 1).

Table 1. Macronutrient content in tested sunflower inbred lines.

Genotype	Variant	Resistance (%)	N	P	K	Ca	Mg
			mg/100g				
PH-BC_1-40A	Control		3820	246.7	3223	3069	1250.0
	Infection	75.0	3582	313.0	3210	3299	1157.0
Ha-48A	Control		4520	295.7	3478	2873	758.7
	Infection	93.9	4425	254.3	3112	2883	1043.0
CMS_3-8A	Control		3854	288.0	2916	2921	1299.0
	Infection	77.8	3310	293.7	2969	2572	1265.0
PR-ST-3A	Control		3691	307.0	3576	2893	866.7
	Infection	73.7	3834	249.0	2914	2745	1075.0
CMS_1-50A	Control		4065	266.7	3646	2796	856.7
	Infection	60.6	3888	273.7	3516	2623	950.0
RUS-RF-OL-168	Control		4133	284.0	4552	2991	887.7
	Infection	60.0	3385	253.0	3181	3694	1397.0
Ha-74A	Control		3093	323.7	3462	3103	1095.0
	Infection	60.0	3290	309.0	3620	3646	1271.0
RHA-583	Control		3412	290.3	4096	2359	619.3
	Infection	71.9	3643	268.7	3645	2132	580.3

Nutrient content in sunflower leaf tissue depended both on genotype and the presence or the absence of infection (Table 1). Genotype dependence of nutrient content in sunflower tissue was also observed by other authors (Saric et al., 1991; Vasic et al., 2001).

Nitrogen concentration in leaves of tested genotypes ranged from 4472 mg/100g (average for both variants – infection, control) in genotype Ha-48A, which was at the same time the one most resistant to sclerotium infection (93.9%), to 3192 mg/100g in genotype Ha-74A (Table 1). There was a low positive correlation between resistance and N content in leaves of control plants, and a high positive correlation between resistance and N content in leaves of infected plants (Table 2). This is not in accordance with the conclusions presented in the work of HuiLian (2004). This author connected increased susceptibility of field crop plants to pathogen attack with the increased nitrogen compound content in the plant. However, facultative parasites, such as *Sclerotinia*, require weak plants to infest and kill in order to survive (Marchner, 1995). Vigorous plant growth stimulated by ample N would suppress infestation by this group of pathogens. This may explain the differences in expression of plant diseases in relation to the nutrition of the host and N content.

Phosphorus content in leaves of control plants was in positive correlation with resistance (Table 2). This is in accordance with the results of Sindhan and Parashar (1996), who found that leaves of groundnut (*Apios americana* Medic.) cultivars resistant to *Cercospora arachidicola* contained more phosphorus than leaves of susceptible cultivars.

Potassium concentration in leaves of control plants ranged from 4552 mg/100g in genotype RUS-RF-OL-168 to 2916 mg/100g in genotype CMS_3-8A (Table 1). Although the results varied, a negative correlation between resistance and K content in leaves of the control, as well as in leaves of infected plants was found (Table 2). Correlation coefficients were moderate and low, respectively, but results clearly showed that a higher K content in leaves has as a consequently lower resistance to sclerotium infection. This is in contrast with the data obtained by Perrenoud (1990) concerning the relationship of K and plant health. This author reviewed some 2450 references and showed that in 70% of all quoted cases K induced a significant reduction in fungal disease incidences. However, in a more recent publication, Mondal et al. (2001) found a negative correlation between K content and disease incidence in soybean (*Glycine max* (L.) Merrill) and sesame (*Sesamum indicum* L.).

Table 2. Correlation of resistance of inbred lines to sclerotia infection with macronutrient content in infected and non-infected sunflower plants.

Macronutrient	Variant	Resistance (%)	Correlation coefficients
N	Control		0.1137
	Infection	75.0	0.5064
P	Control		0.2140
	Infection	93.9	-0.0554
K	Control		-0.3582
	Infection	77.8	-0.1485
Ca	Control		-0.3161
	Infection	73.7	-0.4121
Mg	Control		-0.1917
	Infection	60.6	-0.4040

There was a moderate negative correlation between Ca content in leaves of control and infected plants and resistance to sclerotium infection, meaning that plants with a higher Ca content were more susceptible to the infection (Table 2). This is in contrast with the results obtained by other authors regarding the role of Ca in sunflower resistance and reaction to *Sclerotinia* infection. Antonova et al. (1984) tested chemical composition of leaves and flowers of sunflower plants resistant and susceptible to *Sclerotinia sclerotiorum* and found that flowers of resistant plants contain more Ca.

Infection led to increase in Mg content in leaves of five genotypes (Table 1). Increases in Mg content in infected plants could be a consequence of chlorophyll degradation and disturbed processes of re-translocation and reutilization of Mg ions, since a pathogen attack causes chlorotic to necrotic changes on all plant parts, which are a consequence of chlorophyll degradation (Singh et al., 1998).

Similarly to the results obtained by other authors, nutrient content in leaves of tested sunflower inbred lines was genotype specific. It has also depended on the development of *Sclerotinia* infection, i.e. genotype resistance. There was a high positive correlation between N content in infected plants and resistance, which points to important role of this nutrient in sunflower defence from *Sclerotinia* attack. Moderate negative correlations between K and Ca content in control plants showed that the content of these nutrients in healthy plants could be used as an indicator of cultivated sunflower resistance/susceptibility to *Sclerotinia* infection. Further studies are in progress in order to find out more on the role of nutrients in sunflower response to *Sclerotinia* attack and to test the value of the results of this study on a larger number of sunflower genotypes.

ACKNOWLEDGEMENTS

This work was supported by Ministry of Science, Republic of Serbia.

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Races of *Plasmopara halstedii* on sunflower in separate agrocenosises of Adigeya Republic, Krasnodar and Rostov regions in Russia

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ABSTRACT

During the past four years, the occurrence of *Plasmopara halstedii* and the regional distribution of its pathotypes in some districts of Northern Caucasus and Rostov region were studied. More than 1000 isolates of the pathogen were collected in separate agrocenoses of Adigeya Republic, Krasnodar and Rostov regions in 2004-2007 period. A large-scale identification of these isolates was undertaken. Pathotype characterization, based on sunflower differential lines and evaluated according to a triplet code system, indicated the existence of at least seven pathotypes. Among them, race 330 was predominant. Races 710 and 730 dominated in some fields of Adigeya Republic and Krasnodar region. Races 100, 300, and 310 were only sporadically present. Race 700 was discovered in some fields of Krasnodar region, representing up to 11% of all isolates from these places. At present only races 330, 710 and 730 are economically important in the studied regions of Northern Caucasus.

Key words: downy mildew – *Plasmopara halstedii* – pathotypes ratio – races – sunflower.

INTRODUCTION

Downy mildew of sunflower caused by the pathogenic fungus *Plasmopara halstedii* (Farl.) Berl. et de Tony is a worldwide major disease of this crop. In a recent survey of pathogen race spreading, it was shown that more than 30 pathotypes of this Oomycete on sunflower were discovered in the World (Gulya, 2007). The quantity and composition of pathogen races vary in different countries and are the objective of study by leading phytopathologists (Masirevic, 1998; Molinero-Ruiz et al., 1998; Kormany and Viranyi, 1997; Penaud, 1998; Tourvieille de Labrouhe et al., 2000b; Shindrova, 2000, 2005; Rozynek and Spring, 2000; Shirshikar, 2005). Sunflower is the main oil crop in Russia. Adigeya Republic, Krasnodar and Rostov regions are the territories of intensive sunflower cultivation in Russia. Downy Mildew is one of the most potentially important sunflower diseases here, but for a long period of time a structure of *P. halstedii* population on sunflower in all of Northern Caucasus was a white spot on the world map of pathogen races distribution. This disease was first observed in Russia in the mid 1950s (Novotelnova, 1966). Successful development in this country of a hybrid *H. tuberosus* x *H. annuus* resistant to the disease and breeding based on its new open-pollinated varieties permitted to control the pathogen during three decades. The first information about the appearance of a new virulent pathotype of pathogen in Krasnodar region goes back to the beginning of the 1980s (Tihonov and, Zaichuk, 1981). At that time, the sunflower differential line HA-274 was resistant, but at that time both this differential line as well as the resistant varieties began to be infected. The investigations of Antonova et al. (2000) revealed the presence of races 100, 310 and 330 in Krasnodar region. The favourable weather conditions for disease appearance in 2004-2007 permitted to collect an ample collection of pathogen isolates (about 1000) in different districts of Northern Caucasus, which was preserved at a temperature of -80^{o}C, The aim of this study was to identify these isolates and to determine the race ratio in separate agrocenosises by the use of an international method proposed by a group of scientists (Gulya et al., 1998; Tourvieille de Labrouhe et al., 2000a).

MATERIALS AND METHODS

In order to determine the race variability of sunflower downy mildew in the Northern Caucasus and the ratio of the individual races in separate agrocenosises, expeditions for collecting isolates of *Plasmopara halstedii* from infected plants of different hybrids and varieties were organized in the period 2004-2007. About 1000 isolates of the Oomycete were collected from 14 regions of the Northern Caucasus (Adigeya republic, Krasnodar and Rostov areas) (Table 1, Fig. 1). Leaves from systemically infected plants in the field were harvested and kept in darkness at 6^{o}C in 100% humidity for 12 hour for induction of fungus sporulation. The leaves with sporulation were kept in polyethylene bags at -80^{o}C. At this temperature,

zoosporangia do not lose their viability for some years. For differentiation of pathogen races according to the new nomenclature system, nine sunflower differential lines were used: set 1 – HA 304 (D-1), Rha-265 (D-2), Rha-274 (D-3); set 2: PM-13(D-4), PM-17 (D-5), 803-1(D-6); set 3: HAR-4 (D-7), HAR-5 (D-8), HA-335 (D-9). Races were determined on the basis of the response of the lines from each group (sporulation on the first true leaves) (Tourvieille de Labrouhe et al., 2000a). Seeds of differentials were placed for germination in rolled up filter paper at 25°C. At the radicle length of 1.0-2.0 cm the seedlings were laid in rows in growth plates with wet sterilized sand covered by filter paper (10 seedlings of each line in one plate). The roots of seedlings were covered by strips of filter paper and wet cotton wool. A suspension of zoospores was prepared from frozen spores (the concentration was 10^6 spores/ml) at 16°C; 150 ml of suspension was added to each growth plate and they were kept for 3-5 hours at the same temperature. Plants were grown 7-9 days at 25°C in the daytime and 18°C at night (16 h photoperiod). The growth plates were placed in a wet chamber at 16°C for 12-24 hours for sporulation. Plants with sporulation on true leaves were classified as susceptible. If any of the differential lines displayed partial infection, these lines were re-inoculated a second time, using spores from the universal susceptible or the line in question.

RESULTS

During the period 2004-2007, the climate conditions were favorable for downy mildew on sunflower in more regions of Northern Caucasus. Therefore, a numerous collection of *P. halstedii* isolates (more than 1000) was collected from infected sunflower plants of different varieties, hybrids and lines in 14 areas of Northern Caucasus (Fig. 1).

Fig. 1. Location of districts of gathering *Plasmopara halstedii* isolates from infected sunflower plants: ***K****-Krasnodar region: 1- Krasnodarskiy, 2 - Leningradskiy, 3- Caucazskiy, 4 - Viselkovskiy, 5 - Labinskiy, 6-Kanevskoy, 7 - Novokubanskiy, 8 - Korenovskiy, 9 - Krilovskoy, 10 - Kurganinskiy , 11 - Uspenskiy ;* ***A*** *- Adigeya Republic: 12- Shovgenovskiy;* ***R*** *- Rostov region: 13 -Matveev-Kurganskiy., 14 - Millerovskiy.*

Our identification of collected isolates in accordance with new nomenclature system, proposed by Tourvieille de Labrouhe et al. (2000a), was the first circumstantial investigation of race composition of *P. halstedii* population on sunflower in this part of the world. It revealed 7 races (Table 1). Pathotypes 100, 300, and 310 were only sporadically present in some areas of the above regions. Race 330 was revealed in

all investigated fields everywhere (Table 1). Races 710 and 730 were discovered in 9 and 7 areas, respectively. Race 700 was revealed in Adigeya Republic and in 3 areas of Krasnodar region. All seven races were identified in fields of VNIIMK.

Table 1. *Plasmopara halstedii* races infecting sunflower in some regions of Northern Caucasus during 2004-2007.

District	Year of collection of isolates	Host of pathogen	Races						
			100	300	310	330	700	710	730
		Adigeya Republic							
Schovgenovskiy	2004	Master*	-	-	-	+	+	+	+
		Rostov region							
Matveev Kurgan	2004	R- 453 (Rodnik)*	-	-	+	+	-	-	-
Millerovskiy	2004	Signal**, Donskoy krupnoplodniy*	-	-	-	+	-	+	+
		Krasnodar region							
Leningradskiy	2004	R- 453 (Rodnik)*	-	-	+	+	-	-	-
Caucazskiy	2004	Master*	-	-	-	+	-	-	-
Viselkovskiy	2004	PR64A83,**	-	-	+	+	+	+	+
	2005	R- 453 (Rodnik)*, Signal**							
Labinskiy	2005	R- 453 (Rodnik)*	-	+	-	+	+	+	+
Kanevskoy	2005	Master*, Flagman*, R- 453 (Rodnik)*, Signal**	+	-	-	+	-	+	+
Novokubanskiy	2005	VK 276 B***	-	-	-	+	-	-	-
Korenovskiy	2005	VK-653***	-	+	-	+	-	-	-
Krilovskoy	2006	Konditerskiy (SPK)*, Donskoy krupnoplodniy*	-	-	-	+	+	+	+
Krasnodarskiy	2004-2007	Different varieties, hybrids and lines on the fields of VNIIMK	+	+	+	+	+	+	+
Kurganinskiy	2007	Konditerskiy (SPK)*	-	-	-	+	-	+	-
Uspenskiy	2007	R- 453 (Rodnik)*	-	-	-	+	-	+	-

* open pollinated variety; **hybrid; *** inbred line.

The analysis of the race ratio was carried out for isolates of 24 separate agrocenoses. In Schovgenovskiy district of Adigeya Republic from 23 isolates of fungus, collected on one field from infected plants of open pollinated variety Master, 69.6 % belonged to race 710 and 13.0% were classified as race 330. Races 700 and 730 represented 8.7% each (Table 2). In Millerovskiy district of Rostov region (18 isolates) the highest number corresponded to races 330 (77.7%), 730 (16.7%) and 710 (5,6%). In Matveev Kurgan district, race 330 represented 80% and race 310 was 20%, but the number of isolates was small (6 isolates).

Table 2. The ratio (%) of *Plasmopara halstedii* races of infected sunflower in separate agrocenoses of Adigeya Republic and Rostov region

Location of the field	Year of collection of isolates	Host of pathogen	N. of isolates	Races				
				310	330	700	710	730
Schovgenovskiy district of Adigeya Republic	2004	Master*	23	0	13.0	8.7	69.6	8.7
Millerovskiy district of Rostov region	2004	Donskoy krupnoplodniy*	18	0	77.7	0	5.6	16.7
Matveev-Kurganskiy district of Rostov region	2004	R- 453 (Rodnik)*	6	20	80	0	0	0

*open pollinated variety

Table 3. The ratio (%) of *Plasmopara halstedii* races of infected sunflower in separate agrocenoses in different districts of Krasnodar region

N. of the field	Year of collection of isolates	Host of pathogen	N. of isolates	Races						
				100	300	310	330	700	710	730
Viselkovskiy district										
1	2004	PR64A83**	93	0	0	1.2	23.4	11.2	60.5	3.7
2	2005	R- 453 (Rodnik)*	79	0	0	0	12.7	0	29.1	58.2
3[1]	2005	Signal**	6	0	0	0	50	0	50	0
Kanevskoy district										
4	2005	Flagman*	60	0	0	1.7	91.7	0	3.3	3.3
5	2005	R- 453 (Rodnik)*	63	0	0	1.6	98.4	0	0	0
6	2005	R- 453 (Rodnik)*	12	0	0	0	100	0	0	0
7	2005	Master*	14	7.1	0	0	92.9	0	0	0
8[1]	2005	Signal**	12	0	0	0	100	0	0	0
Krilovskoy district										
9	2006	Konditerskiy (SPK)*	82	0	0	0	90.2	1.2	3.7	4.9
10	2006	Donskoy krupnoplodniy*	15	0	0	0	53.3	0	26.7	20
11[1]	2006	Konditerskiy (SPK)*	11	0	0	0	100	0	0	0
Kurganinskiy district										
12	2007	Konditerskiy (SPK)*	43	0	0	0	97.7	0	2.3	0
Krasnodarskiy district (fields of VNIIMK)										
13	2005	VK-678*** (first planting date)	28	0	0	3.6	89.3	0	7.1	0
14	2005	VK -678*** (second planting date)	45	0	0	2.2	55.6	0	11.1	31.1
15	2005	Different genotypes	30	3.3	3.3	6.7	73.4	3.3	3.3	6.7
16	2005	R- 453 (Rodnik)*	12	0	0	8.3	66.7	0	0	25
17	2005	Different genotypes	10	0	0	0	40	0	30	20
18	2005	Different genotypes	29	0	3.5	10.3	58.6	3.5	17.2	6.9
19	2006	Different genotypes	7	0	0	0	100	0	0	0
20	2006	Different genotypes	15	0	0	0	100	0	0	0
21	2006	Volunteer plants	14	0	0	0	100	0	0	0

[1]Treated fields with metalaxyl-protected seeds; *open pollinated variety; **hybrid; *** inbred line.

Race 330 dominated in 18 out of the 21 studied agrocenoses in Krasnodar region and constituted 40-100% (Table 3). In Viselkovskiy district, two adjacent fields were analyzed. In the first field 93 isolates of the pathogen were collected from infected plants of hybrid PR64A83 in 2004. In the second field (adjacent to the first), 79 isolates were collected from open pollinated variety R- 453 (Rodnik) in 2005. In the first field, race 710 dominated (60.5%), whereas in the second dominated race 730 (58.2%) (Table 3).

Race 730 in the first field only accounted for 3.7%, but in the second field it represented 58.2%. Race 330 was 23.4 % in the first field and 12.7% in the second. Races 310 and 700 were only identified in the first field (1.2% and 11.2%, respectively). In the third field, treated with metalaxyl-protected seeds of hybrid Signal, only 6 isolates were collected and identified, with races 330 and 710 representing 50% each.

Another race composition was found on five fields of Kanevskoy district. Race 330 here was up to 100%. Races 710 and 730 were discovered only in one field, each of them made up to 3.3%. Race 100 made up to 7.1% on another field and race 310 represented 1.6 and 1.7 % in two fields. Races 300 and 700 were not found in these five fields (Table 3).

In Kurganinskiy district, the percentage of race 330 was 97.7% and race 710 constituted 2.3% (Table 3). In all the three studied fields in Krilovskoy district, race 330 also dominated (53.3-100%). From the 82 isolates collected from infected plants of variety Konditerskiy (SPK) races 700, 710 and 730 were also identified, representing only 1.2, 3.7, and 4.9%, respectively. The percentage of race 710 was 26.7% and of race 730 20% in 15 collected isolates from infected sunflower of variety Donskoy krupnoplodniy (Table 3).

All isolates from Krasnodarskiy district were collected from infected plants of different genotypes of sunflower (Russian and foreign open pollinated varieties, hybrids, inbred lines) in VNIIMK`s fields. In all 8 presented agrocenoses, race 330 predominated over the other races. The line VK-678 was planted in one field at two different planting dates. For both dates, apart from race 330, also were present races 310 and 710. Race 730 was not observed among the samples on the first date of planting but it was identified in the samples on the second date of planting (31.1%) . In one of the fields, all the 7 races were observed: races 100, 300, 700 and 710, represented each 3.3% of the samples from this field, races 310 and 730 made up to 6.7% each. In one of the remaining fields, 6 races were discovered; races 300 and 700 constituted 3.5 % each, race 310 was 10.3%, race 710 was 17.2%, and race 730 was 6,9% (Table 3).

DISCUSSION

According to this observation, seven races *of Plasmopara halstedii* were identified in regions of Northern Caucasus during the last four years. The distribution area and the ratio of pathotypes in separate agrocenoses were different. We assumed that at present races 100, 300, and 310 are disappearing here because of the following reasons: firstly, they have been discovered only sporadically; secondly, all their isolates form an extremely poor sporulation even on susceptible differential lines. In addition, races 100 and 300 are the oldest races, at least in Krasnodar territories. Prolonged sunflower breeding for resistance to these pathotypes over the span of some decades must result in their maximal ousting from the populations. At present, the main race which is widely distributed everywhere in the studied territories is 330. This race dominates up to 100% in 18 out of 21 studied agrocenoses in Krasnodar region and in the two studied agrocenosises in Rostov region. But in separate agrocenoses of Adigeya Republic and Krasnodar region, races 710 and 730 are dominant. Race 700 was found in 5 areas and it represented up to 11.2%. Its condition in pathogen population here is not clear now. Maybe it will spread more widely in the future. At present, only races 330, 710 and 730 are economically important in these territories of Northern Caucasus. Strictly speaking, the quantity of races of pathogen depends on the diversity of cultivated sunflower genotypes. The rapid change over the last decades of sunflower crop variety structures in the studied territories, together with a wide distribution of foreign hybrids everywhere in Russia, will change the race structure of *P. halstedii* populations in this country. Data from our investigation showed the necessity to concentrate efforts on the development of native sunflower varieties and hybrids resistant to races 330, 710 and 730. In addition, our investigation will serve as a starting point for controlling the population structure of *Plasmopara halstedii* in Northern Caucasus territories.

ACKNOWLEDGEMENTS

This research was supported by the Russian Foundation of Fundamental Investigations (RFFI) and Administration of Krasnodar region, grant N. 06-04-96641. The authors are grateful to Dr. P. Schindrova (Bulgaria), Dr. T. Gulya (USA), Dr. D. Tourvieille de Labrouhe (France) and Dr. F. Viranyi (Hungary) for sending seeds of sunflower differential lines.

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Differences in some DNA RAPD-loci of *Plasmopara halstedii* races affecting sunflower in Krasnodar region of Russia

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ABSTRACT

Forty-three isolates of *P. halstedii* races 300, 310, 330, 700, 710, 730 affecting sunflower in Krasnodar region were studied by means of 22 RAPD-loci. Primer P38 did not produce amplification in the studied isolates. The other primers produced a total of 92 fragments with an average frequency of 4.4 fragments per primer. Eight primers (L14, Y11, P28, P46, P5, OPM08, OPM20, OPJ15) were not polymorphic among the studied isolates. Thirteen primers (OPB07, OPB17,OPC08, OPC15, OPD 11, OPD 18, OPE 03, OPD 20, OPF09 OPG02 OPG05, OPG06, and OPJ13) allowed the identification of race 300 from the other races, based on the presence of amplified DNA fractions lacking in other races, or the absence of fractions typical for races 310, 330, 700, 710, 730. The resemblance measured between races 330, 700, 710 and 730, counted by means of Jacquard coefficient, was near 1.00. The resemblance between race 300 and the others was 0.29. Accordingly, the genetic distance (D_{xy}) between race 300 and the others was 0.71. These data suggested that local races 310, 330, 700, 710 and 730 in Krasnodar region could not originate from race 300. One primer (OPG06) showed intraracial polymorphism on the presence-absence of a fragment with a length of 1125 bp in all races, except 700 and 710. Monomorphic condition (invariable presence of the 1125 bp fragment) of locus OPG06 in race 710 isolates from five remote districts of Krasnodar region pointed to its stability. The monomorphic condition of this locus (invariable absence of the 1125 bp fragment) in race 330 isolates from Kanevskoy district and polymorphic condition in one isolate from Viselkovskiy district are discussed.

Key words: downy mildew – molecular markers – *Plasmopara halstedii* – races – sunflower – RAPD markers.

INTRODUCTION

The fungus *Plasmopara halstedii* (Farl.) Berl. et de Toni is an obligate parasite of sunflower, causing downy mildew, a worldwide major disease of this crop. The pathogen exists as many physiological races, which over the last few decades has grown into a complex, with at least 36 pathotypes being identified in different countries (Gulya, 2007). Physiological races of obligate parasites are always difficult to differentiate. Different physiological races of this pathogen have been described according to their reactions on various sunflower lines. An international nomenclature based on a series of well defined host plants is starting to be used, which should make it possible to establish the presence of the same races in different continents and to define the specific races in each country (Gulya et al., 1998; Tourvieille de Labrouhe et al., 2000). This international method of *P. halstedii* races differentiation and their new nomenclature application was successfully used by many pathologists from different countries (Rozynek, Spring, 2000; Molinero-Ruiz et al., 2002; Shindrova, 2000, 2005; Shirshikar, 2005; Antonova et. al, 2006; Iwebor et. al., 2005, 2007). But sometimes this cannot guarantee the clear differentiation of some races, especially if they are from different countries. The molecular methods for genomic analysis of this fungus are especially applied at present (Roeckel-Drevet et al., 1997, 2003; Giresse at al., 2007). The relationships between all known races of *P. halstedii* from different countries have been investigated by means of 21 RAPD primers (Tourvieille, 2000; Roeckel-Drevet et al., 2003), but races of fungus from Russia were not used in that investigation. The downy mildew pathogen on sunflower in Russia until recently has been scarcely studied either on racial structure or on the molecular structure of the genome. At this time, a successful control of the disease demands a regular survey of pathogen populations and incorporation of resistance to as many races as possible in sunflower breeding programs.

The objective of our investigation was the analysis by means of RAPD-PCR markers of molecular-genetic polymorphism of *P. halstedii* races present in Krasnodar region of Northern Caucasus.

MATERIALS AND METHODS

The research included 43 field isolates of *P. halstedii* belonging to six races of the pathogen with code numbers: 300, 310, 330, 700, 710, 730 (accordingly a quantity of isolates: 2, 1, 12, 4, 13, 11). The isolates were collected from the affected sunflower plants in different areas of Krasnodar region in 2005-2007. The leaves with sporulation were kept in polyethylene bags at -80°C. The seedling inoculations were implemented by using the method described in these Proceedings (Antonova et al., 2008). The physiological races were determined according to the international nomenclature, which has been proposed by Tourvieille de Labrouhe et al. (2000). All isolates were maintained on seedlings of sunflower open-pollinated variety VNIIMK 8883, which has never been used in breeding for resistance to downy mildew. DNA was extracted from conidial sporulation of the Oomycete on cotyledons of sunflower seedlings; these were artificially infected by zoospores of every isolate separately. Spores were collected and kept at -20°C until DNA extraction, which was performed within 1 month. DNA was extracted by a modified method based on Zolan and Pukkila (1986).

For RAPD-analysis, 22 decamer primers (L14, Y11, P 28, P 53, M 08, M 20, J 15, P38, B 07, B 17, C 08, C 15, D 11, D 18, E 03, D 20, F 09, G 02, G 05, J 13, G 06) were used. The first six primers were used by us early on sunflower (Guchetl et al. 2004). The others have been used for differentiation of 5 races of *P. halstedii* collected from different districts of France (Roeckel-Drevet et al., 1997; Tourvieille et al., 2000). These primers were kindly given to us by those authors. Each 25 μL of reaction volume contained 67 mM tris-HCl, ph 8.8; 16.6 mM $(NH_4)_2SO_4$; 1.5-3.0 mM $MgCl_2$; 0.001 % Tween 20; 0.2 mM deoxynucleoside triphosphates, 10 μM primer; 10 ng template DNA and 1.0 unit *Tag* DNA polymerase (Gosniigenetic, Russia). Amplification was performed in thermocycler (AO DNA-technology, Russia). PCR was conducted at regime standard for RAPD-primers: 1 cycle at 94°C for 2 min (initial denaturation) and 30 cycles – in consecutive temperature change: 1 min. at 94°C (denaturation), 1 min. at 36°C (annealing), 2 min. at 72°C (elongation), 4 min.at 72°C (final elongation).

Electrophoresis of PCR products was carried out in agarose gel (1.5 % agarose, 1x TAE-buffer in horizontal camera during 1.5-2.0 h at I=50 mA, U= 70-90 V; 10 μL of reactionary mixture were introduced in gel together with dye-stuff bromphenol blue. GeneRuler 1 kb DNA Ladder (MBI "Fermentas") was used as marker for DNA fragments lengths. Ethydium Bromide was used for subsequent staining of DNA fragments. Data were documented by means of trans-illuminator and video system (AO DNA-technology, Russia) with computer program "Gel-Imager 2". Experiments were carried out in triplicate.

The differences between isolates were expressed by the presence or the absence of bands on gel corresponding to DNA fragments of the definite length. The resemblance measure between races was calculated by means of Jacquard coefficient (Sneath and Sokal, 1973) using the formula: $\mathbf{J_{xy} = n_{xy} / n_x + n_y}$, where $\mathbf{J_{xy}}$ is the Jacquard's coefficient; $\mathbf{n_{xy}}$, is the number of DNA fragments of patterns **x** and **y** coinciding by their electrophoretical mobility; $\mathbf{n_x}$ and $\mathbf{n_y}$ are the number of amplified DNA fragments of patterns **x** and **y.**

RESULTS AND DISCUSSION

In the first stages of DNA experiments, both the parasite and its host variety VNIIMK 8883 were amplified. The major DNA fragments of the Oomycete and sunflower reproduced always differed by the quantity of nucleotides pairs (Fig. 1). This suggested the correctness of the pathogen sporulation picking up. From 22 RAPD-primers used, one (P38) did not give any amplified DNA spectra. The others produced a total of 92 fragments with average frequency of 4.4 fragments per primer. Eight primers: L14, Y11, P28, P46, P5, OPM08, OPM20, and OPJ15 were not polymorphic in the studied isolates.

Thirteen primers: OPB07 OPB17OPC08, OPC15, OPD 11, OPD 18, OPE 03, OPD 20, OPF09 OPG02 OPG05, OPG06, and OPJ13 allowed the identification of race 300 isolates from other races, based on the presence of amplified DNA fragments lacking in other races, or the absence of fractions typical for races 310, 330, 700, 710, 730 (Fig. 2). These 13 primers produced a total of 76 fragments (from 1 to 12 polymorphic fragments per primer). From them, we identified 62 polymorphic loci. Our results showed that these 62 polymorphic loci only allowed a clear identification of race 300 from the six races studied.

The resemblance measure between race 300 and the others was accounted for by means of Jacquard coefficient (J_{xy}). This method is more suitable for accounting RAPD-data (Link et al., 1995). The resemblance measure between races 330, 700, 710 and 730 was near to 1.00. The resemblance measure between race 300 and the others was defined by a fairly small value of 0.29. Accordingly, the genetic

distance (D_{xy}) between race 300 and the others (which is calculated by the formula $D_{xy}=1- J_{xy}$**)** was 0.71. The resemblance measure between races 300 and 710 in France was 0.88 (Roeckel-Drevet et al., 1997).

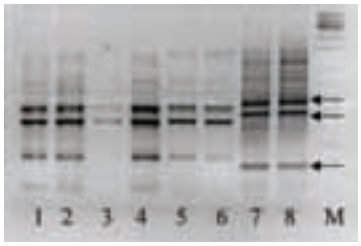

Fig. 1. Amplified DNA electrophoretical spectra of *P. halstedii* and sunflower obtained with primer L 14. Lanes: 1-6 - isolates of *P. halstedii*; 7, 8 – sunflower variety VNIIMK 8883. M – molecular mass marker (GeneRuler 1 kb DNA Ladder, MBI "Fermentas") The arrows show the reproduced DNA fractions having the length: 550 bp, 460 bp and 160 bp (respectively from top to bottom).

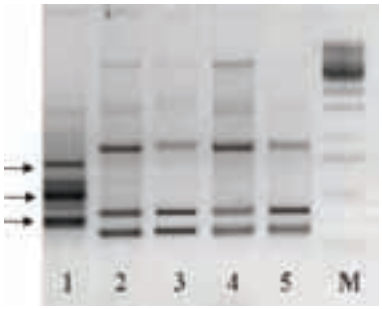

Fig. 2. Amplified DNA electrophoretical spectra of *P. halstedii* obtained with primer OPJ13. Lanes (races): 1 - 300, 2 - 330, 3 – 700, 4 -710, 5 – 730. M – molecular mass marker (GeneRuler 1 kb DNA Ladder, MBI "Fermentas" . The arrows show race 300 DNA fragments with length (from top to bottom) 710bp., 480 bp and 330 bp that distinguished it from the others.

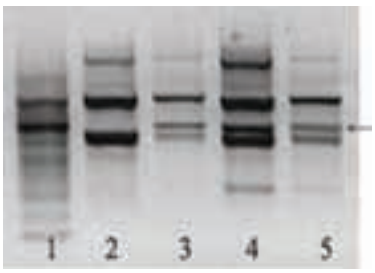

Fig. 3. Amplified DNA electrophoretical spectra of *P. halstedii* obtained with primer OPG06. Lanes (races): 1 - 300, 2 - 330, 3 – 700, 4 -710, 5 – 730. The arrow shows DNA fraction 1125 bp.

Although the race composition of *P. halstedii* population in Russia was not controlled for a long time, since first investigations date back to the beginning of the 1980s, it may be assumed that race 300 appeared in Krasnodar region later than race 100 (Antonova et al., 2000). Apparently, it was introduced into the former USSR with sunflower seeds. Its origin from another continent could explain the the distinctions of race 300 from the others on RAPD-loci studied in our experiments. Our data agreed with investigations of Tourvieille et al. (2000) grouping in separate clusters European and American isolates of this Oomycete.

Only one primer OPG06 produced intraracial polymorphism on the presence-absence of one fraction whose length is 1125 bp (shown as $OPG06_{1125}$) (Fig. 3, Table 1). Two isolates of race 300 which were

Table 1. The characteristic of DNA RAPD-locus $OPG06_{1125}$ of *P. halstedii* races, collected on sunflower from different districts of Krasnodar region, Russia, 2005

Isolate number	Race	District of isolate collection	$OPG06_{1125}$*
1	300	Fields of VNIIMK	1
2	300	Labinskiy	0
3	310	Viselkovskiy	0
4	330	Viselkovskiy	0
5	330	Viselkovskiy	0
6	330	Viselkovskiy	1
7	330	Fields of VNIIMK	1
8	330	Fields of VNIIMK	1
9	330	Fields of VNIIMK	1
10	330	Kanevskoy	0
11	330	Kanevskoy	0
12	330	Kanevskoy	0
13	330	Kanevskoy	0
14	330	Kanevskoy	0
15	330	Kanevskoy	0
16	700	Labinskiy	1
17	700	Labinskiy	1
18	700	Viselkovskiy	1
19	700	Krilovskoy	1
20	710	Fields of VNIIMK	1
21	710	Fields of VNIIMK	1
22	710	Viselkovskiy	1
23	710	Viselkovskiy	1
24	710	Viselkovskiy	1
25	710	Viselkovskiy	1
25	710	Viselkovskiy	1
27	710	Viselkovskiy	1
28	710	Viselkovskiy	1
29	710	Labinskiy	1
30	710	Labinskiy	1
31	710	Kanevskoy	1
32	710	Krilovskoy	1
33	730	Fields of VNIIMK	0
34	730	Fields of VNIIMK	0
35	730	Fields of VNIIMK	0
36	730	Fields of VNIIMK	1
37	730	Viselkovskiy	0
38	730	Viselkovskiy	0
39	730	Viselkovskiy	1
40	730	Viselkovskiy	1
41	730	Viselkovskiy	1
42	730	Viselkovskiy	1
43	730	Viselkovskiy	1

* 0- absence, 1- presence of amplified DNA fragment

collected in different districts of Krasnodar region had different genotypes for this character. The only isolate of race 310 studied showed the absence of fraction. Races 300 and 310, which were found only sporadically in the districts of Krasnodar region, gave an extremely poor sporulation and we failed to collect enough material for analysis. Twelve isolates of race 330 from three districts have shown interesting results. All the six isolates from Kanevskoy district lacked this fraction. All three isolates from fields of VNIIMK have shown its presence and, although two out of three isolates from the Viselkovskiy district did not have it, the third one did. All four isolates of race 700 have shown the presence of fraction 1125 bp in locus OPG06 and they were collected in three districts which are situated quite far from each other (Table 1). The distances between Viselkovskiy district and two others: Krilovskoy and Labinskiy are about 130 and 250 km, respectively. Therefore, despite the small quantity of studied isolates, we presume that for race 700 the presence of this fraction is uniform.

Thirteen isolates of race 710 were collected in five different districts and all of them have shown the presence of this fraction (Table 1). Eleven isolates of race 730 collected in two districts have shown the presence or absence of this fraction. Data of Table 1 show some stability of race 710 in locus OPG06 because the isolates were collected in five different districts and all of them had the fraction 1125 bp. As shown in another manuscript of these proceedings (Antonova et al., 2008), race 330 was predominant in Kanevskoy district, up to 100% in the majority of studied fields, and its isolates from there have shown the condition of locus OPG06 as the absence of the fragment. In some fields of the Viselkovskiiy district, races 710 and 730 predominated, whereas in the others there was race 330. The presence-absence of fraction varied in isolates of races 330 and 730, but it was always present on the isolates of race 710. This gives an idea about the hybridization of races in that district. Apparently, locus OPG06 is not coupled with genes of virulence. In this case, the coexistence in one population of individuals with different allelic conditions is possible.

In conclusion, our study has confirmed the considerable molecular homogeneity previously observed among the French races (Roeckel-Drevet et al., 1997; Tourvieille et al., 2000). Our data suggests that the origin of local races 330, 700, 710 and 730 in Krasnodar region could not be race 300. However, this should be further confirmed, because of the scanty quantity of race 300 isolates available for this study.

Monomorphic condition of locus OPG06 of race 710 isolates from five remote districts of Krasnodar region suggested its stability, i.e. the invariable presence of fraction 1125 bp. Monomorphic condition of locus OPG06 (the absence of fraction 1125 bp.) of race 330 isolates from one district (Kanevskoy) may be connected with this pathotype's prevailing domination there of up to 100% in the majority of studied agrocenoses. And we suppose that for "pure" race 330 the absence of fraction 1125 bp in OPG06 locus is a constant character. The polymorphic condition of this locus revealed in isolates of race 330 from Viselkovskiy district may possibly be explained as the result of hybridization between races 330 and 710 or 730 in that place.

ACKNOWLEDGEMENTS

We wish to thank Dr. D. Tourvieille de Labrouhe and Dr. J. Tourvieille for providing the RAPD- primers used in this study. This research was supported by the Russian Foundation of Fundamental Investigations (RFFI) and Administration of Krasnodar region, grant 06-04-96641.

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Relations between spring rainfall and infection of sunflower by *Plasmopara halstedii* (downy mildew)

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ABSTRACT

The incidence of spring rainfall on the severity of primary downy mildew attack of sunflower was studied in field trials with staggered sowing dates. Infection potential of soil depended partly on rainfall probably because, in damp conditions, oospores germinate and must infect sunflower rapidly. In contrast, under dry conditions, they remain dormant and thus maintain their infection potential. Disease risk appears greatest if there is heavy rainfall when sunflower seedlings are at their most susceptible stage, between germination and emergence. Heavy rainfall before sowing had no effect on percentage of diseased plants and heavy rainfall after seedling emergence did not increase primary downy mildew attack.

Key words: disease risk epidemiology – downy mildew – *Helianthus annuus* – infection potential – soil test.

RESUME

La mise en place de semis décalés sur deux années présentant des conditions climatiques différentes a permis de préciser l'influence des précipitations printanières sur l'expression de la forme la plus dommageable du mildiou qui est l'infection primaire tellurique. D'une part, le potentiel infectieux du sol évolue au grès des pluviométries. Les pluies continues épuisent le stock d'inoculum alors qu'une période sèche fait grimper le pouvoir infectieux de sol au niveau de l'horizon correspondant au lit de semence. D'autre part, le risque mildiou est étroitement lié à la présence de pluies abondantes au moment où la plantule présente une forte sensibilité aux infections telluriques. C'est-à-dire entre le début de la germination et l'émergence des cotylédons. Les fortes pluies qui interviennent avant la germination n'ont que peu d'impact sur le taux de plantes malades. Les fortes pluies qui arrivent après la levée n'augmentent pas le nombre d'infections telluriques. L'utilisation de ces informations épidémiologiques devrait être intégrées à la construction d'un modèle d'analyse de risques.

Mots clés: analyse de risque –épidémiologie – *Helianthus annuus* – mildiou – potentiel infectieux – test sur terre.

INTRODUCTION

Plasmopara halstedii is a soil-borne parasite, which remains in the soil in the form of oospores. These spores are produced by sexual reproduction and may remain dormant but viable for up to 10 years. Under favourable conditions, oospores germinate to give zoosporangia which, in the presence of free water, liberate mobile zoospores. These cause primary infections of sunflower radicles, leading to systemic attacks that cause most loss to the crop. Rainfall is a major climatic factor determining disease risk. Delos et al. (2000) considered that rainfall just before or after sowing was the most favourable for downy mildew infections of sunflower. To determine in more detail the effect of rainfall on percentage attack, in 2006 and 2007, sunflowers were sown at weekly intervals and detailed records were made of rainfall, soil temperature and numbers of plants showing systemic downy mildew symptoms. The infection potential of the soil in the fields concerned was measured by a growth chamber test developed by Tourvieille de Labrouhe and Walser (2005). Correlations between the different factors were determined in order to define those that should be included in models predicting disease risk.

MATERIALS AND METHODS

Sunflower genotypes

The inbred sunflower line GB (INRA, Clermont-Ferrand) was used both for soil tests in the laboratory and to measure downy mildew attack in the field. This line has no known downy mildew resistance gene.

Field trials

Trials were carried out in fields near Clermont-Ferrand (Auvergne) naturally infected with race 710 of *P.halstedii.* Each year, 10 zones were defined in the field and, in each, one 3m row was sown with the inbred line GB every week from 21st of March to 6th of May in 2006 (8 sowing dates) and from 13th of March to 15th of May in 2007 (10 sowing dates).

Observations of weather conditions

Rainfall was obtained from Météo-France at Clermont-Ferrand airport (at 1km). Soil temperature was measured with a recorder (xvacq de TMI Orion) measuring the temperature at a depth of 3cm every hour.

Field observations

The trials were observed each week from seedling emergence to 2 pairs of leaves. The numbers of plants showing systemic downy mildew symptoms resulting from primary infections through the roots were counted.

Soil test

The day before soil sampling, sunflower seeds were germinated at 100% RH after soaking in water for 2 hours. Soil was sampled at a depth corresponding to that at which sunflower seeds are normally sown. The samples were placed in trays and the germinating seed sown at a depth of 1cm. After 48h at 18°C, the trays were immersed in water for 8h. They were then incubated for 12 days at 18°C and 12000 lux light 16h/24. To determine presence of downy mildew, the trays were covered with a plastic bag to obtain 100%RH for 48h and then observations were made of sporulation on cotyledons and true leaves (Tourvieille de Labrouhe and Walser, 2005).

RESULTS

Weather conditions

Rainfall, mean daily temperatures and sowing dates are presented in Fig. 1 and 2.

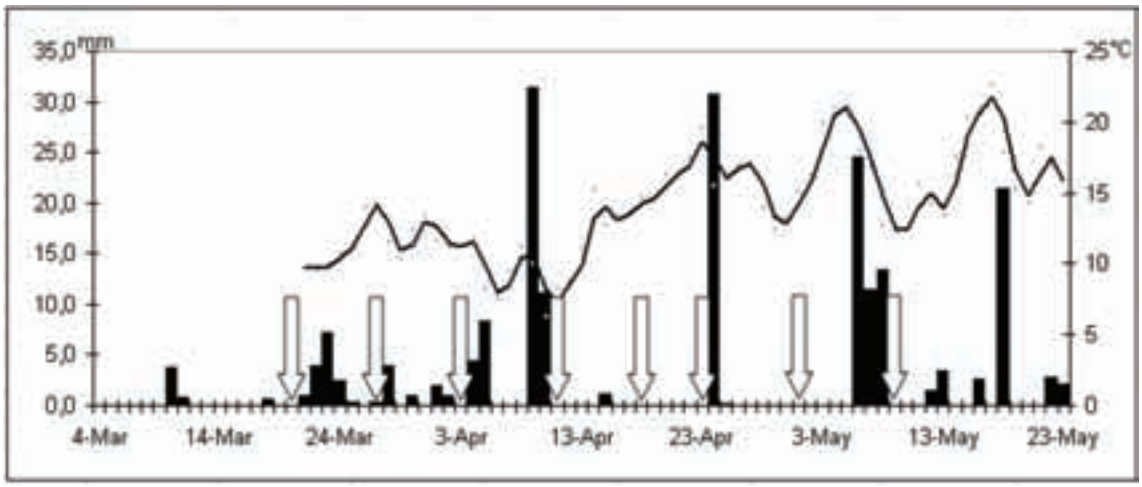

Fig. 1. Soil temperature and rainfall in 2006. Arrows indicate sowing and soil sampling dates

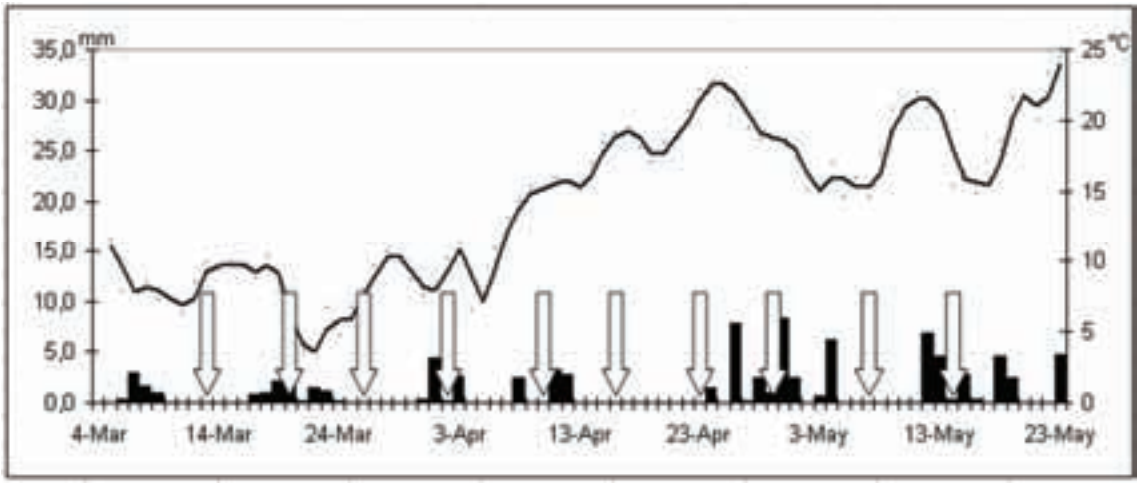

Fig. 2. Soil temperature and rainfall in 2007. Arrows indicate sowing and soil sampling dates

Weather conditions were very different in 2006 and 2007. In 2006, the period studied was cool (mean temperature 15°C) with some heavy rain (178mm, including 42mm 9-10/4, 31mm 24/4, 49mm 6-8/5 et 21mm 18/5). 2007 was much warmer (mean temperature 18°C) and drier (less than 100mm, with 12mm 29/4-1/5 and 14mm 12-15/5).

Variation in downy mildew attack according to sowing date
Table 1 presents percentage primary attacks in 2006 and 2007.

Table 1. Mean percentage downy mildew attack for each sowing date (10 plots for each date)

2006		2007	
Sowing date	% attack	Sowing date	% attack
		March 13rd	0.0
March 20th	16.3	March 20th	1.1
March 27th	44.0	March 26th	0.4
April 3rd	25.1	April 2nd	0.0
April 11th	32.5	April 10th	2.2
April 18th	23.3	April 16th	13.2
April 23rd	34.9	April 23rd	12.9
May 1st	16.0	April 30th	2.0
May 9th	11.6	May 7th	0.2
		May 14th	0.8

In 2007, only 2 dates (16/4 and 23/4) showed more than 10% attack (with a maximum of 39% for one plot), in contrast with 2006 when mean attack was 11 to 44%, with a plot maximum of 83%. Both years showed a considerable variation between sowing dates.

Soil infection potential according to sampling date
Fig. 3 and 4 show the variations in infection potential measured by the laboratory soil test. Potential infection varied considerably according to the zones in the field where soil was sampled but also according to sampling date. The lowest levels were found in March, and in April the greatest potentials were observed for 2 dates: 3/4 and 23/4 in 2006 and 2/4 and 23/4 in 2007.

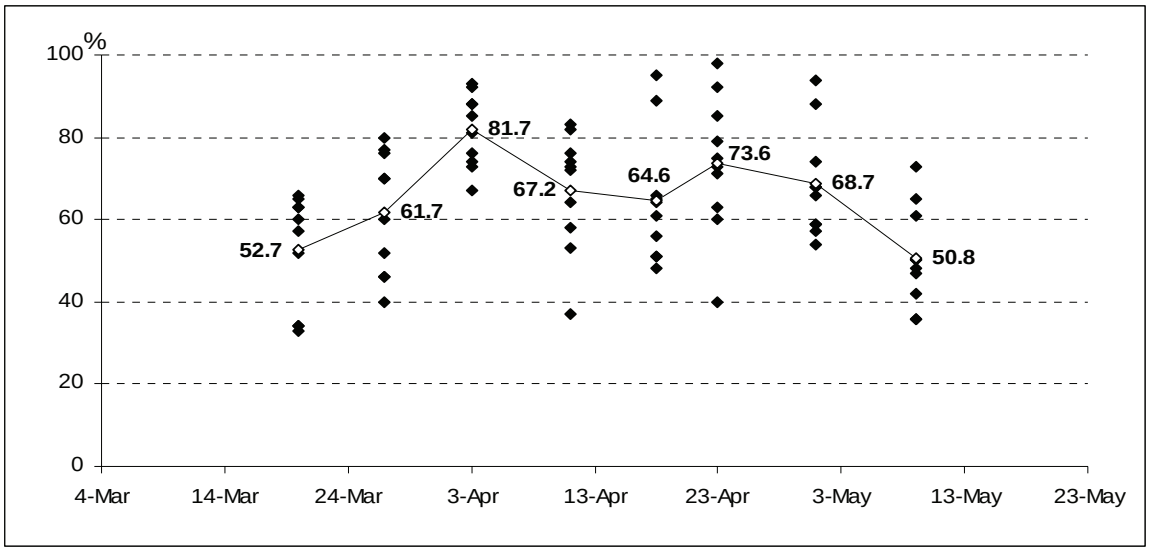

Fig. 3. Variation in percentage of seedlings showing downy mildew symptoms according to date of soil test (10 samples per date) for 2006

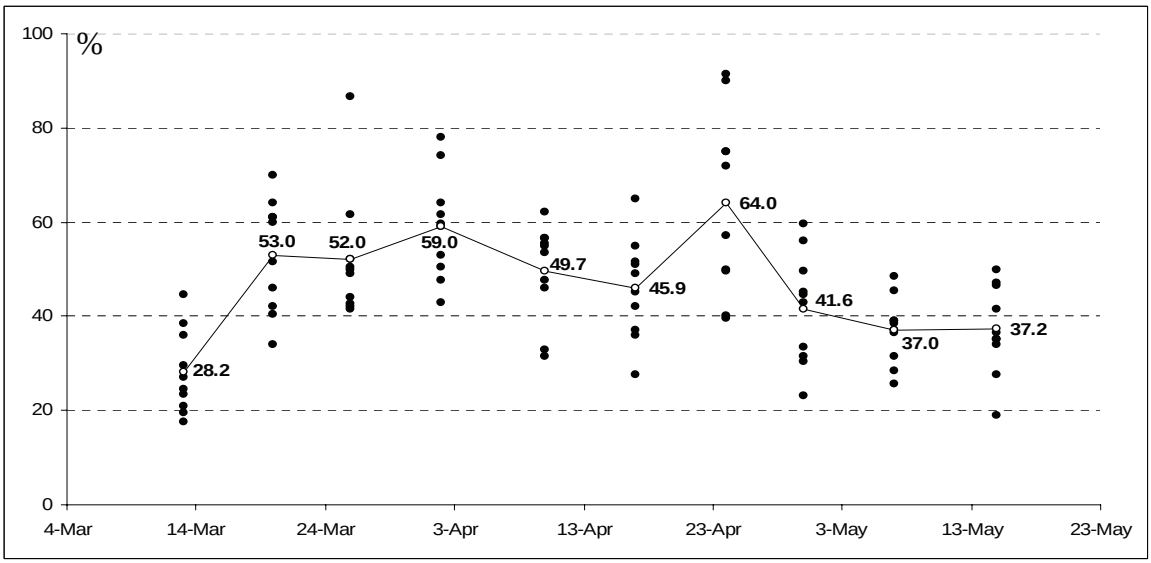

Fig. 4. Variation in percentage of seedlings showing downy mildew symptoms according to date of soil test (10 samples per date) for 2007.

Relations between rainfall and soil infection potential

There was a negative relation between rainfall and soil infection potential. This was confirmed by comparison between total rainfall 10 days before sampling and the percentage of diseased plants in soil tests (Table 2). The negative correlation coefficient was highly significant in 2007, an unfavourable year for downy mildew, especially if only April is considered.

Table 2. Correlation coefficients (Pearson) between rainfall and soil infection potential

	2006		2007	
	March-May	April	March-May	April
Number of replicates (10 plots by date of sowing)	80	40	100	50
Correlation coefficient				
- Infection potential / rainfall 10 days before sampling	r= - 0.166	r= -0.337*****	r= - 0.416******	r= -0,513******
- Rate of attack / rainfall between "sowing + 166 h. with > 7°C" and "sowing + 360 h. with > 7°C"	r= 0.383******	r= 0.403******	r= 0.513******	r= 0.522******

***** significant at p = 0.05, ****** significant at p = 0.01

Relation between rainfall and downy mildew attack in the field

Correlations were calculated between percentage of primary attack and rainfall in the pre-emergence period taking into account soil temperature, which is important for rapid emergence The closest correlation was with rainfall in the period from sowing +166h at above 7°C and sowing + 360h at 7°C (Table 2). For both years, the correlation coefficients were highly significant.

DISCUSSION

The effect of rainfall on the level of downy mildew attack was analysed for 2 very contrasting years. In neither year was there any water deficit, but whereas 2006 had some days of heavy rain interspersed with dry periods, in 2007 there were no heavy rains but regular damp periods. These differences are useful to make general conclusions.

The fields used in 2006 and 2007 had shown comparable levels of downy mildew attack in preceding years, suggesting that they should have similar inoculum potentials (Tourvieille de Labrouhe et al., 2008). The present results indicate quite clearly that the risk of downy mildew attack depends closely on pattern and intensity of rainfall. Délos et al. (2000) reported that rainfall ought to be sufficient to give the free water in the soil necessary for zoospore movement. The low levels of attack in March 2006 and 2007 may be explained not only by lack of rainfall but also low infection potentials, as measured by the soil test. The limiting factor could be the level of inoculum maturation, which depends on soil temperature.

The low levels of attack on the last sowing dates, in May, when there was considerable rainfall, especially in 2006, also suggest that the limiting factor was also a low level of inoculum. This was confirmed by soil tests. It appears likely that the inoculum present around seeds had been used up by the end of April. Infection potential varied between weeks, and soil samples taken after a dry period (when zoosporangia may not have been produced) always showed an increase in infection potential. This observation was confirmed by the negative correlation between rainfall in the 10 days preceding soil sampling and the infection potential of this sample. As suggested by Délos et al. (2000), rainfall may be necessary to obtain free water in the soil for zoospore movement, but it could also be suggested that rainfall leads to production of inoculum which can only cause infection over quite a short period (Goossen and Sackston, 1968), so that none remains in later weeks. Thus, in addition to a variable level of infection potential over the whole period, with a maximum in mid-April, (Tourvieille de Labrouhe et al., 2008), daily variation may occur according to soil humidity. The trials have not been made under very dry conditions so no data is available as to the length of time necessary for oospores to produce zoosporangia when conditions become favourable for the parasite. It would be useful to carry out laboratory tests on soil samples subjected to variable periods of drying.

Délos et al. (2000) reported that attack levels were closely correlated with rainfall from 5 days before sowing to 5 days after sowing. The present results did not show this correlation. Taking into account minimum growth temperature (6°C; Merrien, 1992) and sunflower germination optimum (8°C; Anonymous, 2003), a close relation appeared between rainfall at sowing date + 166h above 7°C (beginning of radicle growth) and sowing date + 360h at above 7°C (emergence). This is generally equivalent to 5 to 15 days after sowing, later than that suggested by the earlier results.

These epidemiological results should help in the construction of a model concerning disease risk. The probability of the appearance of plants showing downy mildew symptoms depends on many factors, of which the following appear to be the most important:

- The level of infestation of the field , which will depend on the number of diseased plants in the preceding sunflower crop (Tourvieille de Labrouhe et al., 2008).
- The use of resistant sunflower varieties and the development of downy mildew races virulent on these varieties (Vear et al., 2007).
- Seed dressing with fungicide and resistance of downy mildew isolates to this fungicide (Gulya, 2000).
- crop management practices which could affect disease development (Covarelli et Tosi, 2007; Escande et al., 2007).
- Rainfall between germination and emergence.
- Soil infection potential on level of seed bed.

ACKNOWLEDGEMENTS

This work was carried out with the financial support of the ANR- Agence Nationale de la Recherche (The French National Research Agency) under the "Programme Agriculture et Développement Durable", project "ANR-05-PADD-0705, Cedre"

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Structural aspects regarding formation and emission of *Diaporthe* (*Phomopsis*) *helianthi* ascospores

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ABSTRACT

Ascospores are the main infection source of Phomopsis in sunflower. However, the concept of their structure is still being contradicted and is not complete enough. The objective of this work was to study the structural organization of the ascospore formation and emission for the sunflower *Phomopsis* pathogen. Perithecia of the fungus both in their intact and crushed condition were studied using light microscopes, digital photography and computer-aided analysis. It was determined that 16 ascospores were produced within an ascus and that was twice as many as those considered earlier. The ascospores are pressed out of the water-swollen perithecium wall in the form of a colorless sphere, whose membrane has a granular structure. The infection source contained within the sphere is transmitted by wind.

Key words: ascospore – fungus – perithecium – Phomopsis – structure – sunflower.

INTRODUCTION

Severe infection of sunflower stems caused by Phomopsis (*Diaporthe helianthi* Munt.-Cvet. et al.) was first observed in Yugoslavia (Mihalicevic et al., 1980) and quickly spread in other countries. The severe damage caused by this disease prompted general interest in the Phomopsis pathogen biology. It was determined that the fungus penetrated into the plant through the leaves, spread along the vessels and then entered the stem (Petrov et al., 1981; Bertrand and Tourvieille, 1987). Pathohistological bases of the fungus penetration and resulting stem tissue changes (Muntanola-Cvetkovic et al., 1989) were studied. Nowadays, it is recognized everywhere that ascospores developing in perithecia are a main infection source. Perithecia, of an irregular round shape and 150-430 x 180-850 μm in size, are produced on overwintering plant residues and contain elongated clavate asci (44-67.5 x 4.5-12.0 μm in size), each of which includes 8 ascospores (Assemat and Fayret, 1988; Yakutkin, 1988). The ascospores, which are colorless, with one partition, of an elongated elliptic shape, 15-17 x 5-7.5 μm in size, slightly pressed in the middle beside the partition, have two equal cells containing generally two fat drops per cell and, as some researchers consider, have constricted ends (Maric et al., 1982). According to other scientists' observations they have rounded ends (Assemat and Fayret, 1988) and may reach 7.5 μm in length (Yakutkin, 1988). It is accepted that the ascospores are thrown out from the perithecium to the height of about 3 mm above the plant residue surface. Sometimes, a mucoid drop or a white mass containing ascospores form on the rostrum apex (Maric et al., 1982; Yakutkin, 1988).

The structure of fruit bodies, conidia and ascospores serves as a basis for fungus identification. However, until now their understanding has been incomplete and rather contradictory.

The objective of this work was to study the structural organization of the ascospore formation and emission for the sunflower Phomopsis pathogen.

MATERIALS AND METHODS

For two years, the fungal fruit body formation was observed in the Phomopsis-affected seeds and stems of the sunflower cultivars Rodnik and Berezansky, the hybrids Fly (Monsanto) and Melody (Syngenta), as well as the stems of *Sonchus oleraceus* (L.) Scop. by keeping them under natural or storage or stationary (refrigerator) conditions.

The stem fragments of the above plants and sunflower seeds with the Phomopsis infection symptoms were washed with water, disinfected with ethyl alcohol; afterwards, the stems were flamed with a gas-stove burner and the seeds were washed with sterile water and placed on sterile wet filter paper inside Petri dishes, which were incubated in the environmental chamber (Sanyo), at 25°C, 16-hour photoperiod

(3000 lux) and 80% of humidity. A part of the experiment was conducted under the same conditions but at the night temperature of 12-15°C.

Intact fruit bodies of Phomopsis forming on the stem or on seeds were examined with a stereozoom trinocular microscope (MLS) and perithecia isolated from the plant substrate and crushed – with a laboratory (ML2300) microscope and trinocular microscopes (Meiji, Japan). Their typical structures were photographed with the digital cameras Canon Digital (Canon) and Cyber-short (Sony) and CCD Digital Microscope camera with software. The pictures were analyzed and processed on a computer.

RESULTS

It was determined that the content of the clavate ascus transformed from an undifferentiated condition to the ascospore formation. The ascospore formation happened stepwise. First, 8 colorless structures with an elongated elliptic shape and slightly deppressed beside the partition were produced within the asci. Each of these structures had two equal cells containing generally two fat drops or, although seldom, one fat drop, and constricted ends (Fig. 1a). Their size exceeded 10 µm, but did not reach 20 µm. They did not leave the asci, which were still so firm that they did not collapse while the preparation was produced by crushing the perithecium on the microscope slide. Being in the ascus, the structures started to change: their membranes becoming thinner and their ends rounded (Fig. 1b). Further, each cell of these structures transformed to a bicellular colorless structure of an elongated elliptic shape, depressed in the middle near the partition, with two rounded fat drops – one at each of the opposite ends (Fig. 1a). These structures were identified by us as ascospores, and the initial ones were called biascospores. Two bicellular ascospores, whose length did not exceed 10 µm, were generated by each biascospore. The biascospore cell that had one fat drop turned into a unicellular ascospore with one fat drop in its center (Fig. 1c).

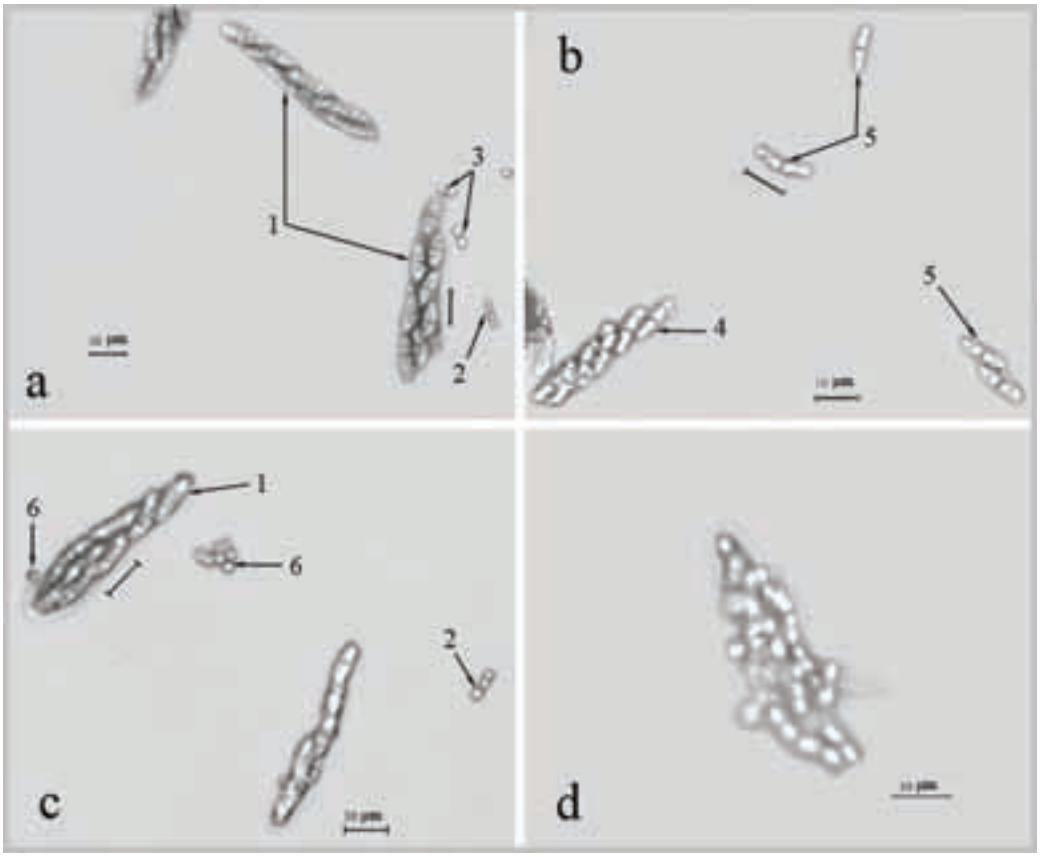

Fig. 1. Structures emerging from a crushed perithecium at their different developmental stages: 1- intact asci with biascospores; 2- ascospores still forming a pair and connected by a thin layer of a common membrane; 3 – bicellular ascospores; 4- a collapsed ascus with biascospores that already have rounded ends; 5- biascospores with rounded ends; 6- unicellular ascospores; d- a collapsing ascus with ascospores (ML2300) x 400.

The transformation of biascospores to ascospores did not happen within an ascus simultaneously, but sequentially: it started at the narrow end and finished at the broad part (from bottom to top). At that period the asci, which developed irregularly within the same perithecium, became fragile because of the thinning of their membranes especially at the sites where the ascospores had already been produced. Thus, the following structures emerged from the crushed perithecium at that period: intact asci with biascospores (Fig. 1a); collapsed asci with biascospores already having rounded ends; ascospores keeping to the arrangement in the form of the ascus already collapsed (Fig. 1d); ascospores still united into a pair by a thin layer of a common membrane (Fig. 1a; Fig. 1c); separate ascospores, generally bicellular and rarely unicellular. 16 ascospores developed in each ascus. No paraphyses were identified. As the last biascospore turned into two ascospores, the ascus membrane collapsed (Fig. 1d). From the preparation generated from a crushed ripe perithecium only free ascospores emerged (Fig. 2). Most of them were bicellular. The quantity of unicellular ascospores is, as a rule, not high and depends on the perithecium formation conditions. For example, during a drought, the number of unicellular ascospores increases.

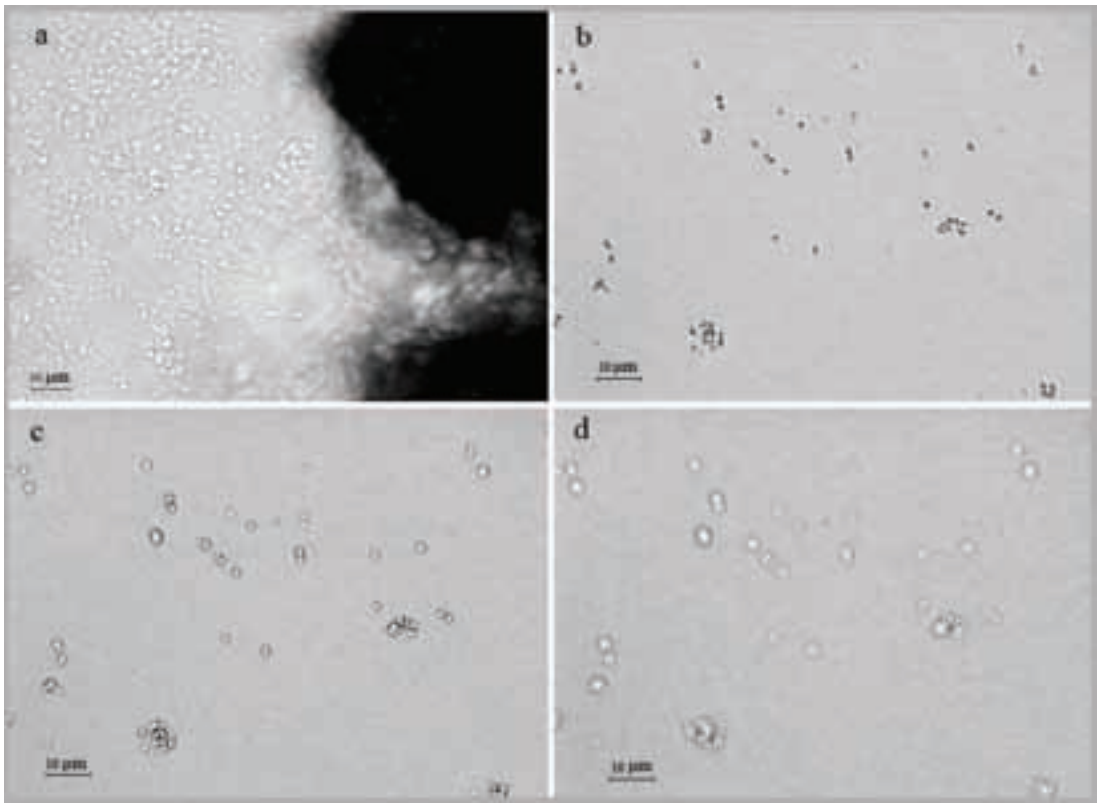

Fig. 2. The fragment of a ripe perithecium of *Diaporthe helianthi* photographed with different sharpnesses of the microscope: a- ascospores emerging from the crushed perithecium, (ML2300) x 400; b- on its surface; c- inside a cell; d- with optical border effect on the membrane and partition, CCD camera x 400.

Before the ascospore emission, a colorless transient sphere (Fig. 3a) with a granular structure of its membrane and smooth surface (Fig. 3b) appeared at the rostrum apex under the pressure of the perithecium walls swollen with water. The ascospores (Fig. 3c) started to be pressed into this sphere. The size of the sphere was from 150 to 200 μm. The color of the filled sphere changed from beige to bright yellow and depended on color and size of fat drops in ascospores, the larger the drops the yellower the sphere (Fig. 3d). The filled spheres became detached from the rostrums and fell into the humid chamber (Fig. 4). Under natural conditions they are caught by ascending air currents and borne by the wind.

Thus, it was determined that 16 ascospores developed in the perithecium ascus of *D. helianthi* which was twice as many as had been considered earlier. Bicellular, or more rarely unicellular, ascospores developed as pairs, one pair per each cell of a bicellular biascospore, and each ascus contained 8 biascospores. The ascospores were pressed by the water-swollen perithecium walls into a colorless sphere, whose membrane had a granular structure and whose surface was not smooth. The infection was transmitted by wind together with the sphere.

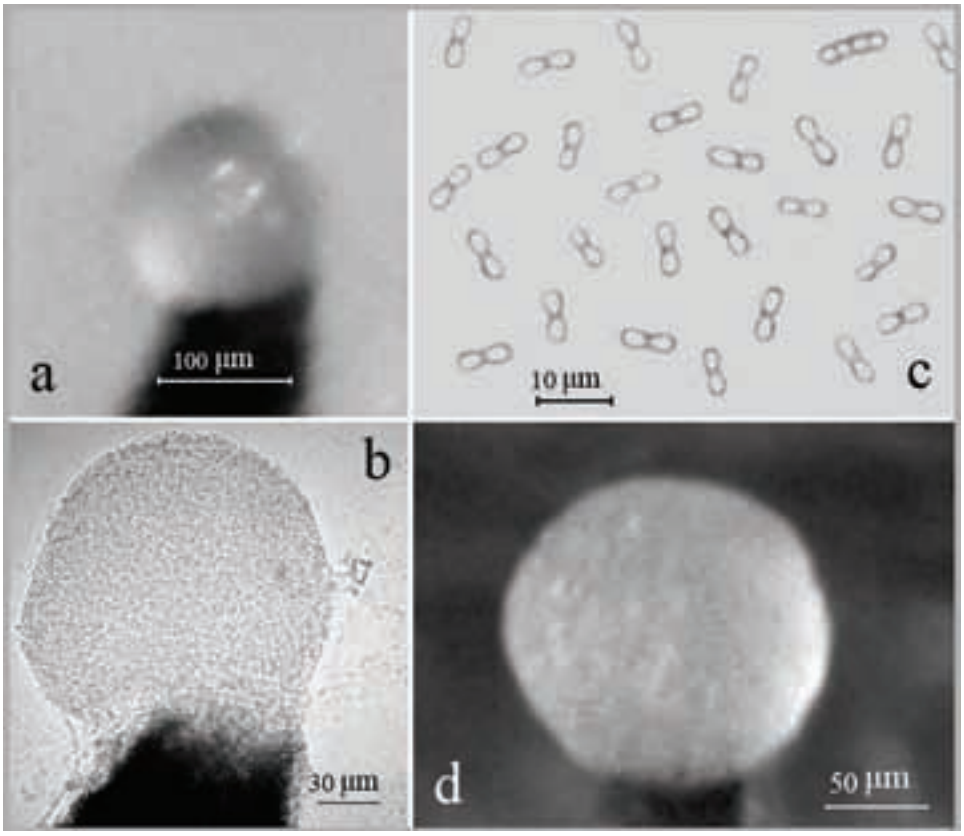

Fig. 3. Apexes of rostrums with spheres: a- an empty sphere under the stereozoom microscope. MLS x 12; b- membrane of an empty sphere. (ML2300) x 400; c- ascospores. (ML2300) x 400; d- a sphere filled with ascospores. MLS x 42.

Fig. 4. Appearance of the *Diaporthe helianthi* infection source from perithecia. MLS x 12: 1- a sphere filled with ascospores; 2 – a sphere detached from the rostrum; 3- rostrum.

DISCUSSION

Earlier unknown phenomena have been demonstrated: spreading the infection source by spheres and a stepwise formation of ascopspores including their development within the larger structures (biascospores) formed earlier in an ascus. According to their structure the biascospores correspond to spores, and depending on their function – to additional asci or capsules as they are not spread from the perithecium and do not affect plants.

The results presented differ from the already known concepts regarding the structure and formation of the *D. helianthi* ascospores. However, they solve the contradictions described in the introduction to this paper. Now there are no doubts that bicellular ascospores have rounded ends, which contain one fat drop and reach about 10 μm in length. It should be noted that the formation of ascospores happens stepwise. This fact was unknown earlier and conventional approaches were used here: some researchers took larger biascospores for ascospores, others – bicellular ascospores with rounded ends. These contradictions resulted in indicating significantly different sizes and forms of the ascospore ends in the world literature.

It is not clear what the reason for this stepwise formation of ascospores within the *D. helianthi* asci is. Why isn't the fungus spread by biascospores that have a thicker membrane (i.e., are better protected)? Evidently, the answer to this question is concealed in the perfect stage characteristics of the fungus.

The infection distribution by spheres does not exclude the emission of ascospores from the perithecium as described above. It is possible that they coincide. The spheres could be easily taken for mucoid drops mentioned in earlier publications. Moreover, the filled spheres subjected to internal pressure easily collapse at the slightest pressure from outside and it is impossible to detect their membranes. However, some empty and full drop-shaped structures were noticed on the rostrum apexes. But a drop cannot be empty – then, it is nothing but a membrane. This phenomenon has been shown by us. The formation of spheres on the rostrum apexes often occurs under nightly temperature drop conditions. It should be noted that the spheres are a very convenient means of transporting ascospores because the infection source is well protected inside them. Due to the spheres there may be a high concentration of spores on the leaves of infected plants. Though the sphere is large and contains a great number of ascospores it cannot land for a long time, as its surface is not even.

ACKNOWLEDGEMENTS

This work was supported by the ISTC Grant #3034. The authors express their gratitude to Vladimir Ismailov, #3034 ISTC Project Manager, for offering an opportunity to conduct this research, as well as to Olesya Khudyakova, Junior Research Scientist, for her assistance.

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Research on a growth chamber test to measure quantitative resistance to sunflower downy mildew

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ABSTRACT

A test on sunflower seedlings in a growth chamber would facilitate measurement of factors determining quantitative resistance to downy mildew compared with the complex field trials necessary at present. Tests based on the usual method to determine major gene resistance were made on genotypes with no efficient major gene. Observations were made of the percentage of plants showing either damping off or sporulation on cotyledons and true leaves, with the aim of representing the percentage of systemically diseased plants observed in field trials. It was found that radicle length needs to be between 3 and 10mm at infection to obtain the closest correlation between field and growth chamber results for inbred lines. For hybrids, there was no significant correlation with field results and hybrids made between one tester line and varied inbreds showed reduced variability compared with per se values. Use of this test to understand quantitative resistance and to improve durability of resistance is discussed.

Key words: germination – *Helianthus annuus* – *Plasmopara halstedii* – quantitative resistance – sporulation

RESUME

Afin de phénotyper des génotypes de tournesol pour leur caractère de résistance partielle non-race spécifique, nous avons recherché un test en laboratoire. L'intérêt du protocole de notation a été validé par comparaison avec le comportement des génotypes observé en infections naturelles en absence de toutes méthodes de lutte culturale ou chimique. Les tests sont réalisés en chambre de culture dans les conditions identiques à celles qui sont utilisées pour la caractérisation des résistances monogéniques dominantes**.** Nous utilisons un pathotype virulent vis-à-vis des génotypes en évaluation. Les observations portent sur l'importance des symptômes: la fonte de semis et l'étendue des sporulations sur les organes aériens. Le taux de plantules présentant des symptômes forts et caractéristiques de mildiou lors du test en laboratoire est bien corrélé avec le taux de plantes montrant une infection primaire (infection tellurique) lors des observations réalisées en plein champ. L'intérêt de ce test en laboratoire pour sélectionner des variétés de tournesol présentant un bon niveau de résistance non race spécifique au mildiou est discuté.

INTRODUCTION

Downy mildew resistance in sunflowers has mainly been based on use of major resistance genes, denoted *Pl*. However, selection pressure on the parasite, *Plasmopara halstedii* has led to the appearance of new races (Tourvieille de Labrouhe et al., 2005) which could cause reduced crop yields in areas where weather conditions at sowing are favourable to the disease (Délos et al., 2000). To obtain more durable resistance, research was made for quantitative resistance and field trials showed high levels of partial resistance in cultivated sunflower which would be useful in breeding (Tourvieille de Labrouhe et al., 2008). However, field trials with downy mildew are complex and limited to the naturally occurring race. To make possible studies of reaction to different races, or large scale early breeding tests, a laboratory test on sunflower seedlings, measuring frequency or extent of downy mildew symptoms, is required. This paper reports experiments measuring the frequency of sporulation on the first true leaves, and the effects of radicle length when infected, for both inbred lines and hybrids. The results were compared with those obtained in field trials.

MATERIALS AND METHODS

Plasmopara halstedii race. Race 710 was shown to be naturally present in the field at Clermont-Ferrand, by observation of differential lines (Gulya et al., 1998). In the growth chamber, the same race was used.

Sunflower genotypes. Two series of genotypes were used (Table 1):

- 44 inbred lines and 45 hybrids obtained from crosses between these lines, considered as representing the variability present in modern cultivated sunflower.
- 40 recombinant inbred lines (RIL) chosen, among a population obtained from a cross between 2 INRA lines, XRQ and PSC8, for their diversity of reaction in downy mildew field trials. They carried either no *Pl* gene or *Pl*2, which is not effective against race 710. Hybrids between these lines and a very susceptible line GB (Vear et al., 2006) were also studied.

Table 1. Numbers of inbred lines and hybrids used in each experiment

Germination stage	Radicle length	Inbred - hybrid comparison	Heredity
44 inbreds	40 RIL	40 RIL	11 inbreds
45 hybrids	40 GB x RIL	40 GBxRIL	28 hybrids

Field trials. Methodology used was that of Tourvieille de Labrouhe et al. (2008). The level of attack of each genotype was defined according to the percentage showing damping off, yellowed leaves or dwarfing, characteristic of primary downy mildew attacks (Tourvieille de Labrouhe et al., 2000). Three to 4 weeks after sowing (cotyledon stage), the number of plants emerged in each plot was counted (including those showing symptoms of damping off). Two to 3 weeks later (2-3 pairs of leaves), the number of healthy plants per plot were counted (rather than the number of diseased plants since some of these had already withered). Percentage infection was then calculated (from 100-% healthy plants).

Growth chamber experiments. Growth chambers, in accordance with quarantine regulations, had 16h light (12000 lux) with a temperature of 18±1°C and 65-90% RH. Methodology was based on that of Roche et al. (2005) for testing major gene resistance. Sunflower seeds with radicle lengths between 1 and 30mm were soaked for 3h in fresh zoosporangia suspensions (100,000/ml), obtained from infected seedlings covered with polythene bags for 48h. For the germination stage trial, germinating seeds were divided into 2 groups: those with radicles of <5mm and those with radicles of >5mm. For the radicle length trial: germinated seeds were photographed before infection to permit measurement of radicle lengths. Seedlings were then pricked out in trays with Klasmann Seedlingsubstrat NF U 44-551 compost.

After 12 days, seedlings were maintained at 100%RH for 48h. Since all genotypes were susceptible to race 710, they all showed some sporulation on the shoot. However, symptom intensity varied and plants were placed in 1 of 3 classes: 1= Damped off (rotting before or after emergence); 2= Sporulation on cotyledons and at least 1 true leaf; 3= More or less sporulation on cotyledons but no sporulation on true leaves. From these observations, the percentage of "completely susceptible" (%CS) was calculated from the sum of classes 1 and 2 compared with the total.

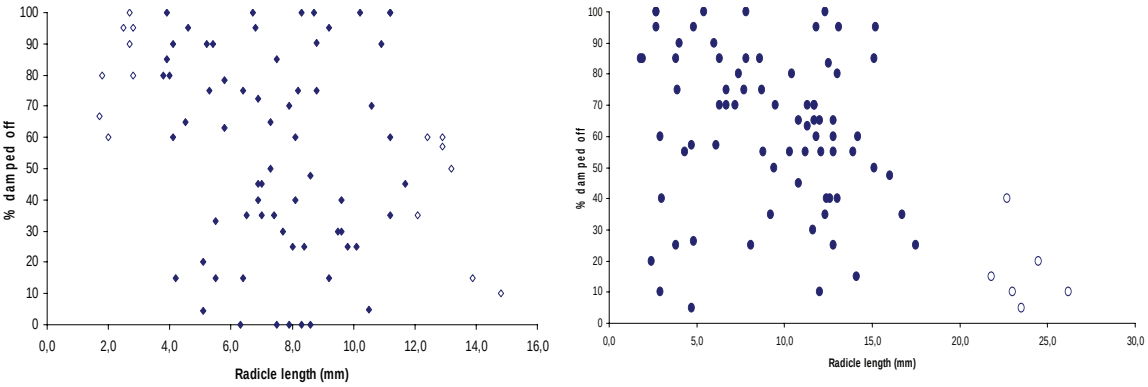
RESULTS

Effect of germination stage. The %CS was significantly higher for seedlings with radicles <5mm (Table 2) but the results of the 2 series were significantly correlated (Pearson correlation): inbred lines r= 0.774****** , hybrids: r= 0.572******. However, 7 inbred lines and 15 hybrids showed more symptoms with radicles >5mm.

Table 2. Percent of seedlings showing complete susceptibility (%CS) according to germination stage when infected

		Radicle length	
		< 5 mm	≥ 5 mm
44 inbred lines			
	Mean	73.1%	58.2%
Extremes			
	inbred F340	100.0	35.7
	inbred MO502	44.0	65.0
45 hybrids			
	Mean	65.2%	52.8%
Extremes			
	hybrid CD x 90R18	100.0	0.0
	hybrid SL x PAZ2	47.6	75.0

Effect of radicle length on proportion of damped off seedlings. Fig. 1a and 1b present the relations between radicle length measured from photographs before infection and proportion of damped off seedlings of RIL and their hybrids, respectively. For the RIL, the 8 genotypes showing the shortest radicles (<3mm) were all damped off at >60% whereas the 8 with radicles of >12mm always showed less than 60% damping off.



a.) b.)

Fig. 1. Proportion of damping off according to radicle length: a.) 40 RIL; b.) 40 hybrids (2 replications)

The hybrids showed a more rapid germination than the inbred lines and there was no clear effect of short radicles, but the hybrids with long radicles (>16mm) again had a low level of damping off (<50%).
Comparison RIL - hybrids: Since germination rate appeared to be important in measurement of quantitative resistance, the vigour provided by hybrid seed could help to provide uniformity between genotypes. The reactions of 40RIL were thus compared with those of their hybrids with GB (Fig. 2). The hybrids showed a greater proportion of CS (68.2%) than the RIL (56.2%), probably related to the high susceptibility of GB. As could be expected from crosses with a single tester line, there was less variation among the hybrids than among RIL, but the results were significantly correlated (r= 0.392*****).

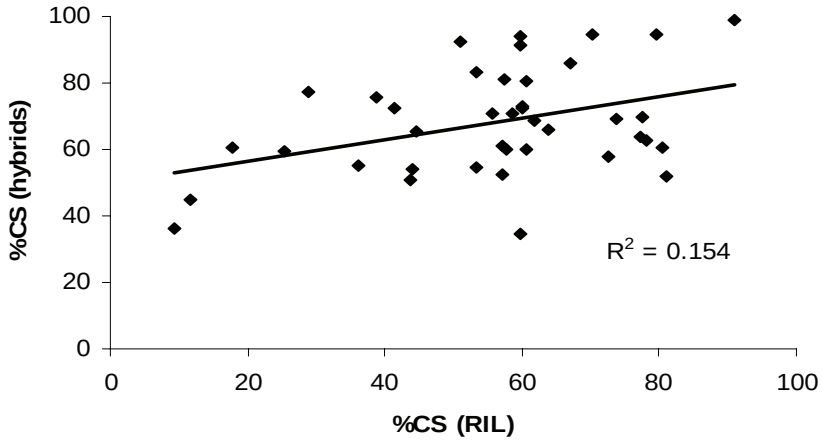


Fig. 2. Relations between 40 RIL and 40 (GB x RIL) hybrids: mean % completely susceptible in 4 tests.

Choice of test conditions according to relations between growth chamber tests and field trials. Fig. 3a and 3b show that when the germinated seed was divided into 2 groups, radicles <5mm or >5mm, the closest correlation between %CS in growth chamber and % infection in the field was obtained with radicles <5mm. For the RIL and their hybrids, where radicle length was measured at infection and long radicles showed little damping off whereas short radicles of inbred lines showed a high level of damping

off, correlations were made with field attack, including and excluding the genotypes concerned. Exclusion of seedlings which had probably not been infected correctly did not exclude extreme reactions and improved the correlation for inbred lines but made no difference for hybrids (Table 3). In addition, when reactions of inbred lines in the field were compared with those of their hybrids under test, there were no significant correlations. It may thus be concluded that to judge inbred lines it is better to test the lines than hybrids made with a single tester genotype.

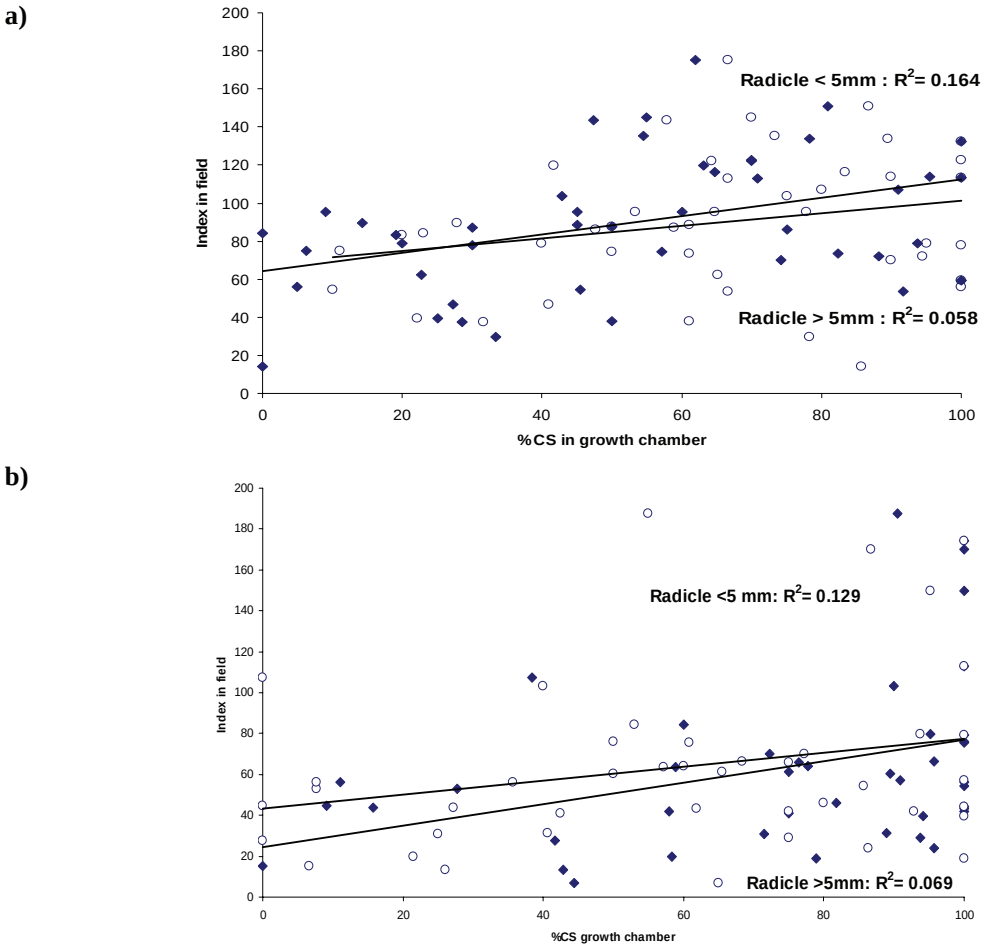


Fig. 3. Comparison of downy mildew attack on 45 hybrids (a) and 44 inbred lines (b) in the field and in the growth chamber test according to radicle length in the test.

Table 3. Correlations between percent downy mildew attack in field trials and in growth chamber tests, including and excluding genotypes with very short or very long radicles when infected in the growth chamber

	Complete series		Excluding genotypes with very short or very long radicles.	
	Nb genotypes	Pearson correlation	Nb genotypes	Pearson correlation
RIL in growth Chamber[(2)] / RIL in field trials[(1)]	40	r= **0.484****	35	r= **0.548****
Hybrids in growth Chamber[(2)] / Hybrids in field trials[(1)]	40	r= 0.188 ns	34	r= 0.274 ns
Hybrids in growth Chamber[(2)] / RIL in field trials[(1)]	40	r= 0.214 ns	35	r= 0.248 ns

[(1)] % attack compared with mean of 4 check lines (4 replications of 30 plants)
[(2)] % completely susceptible plants (%CS) (2 replications of at least 10 plants)

Inheritance of percent completely susceptible plants in growth chamber tests. The 28 hybrids from a factorial cross of 7 female lines and 4 restorers, and the parental lines were tested in the growth chamber. Results are presented in Table 4.

Table 4. Percent completely susceptible plants in growth chamber tests on hybrids of a factorial cross and their parental lines.

Females \ Males	83HR4	PR56	PAZ2	90R18	Mean hybrid value	Inbred line
FU	61.1	4.2	11.1	22.2	24.7	15.8
FRIGA	80.0	42.9	10.0	100.0	58.2	38.5
IR	66.7	78.3	40.9	85.7	67.9	100.0
GX	58.8	31.6	90.0	65.2	61.4	89.5
SL72	100.0	83.3	47.6	100.0	82.7	100.0
GU	86.7	75.0	89.5	95.0	86.5	90.5
HA89	40.0	23.1	20.0	100.0	45.8	100.0
Mean hybrid value	70.5	48.3	44.2	81.2		
Inbred line	100.0	57.9	11.1	95.7		

The female line FU appears best with hybrids showing low percent of completely susceptible plants, and this is also true for the restorers PR56 and PAZ2. The mean parent - hybrid correlation was highly significant (r= 0.617**) (Fig. 4).

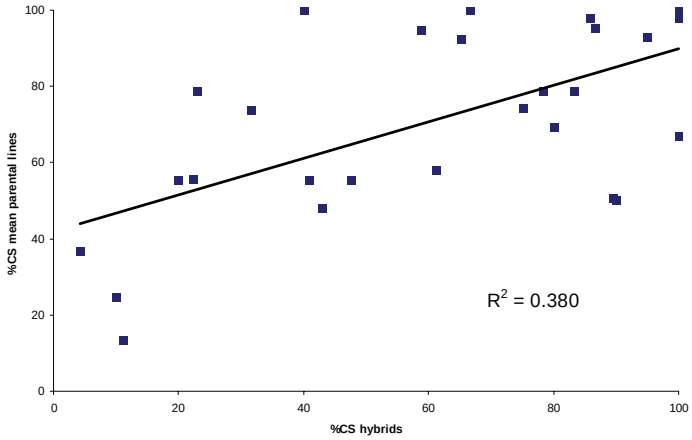


Fig. 4. Relations between percent completely susceptible plants in growth chamber test of hybrids compared with parental inbred lines

DISCUSSION

Horizontal reactions (according to Robinson, 1973) of sunflower genotypes to parasites depend on many environmental factors. It is often difficult to design a laboratory test which gives a good indication of the mean response from multi-location trials over several years in the field (Ladsous et al., 1991; Viguié et al., 2000; Eva, 2002; Serre et al., 2004). However, in controlled conditions, it may be possible to obtain more detailed knowledge of the processes involved in resistance even if the results of a single test do not represent the overall field reaction. The results reported here concern the probability that a systemic downy mildew attack will develop after infection of sunflower seedlings at their most susceptible stage under environmental conditions favourable for the disease.

The percentage of completely susceptible plants for each genotype depends clearly on radicle length at infection. This is probably because the pathogen infects plant tissues through the extremities of root hairs (Allard, 1978) and to provoke symptoms characteristic of systemic infection, it must reach the apical meristem very quickly. The test described here measures this possibility and the results show that for the germination stage to be the most uniform possible, infection should be made of seedlings with radicles

measuring between 3 and 10mm. This measurement of the proportion of completely susceptible seedlings in the growth chamber is indicative of reaction in field trials for many inbred lines, but it does not represent all possible resistance factors. Genotypes such as “IR” and “90R18”, which have high levels of resistance in the field, appear very susceptible in the growth chamber. Factors other than the germination rate are certainly involved; some examples could be resistance to infection or tissue receptivity (Mazeyrat et al., 1999). These cannot be studied in the field and will require additional growth chamber or laboratory tests.

In 2000, changes in downy mildew races in France suggested that sole use of monogenic resistances was a strategic error (Tourvieille de Labrouhe, 2000) and more recently it has been confirmed that major gene resistance alone does not provide durable control of downy mildew (Tourvieille de Labrouhe et al., 2005). Quantitative resistance is most often non-race specific and the levels observed in cultivated sunflowers suggest that this type of resistance should be used in breeding, at least to complement major genes. The test proposed here is a first step in understanding this resistance and making routine breeding programmes for this character possible.

ACKNOWLEDGEMENTS

This study was supported by Promosol and by MAP (Ministry for Agriculture and Fishing) within the framework of the program CAS DAR.

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Can management of *Pl* genes influence aggressiveness in *Plasmopara halstedii* (sunflower downy mildew)?

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ABSTRACT

Evolution of aggressiveness in populations of race 710 of *Plasmopara halstedii* was measured under different strategies of *Pl* gene management: mixture, alternation and monoculture of major resistance genes in comparison with a population under no selection pressure. Two sunflower lines showing different levels of non-race-specific resistance were used to measure four aggressiveness criteria: length of latent period, sporulation density, percentage infection, and hypocotyl length. The sunflower inbred line BT, rather susceptible in the field, presented a higher percentage infection, a higher sporulation density, a lower latent period and less reduced hypocotyl length than inbred line FU, which has greater resistance in the field. Differences were observed between *P. halstedii* populations under different strategies of *Pl* gene management. Strains multiplied under varietal mixtures gave the greatest sporulation densities and shortest hypocotyl lengths, those multiplied under alternation gave a reduced latent period and shorter hypocotyl lengths compared with those not influenced by selection pressure. There were no significant differences between populations multiplied under monoculture of resistance genes and those under no selection pressure. These differences appear to be linked to the number of diseased plants present. The results suggested that the method of *Pl* gene management affects aggressiveness because it determines the number of susceptible plants harbouring the parasite. Applications of these strategies of *Pl* gene management are discussed.

Key words: alternation – mixture– monoculture – pathogenicity – *Pl* gene

RESUME

L'évolution de l'agressivité des populations du profil 710 de *Plasmopara halstedii* a été mesurée sous différentes stratégies de gestion de gènes *Pl*: l'alternance, l'assemblage et la monoculture de source de la résistance en comparaison avec une population n'ayant subit aucune pression. Deux génotypes du tournesol présentant des niveaux différents de résistance non-race spécifique ont été utilisés pour mesurer quatre facteurs de l'agressivité: le taux de réussite de l'infection, la durée de latence, la densité de sporulation et la longueur de l'hypocotyle. Les souches récoltées sous les systèmes de l'alternance et de l'assemblage présentent des durées de latence les plus courtes significativement sur le génotype résistant et des longueurs de l'hypocotyle les moins grandes sur le génotype le plus sensible par rapport aux souches multipliées sous la monoculture de source résistant. De plus, les souches récoltées sous le système de l'alternance présentent des densités de sporulation plus élevées sur les deux génotypes. Cette évolution semble directement liée à la présence de nombreuses plantes malades dans ces dispositifs. Nos résultats suggèrent l'existence d'un impact du mode de gestion des gènes *Pl* sur l'évolution de l'agressivité. Seules les stratégies qui maintiennent des effectifs de la population parasitaire assez élevés permettent une évolution de l'agressivité de *P.halstedii*. Les résultats sont discutés aux regards à la mise en œuvre de ces méthodes de gestion.

Mots-clés: alternance – assemblage – monoculture – pathogenicité – *Pl* gene

INTRODUCTION

Selective effects on pathogenicity due to host resistance are an important aspect of plant-pathogen interactions, which can be divided into two parts: virulence (specific disease-causing abilities) and aggressiveness (non-specific disease-causing abilities) according to Van der Plank (1968). There have been many reports concerning increase of virulence in relation to host resistance in pathogens of economically important crops (McDonald and Linde, 2002). Similarly, Gandon and Michalakis (2000) predicted that increased levels of quantitative host resistance may select for increased aggressiveness of parasites, leading to increased crop losses. Cowger and Mundt (2002) showed that wheat cultivars with good partial resistance selected more aggressive isolates of *Mycospharella graminicola*. However, this is not always true, Sullivan et al. (2005) reported that tobacco cultivars with high levels of quantitative resistance did not select for more aggressive isolates of *Phytophthora parasitica* var. *nicotiana*e. Also, Flier et al. (2007) showed that, following large-scale introduction of more resistant potato varieties in organic production systems in Europe, there was no shift towards increased levels of aggressiveness of *Phytophthora infestans* populations.

Plasmopara halstedii (downy mildew) is a pathogen specific to sunflower, present in most areas of the world where this crop is grown. It shows physiological races (pathotypes) capable of infecting a variable range of sunflower genotypes. The nomenclature of these races is based on the reaction of a series of differential lines (Gulya et al., 1998). Race specific resistance is controlled by major genes, denoted *Pl*. Tourvieille de Labrouhe et al. (2005) showed that whatever the method of management (mixture, alternation, monoculture) of *Pl* genes, their selection pressure led to appearance of new virulences.

This paper reports studies of levels of aggressiveness in 3 populations of *P. halstedii*, race 710, obtained under different strategies of *Pl* gene management: mixture, alternance and monoculture, in comparison with a population obtained in the absence of any effective *Pl* gene.

MATERIALS AND METHODS

Sunflower genotypes

Four quasi-isogenic hybrids were used, obtained from crosses of 2 forms each of two inbred lines:

- L1a: carrying resistance gene *Pl2*, resistant to race 100 and susceptible to race 710,
- L1b: carrying resistance genes *Pl2* and *Pl8*, resistant to races100 and 710,
- L2a: carrying no known resistance gene,
- L2b: carrying resistance gene *Pl6*, resistant to races 100 and 710.

The four hybrids were produced as follows: H1= L1a x L2a, H2= L1a x L2b, H3= L1b x L2a and H4= L1b x L2b.

P. halstedii strains present in the soil were trapped with a sunflower hybrid (Airelle), carrying no downy mildew resistant gene. To characterise aggressiveness of *P. halstedii* strains, two inbred lines not carrying any *Pl* gene and known to have different levels of non race specific resistance (Vear et al., 2007) were studied: FU and BT.

Experimental protocol

The protocol was developed by Tourvieille de Labrouhe et al. (2005) to determine durability of resistance. Four plots constituted by netting cages were maintained with climate conditions favourable for expression of disease. Plot P1 was planted in four consecutive years with H1 (no effective resistance against race 710). Plot P2 was planted all years with an equal mixture of the four hybrids. Plot P3 was planted in first year with H1, then successively with H2, H3 and H4. Plot P4 was planted with H2, resistant to race 710, in all 4 years.

P. halstedii *strains*

After 4 years, *P.halstedii* strains were collected from soil according to the method described by Tourvieille de Labrouhe et al. (2008) and their virulence profile characterised by the method of Gulya et al. (1998). For plots 1, 2 and 3, four strains were analysed and for P4, 3 strains.

Measurements of aggressiveness

The protocols developed by Sakr et al. (2008) were used, determining:

- Length of period between infection and sporulation on 80% of infected plants = latent period,
- Maximal sporulation density on cotyledons obtained 12 and 13 days after infection = sporulation density,

- Percentage infection = % infection,
- Hypocotyl length 13 days after infection, calculated by a percentage of the hypocotyl length of healthy plants = hypocotyl length.

All tests were carried out in growth chambers respecting European regulations (No 2003/DRAF/70).

Statistical Analyses

All statistical analyses of the phenotypic data were performed using StatBox 6.7® (GimmerSoft) software. To compare strains and genotypes, there were 2 replications for sporulation density and 3 replications for percentage infection, latent period and hypocotyl length. To compare each characteristic in the different plots, the means of each strain were used as replications in one-way analyses of variance (ANOVA). The Newman-Keuls test was used to compare the means at P=0.05

RESULTS

Changes in percentage attack in the 4 plots

Data are presented in Table 1.

Table 1. Changes in downy mildew attack in 4 plots observed over 5 years.

Plots	**2001**[1]	**2002** (*)	**2003** (*)	**2004** (*)	**2005**
P1					
% diseased plants	71.5	37.4	75.4	60.3	
Number of diseased plants	203	125	215	194	
% of race 710	100	100	100	100	84.0
P2					
% diseased plants	13.9	6.5	9.9	15.1	
Number of diseased plants	43	19	33	51	
% of race 710	100	100	81.0	91.3	48.9
P3					
% diseased plants	75.2	1.1	1.5	1.1	
Number of diseased plants	236	4	5	11	
% of race 710	100	100	100	9.1	12.5
P4					
% diseased plants	2.7	1.1	4.9	14.8	
Number of diseased plants	10	4	16	52	
% of race 710	100	100	16.7	30.0	34.5

[1]Tourvieille de Labrouhe et al. 2005

Table 1 shows that total numbers of diseased plants differed between plots (from 737 for plot P1 to 82 for plot P4). There was a continued reduction in percentage of *P.halstedii* samples of race 710 especially in the absence of susceptible sunflower genotypes in plots P3 and P4. Nevertheless this race was present in soil samples taken in 2005 from all plots.

Comparison of aggressiveness of 15 strains of race 710 on inbred lines FU and BT

The two sunflower lines gave a significantly different response (Table 2).

Table 2. Anova on aggressive criteria of 15 strains of *P. halstedii* measured on two sunflower lines.

% infection	Line effect			Strain effect			Interaction		
	BT	FU	Significant	Mini	Maxi	Significant	Min.	Max.	Significant
	100%	99.3%	P<0.001	98.6%	100%	NS	97.2%	100%	NS
Sporulation density (zoosporangia per cotyledon)	963 10^5	788 10^5	P<0.001	677 10^5	1264 10^5	P<0.001	562 10^5	1343 10^5	NS
Latent period (days)	8.1 d.	9.0 d.	P<0.001	8.3 d.	8.9 d.	NS	7.8 d.	9.7 d.	NS
Hypocotyl length (% of length of healthy plants)	33.0%	40.1%	P<0.001	31.1%	40.3%	NS	26.7%	43.7%	NS

The inbred line BT showed a higher percentage infection, a higher sporulation density, a shorter latent period and less reduced hypocotyl length than FU. The 15 strains appeared as being homogeneous for all criteria analysed except spore density. There was no interaction between parasite strains and host genotypes.

Comparison of strain aggressiveness in each plot

Plot P4 was not distinct from P1 whereas P2 presented greater mean sporulation density and reduction in hypocotyl length, and P3 showed a shorter latent period and greater reduction in hypocotyl length (Table 3).

Table 3. Comparison of means observed for isolates from each plot compared with P1 (no effective *Pl* gene).

	% infection		Latent period (days)		Sporulation density (zoosporangia per cotyledon)		Hypocotyl length (% of length of healthy plants)	
	mean	reference[1]	mean	/reference[1]	mean	/reference[1]	mean	/reference[1]
P1 (reference)	99.65		8.83		8.15		40.00	
P2	99.84	NS	8.55	NS	10.89	S	35.11	S
P3	99.86	NS	8.41	S	8.30	NS	35.10	S
P4	99.09	NS	8.71	NS	7.32	NS	38.03	NS

[1]Test of Newman Keuls, P=0.05

DISCUSSION

The presence of strains of race 710 in plots not grown with a susceptible genotype for 3 (P3) or 4 years (P4) trapped by a susceptible genotype in soil tests may be explained by the maintenance of the inoculum in the soil and/or hybrid seed impurities susceptible to isolates sampled in 2005. With the first hypothesis, the evolution of parasitic populations may depend on characters linked to fitness but independent of aggressiveness, such as their capacity to survive for a long time as oospores. With the second hypothesis, the level of susceptible seed impurities would be the important factor which intervenes in the evolution of parasitic populations.

Study of the reaction of two inbred lines to 15 strains underlined their differences in behaviour. The very good resistance of inbred line FU observed in the field was confirmed by the measurements of aggressiveness criteria described by Sakr et al. (2008). These methods can be used to characterise non-race-specific partial resistance since there were no interactions between genotypes and strains. For the 15 strains analysed, only sporulation density varied (from 1 to 2), overall, the *P. halstedii* strains appeared to be quite homogeneous.

Comparison of parasite populations isolated from the 4 plots showed that strains of race 710 from plot P4 (monoculture of *Pl*6) were not different from the population isolated from P1, with no efficient *Pl* gene. This could be explained on one hand by selection of strains which survive in the soil, independently from the factors of aggressiveness measured, or, on the other hand, by a weak level of parasitic multiplication linked to a small number of plants susceptible to race 710, thus giving incomplete expression of parasitic diversity. This second hypothesis appears most likely because the number of plants infected with race 710 was always very low in plot P4. Plot P2 was grown with a mixture of different hybrid forms, giving 25% of plants susceptible to race 710, one third of which contributed to parasitic multiplication (Table 1). Compared with plot P1, and with few infected plants, it is reasonable to suggest that isolates with a high sporulation capacity could have been favoured and may have caused the secondary infections shown by 20% of infested plants in this plot between 2001 and 2004 (Tourvieille de Labrouhe et al., 2005). These secondary infections contributed to the stock of inoculum which may explain why strains isolated from this plot showed a significantly higher sporulation density. In plot P3 (alternation), the abundant downy mildew population created in the first year, from more than 230 diseased plants, was confronted with new resistance genes every year but race 710 remained in 2005, although at a lower level than in the other 3 plots. This population evolved towards increased aggressiveness as measured by latent period. Compared with plot P4, it had a wider genetic base. Differences in aggressiveness, as compared with plot P1, were weak but significant for latent period, suggesting that, from a similar number of diseased plants, different aggressiveness factors could be

selected if the number of diseased plants is small. The 2 plots that significantly differed for either latent period or sporulation density (i.e., P3 and P2) also differed for hypocotyl length.

It is commonly admitted that non-race specific partial resistance applies selection pressure on parasitic populations, which may lead to more aggressive strains. An example was maize resistance against *Cochliobolus heterostrophus* (Kolmer and Leonard, 1986). In contrast, many authors report that use of race specific resistance does not lead to modifications in aggressiveness. Sullivan et al., (2005) showed that race specific resistance in tobacco did not exert a selective effect on aggressiveness of *Phytophthora parasitica* var. *nicotiana*e and in the pathosystem *Venturia inaequalis* / apple, Parisi et al. (2004) found that virulent strains taken from cultivars carrying vertical resistance genes were highly aggressive. Since the four sunflower hybrids in the present study were isogenic except for their *Pl* genes, it appears reasonable to consider that selection pressure was mainly applied on criteria linked to virulence (Tourvieille de Labrouhe et al., 2005). The results obtained showed positive effects of certain modes of *Pl* gene management on aggressiveness factors. This effect no doubt depends more on the number of susceptible plants than on direct selection pressure of monogenic resistances. It could be suggested that management of *Pl* genes which reduce the number of susceptible plants, limits selection pressure for more aggressive strains, but increases the risk of appearance of new virulence. In contrast, management modes which lead to a non negligible number of diseased plants (mixtures and alternation), may slow down the appearance of new virulence (Tourvieille de Labrouhe et al., 2005), but could favour more aggressive strains. This conclusion must be taken into account in the choice of methods to obtain durable control of sunflower downy mildew with both race-specific and non-race-specific resistance.

ACKNOWLEDGEMENTS

We would like to thank the Syrian Commission of Energy Atomic for a Doctoral Scholarship favouring the first author. This work was carried out with the financial support of the ANR- Agence Nationale de la Recherche (The French National Research Agency) under the Programme Agriculture et Développement Durable, project “ANR-05-PADD-0705, Cedre”, and the French Centre Technique Interprofessionnel des Oléagineux Métropolitains (CETIOM).

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Morphological and molecular identification of *Diaporthe helianthi* from *Xanthium italicum*

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ABSTRACT

Up to the present time, *Diaporthe helianthi* has not been reported outside the genus *Helianthus* in Croatia. This pathogen has been recently recovered on *Xanthium italicum* (cocklebur) in Slavonia and Baranja County. Isolates of *Diaporthe* sp. originating from *X. italicum* were studied and compared with *D. helianthi* isolates from sunflower. Phylogenetic analysis of nuclear ribosomal DNA internal transcribed spacer sequences (ITS1 and ITS2) showed that *X. italicum* is a new host for *D. helianthi*.

Key words: *Diaporthe helianthi* – identification – *Xanthium italicum*.

INTRODUCTION

The cultivated sunflower (*Helianthus annuus* L.) is the most important annual crop grown for edible oil in Croatia. Stem canker caused by *Diaporthe helianthi* Munt.-Cvet. et al. (anamorph *Phomopsis helianthi* Munt.-Cvet. et al.) is one of the most important sunflower diseases. The pathogen was identified in former Yugoslavia for the first time in 1980 (Mihaljcevic et al., 1980; Muntanola-Cvetkovic et al., 1981) and nowadays this disease has spread to many other countries. The fungus can cause yield losses up to 40% (Demazure, 1995) and reduce oil content (Franco and Morales, 1997) in the case of environmental conditions being favourable for infection.

The occurrence of fungi from *Diaporthe*/*Phomopsis* genera on weeds has been studied over a long period. Weeds as alternative hosts of *Diaporthe*/*Phomopsis* have a very important role as a potential source of inoculum for cultivated plants (Roy et al., 1997; Mengistu and Reddy, 2005; Vrandecic et al., 2006). Mihaljcevic and Muntanola-Cvetkovic (1985) reported *Phomopsis* spp. on 15 plant species, among which were *Xanthium strumarium* L. and *X. italicum* Moretti. Nikandorow et al. (1990) determined *X. spinosum* L., *X. orientale* L. and *X. occidentale* L. to be hosts of *Phomopsis* species. Muntanola-Cvetkovic et al. (1996) identified two *Diaporthe/Phomopsis* species on *X. italicum*. Until recently, sunflower was the only known host for *D. helianthi*. Piven' et al. (2000) stated that *Cyclachaena xanthiifolia* (family *Astraceae*) are potential alternative hosts for *Phomopsis arctii* (Lasch) Nitschke (*Diaporthe arctii*) and *P. helianthi*. This paper presents results of the study on morphological, cultural and biomolecular characterization to identify *Diaporthe/Phomopsis* isolate obtained from *X. italicum*.

MATERIALS AND METHODS

Isolates used in this study (Table 1) were obtained from naturally infected living plants or overwintered residues of *X. italicum* and sunflower plants from location in Slavonia and Baranja County (Croatia). *X. italicum* plant tissues with symptoms of infection with *Diaporthe*/*Phomopsis* were disinfected and small tissue pieces were placed in Petri dishes on moist filter paper or directly on potato dextrose agar (PDA). Petri dishes were kept at 25°C with 12 h light/dark regime. Isolation of *D. helianthi* isolates was performed by transferring mycelia or pycnidia with conidia exudates on PDA. In order to examine microscopic features and cultural characteristics, pure cultures were kept in a thermostat (25°C, 12 h light/dark). Morphological and cultural characteristics of *Diaporthe/Phomopsis* isolates from *X. italicum* were compared with isolates of *D. helianthi* from sunflower.

DNA extraction was made following Cenis (1992). The standard PCR conditions for ITS475 primers are described in White et al. (1990). Purified PCR products were sequenced in both directions using primers ITS4 and ITS5 (Gene Lab – ENEA, Roma). Sequences were aligned by CLUSTAL W (Thompson et al., 1994) and manually adjusted by Chromas (version 1.45). Additional *Diaporthe/Phomopsis* sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov) and added to alignment. The *Colletotrichum coccodes* (Wallr.) Hughes and *Colletotrichum dematium* (Pers.) Grove were included as an outgroup. Alignment gaps were treated as missing data. Phylogenetic analysis was

conducted by UPGMA (Kimura 2-parameter model) methods using MEGA version 3.1. (Kumar et al. 2004). Bootstrap analysis for 1000 replicates was done to evaluate tree topologies.

Table 1. Isolates used in this study

Isolate	Species	Host	Origin	Reference	GenBank numbers
CBS592.81	*D. helianthi*	Sunflower	ex Yugoslavia	Rekab et al. (2004)	AY705842
IMI318865	*D. helianthi*	Sunflower	ex Yugoslavia	Rekab et al. (2004)	AJ312363
Dh95004	*D. helianthi*	Sunflower	France	Say-Lesage et al. (2002)	AF358438
Dh95016	*D. helianthi*	Sunflower	France	Say-Lesage et al. (2002)	AF358435
F1	*D. helianthi*	Sunflower	France	Rekab et al. (2004)	AJ312350
A3	*D. helianthi*	Sunflower	Argentina	Rekab et al. (2004)	AJ312364
Xa3	*D. helianthi*	*X. italicum*	Croatia	This study	
Xa5	*D. helianthi*	*X. italicum*	Croatia	This study	
Su5/04	*D. helianthi*	Sunflower	Croatia	This study	
Su12/05	*D. helianthi*	Sunflower	Croatia	This study	
978	*C. coccodes*	Pepper	Italy		AM422215
AR3563	*C. dematium*	*Lirope muscarii*	Mexico	Farr et al. (2006)	DQ286154

RESULTS AND DISCUSSION

After 8 days, colonies of *Diaporthe/Phomopsis* from *X. italicum* on PDA formed less abundant white mycelium, the aerial part plenty of it, sometimes with narrow greenish-yellow areas. Reverse of culture was whitish to beige color and had in the beginning light brown scattered spots, which later turned dark brown. The pycnidia formed in simple stromatic structures usually aggregate, rarely solitary, measuring 240-450 x 230-380 µm. Conidia only of ß-type, 24.4 x 1.8 µm. After 30-40 days isolates from *X. italicum* (Xa3 and Xa5) and isolate Su5/04 from sunflower formed spare globose perithecia. Biometrical values of perithecia (isolates from *X. italicum*) were 290 x 280 µm. Asci hyaline, elongated-elliptical, 8-spored, 37.1-59.8 x 5.8-10.5 µm (av.= 47.3 x 7.8), ascospores irregularly biseriate, subelliptical, slightly constricted at the septum, 1-septate, 9.2-16.2 x 2.2-5.5 µm (av.= 12.5 x 3.2). Comparing cultures characteristics and biometrical values of *Diaporthe/Phomopsis* from *X. italicum* and *D. helianthi* from sunflower, no differences were determined.

Comparing our isolates from Croatia with the *Phomopsis* sp. isolates from *X. italicum* (XIT-2) described by Muntanola-Cvetković et al. (1996), similarities are established in symptoms, pycnidia formation and biometrical values of ß conidia, as well as in development and the characteristics of teleomorphic stage. Our isolates did not form pycnidia containing α conidia either in natural environment or in laboratory. Muntanola-Cvetković et al. (1996) found this type of conidia in the majority of cultures, although always in a small number.

The sequence analyses of rDNA-ITS *Diaporthe/Phomopsis* isolates using UPGMA methods revealed that our isolates from *X. italicum* (Xa3 and Xa5) group together (100% bootstrap support) with the isolates of *D. helianthi* from sunflower (Su5/04 and Su12/05) and with *D. helianthi* isolates from former Yugoslavia (AJ312363 and AY705842), France (AF358438, AJ312350 and AF358435) and one from Argentina (AJ312364) which Rekab et al. (2004) marked as *D. helianthi s. str*. All isolates from France and former Yugoslavia originated from countries where severe epiphytotics of sunflower stem canker were reported. On the basis of morphological and molecular characteristics, the fungi isolated from *X. italicum* were identified as *D. helianthi*.

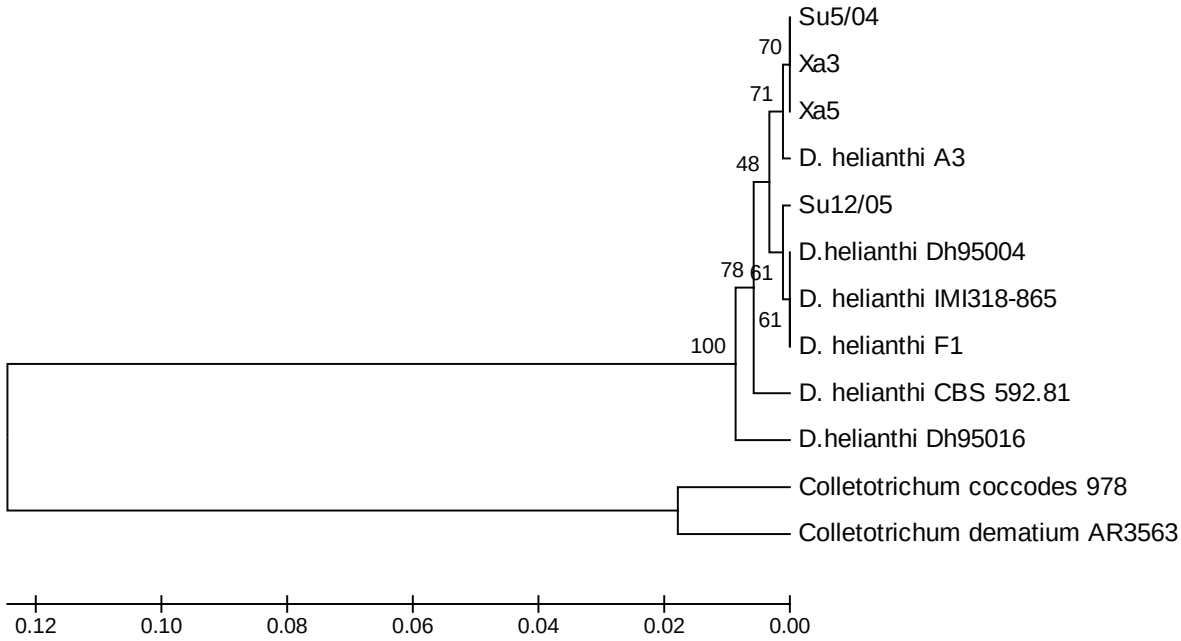

Fig. 1. Molecular phylogenetic tree based on ITS1-5.8S gene-ITS2 sequences using UPGMA -Kimura 2-parameter model. Numbers above each branch represent percentages of 1000 bootstrap repetitions. *C. coccodes* (AM422215) and *C. dematium* (DQ286154) were used as an outgroup. The scale bar shows the number of substitutions per site.

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Origins of major genes for downy mildew resistance in sunflower

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ABSTRACT

New sources of major gene resistance to sunflower downy mildew were compared with known resistance genes. All genes appear to come from crosses with wild *Helianthus*, and most frequently from wild *H. annuus*. The gene *Pl6* has been found in many different ecotypes but resistances which segregate independently from this gene have also been obtained. Genes considered as different may be the result of intra-cluster recombinations. Only 1 or perhaps 2 genes have been obtained from *H. argophyllus*. Identification of genes from *H. tuberosus*, is not complete, possibly because these sources show downy mildew sporulation on cotyledons. Some other annual species also show major gene type resistances. It is concluded that knowledge of these sources is important, both for their use in breeding and also to distinguish between major gene and quantitative resistance.

Key words: introgression – *Pl* genes – *Plasmopara halstedii* – resistance tests – segregation.

INTRODUCTION

Over 40 years, there has been considerable search for new genes giving resistance to new downy mildew races when these appear. Well known genes have been shown to be clustered: firstly *Pl1, Pl2, Pl6* and *Pl7* (Mouzeyar et al., 1995; Roeckel-Drevet et al., 1997; Mestries et al., 1998) on LG 8 (Cartisol LG1), secondly *Pl5* and *Pl8* (Bert et al., 2001; Radwan et al., 2004), on LG13 (Cartisol LG6) and more recently *Plarg* (Dussle et al., 2004) on LG1 (CartisolLG13). The *Pl2/Pl6* cluster was found to have a structure TIR-NBS-LRR (Bouzidi et al., 2001), typical of specific resistance genes producing hypersensitive reactions whereas the *Pl5/Pl8* cluster was shown to be NBS-LRR-CC (Radwan et al., 2002). *Pl6* has been found to show within-gene segregation leading to separation of resistances to races 703 and 710 from those for races 100 and 304 (Vear et al., 1997). Many other ecotypes of wild *H. annuus* or other wild species, in particular *H. argophyllus* have been tested and resistances introgressed into cultivated sunflower. This requires a large effort, and before being able to say whether a gene is new, it is necessary to have the homozygous form, and then make crosses with known genes. So the question may be asked as what wild species give the greatest probability of finding new and useful genes. This paper compares origins in cultivated sunflower, wild *H. annuus* and *H. argophyllus* and some other species, both from the bibliography and from recent genetical analyses made by INRA.

MATERIALS AND METHODS

Material: All the cultivated sunflower lines: resistance sources, race differentials and susceptible lines used for test crosses are maintained by INRA, together with collections of wild *H. annuus* and other *Helianthu*s species, interspecific pools and introgressions. For genetical analyses, it was necessary for lines to be homozygous for downy mildew resistance and to produce sufficient seed for tests to be made with several downy mildew races. Test cross progenies were obtained by crossing new resistance sources with lines carrying known genes and then crossing the F_1 hybrid with a completely susceptible line. These progenies were then tested to determine whether they segregated for downy mildew resistance (no segregation: same gene or closely linked; 3R/1S: 2 independent genes).

Downy mildew resistance tests: Classic seedling tests on germinated seed (Tourvieille de Labrouhe et al., 2000) were carried out in growth chambers approved for manipulation of downy mildew races observed in France. Large scale tests, with 100-300 seedlings per progeny, to determine genetical segregation were made with races 710, 304, and 703. Test of resistance to other races were made on 20-30 plants/genotype, with races 100, 314, 334, 704 and 714.

RESULTS

Resistance sources: Table 1 presents knowledge of origins and *Pl* genes in the species most commonly used as resistance sources.

Resistance from cultivated sunflower: In these sources, resistance did not come directly from wild *Helianthus*. However, the ancestors reported suggest that downy mildew resistance came from crosses with wild species at some time, although there are some specificities that have not appeared in recent crosses. The lines nearly all appear to be traceable to a Canadian line 953 involving wild *H. annuus* (Fick and Zimmer, 1974), with crosses made either at Morden or by INTA in Argentina. *Pl1* came from 953-102-1-1, and was included in varieties such as Advent, from which Vrânceanu and Stoenescu (1970) obtained AD66, but also Rha265 and RHA266. *Pl2* came from both 953-102 giving RHA274, and 953-88 as HA61. *Pl2* was widely used, but new sources have not been reported, perhaps they have not been retained since they do not provide resistance to recent downy mildew races. Although partly from the same origin, downy mildew resistance from Argentinean populations appears quite specific because, at least in the resources held and lines developed at INRA, they are all resistant to race 710 but susceptible to race 703. According to the ancestry detailed by Romano and Vazquez (2003), resistance in the open pollinated variety Guayacan, and so the pool USDA HAR5 and INRA line QHP1, also came from the Canadian line 953-102. In contrast, resistance in the varieties Charata (which gave HAR4) and Caburé probably came from an Argentinean interspecific pool with Russian open pollinated varieties crossed with *H. annuus*, *H. argophyllus* and *H .petiolaris* in 1955/56. It seems likely that in the multiplication of these open pollinated varieties some intercrossing must have occurred, to have spread the gene which we refer to as "Pl_{QHP1}". In the development of the USDA or INRA lines, resistance to race 710 was certainly retained because that was the most useful resistance, but the combination with susceptibility to race 703 is quite specific and not known for any other resistance source. This resistance segregates as a single dominant gene, giving clear resistance; it also segregates in test crosses with *Pl6* (in spite of apparently having a common origin with *Pl1* and *Pl2)* and also *Pl5*, and *Plarg* (Table 1). Bulk segregant analyses have been made, but this gene has not yet been located on a linkage group.

Resistance directly from wild Helianthus annuus

(i) Pl6: Miller and Gulya (1991) developed HA335 and HA336 from crosses with wild *H. annuus* from Texas with resistance to all known races except those named "xx4", such as 304, 314 and 334, observed in France. Their resistance gene *Pl6* was located in the cluster with *Pl1* and *Pl2*. More recently, in collaboration with J. Miller, we found that 2 other USDA origins probably also carry *Pl6* (Table 1), both being resistance to race 710 and susceptible to race 304: "TP5", from a Californian *H. annuus* and HA459 (from a Texas *H. annuus)*. Some origins from crosses made at Montpellier, with wild *H. annuus* in the 1990s also appeared to carry *Pl6*, for example ecotype MPHE-519 from Arizona More recently, further crosses have been made with a wide range of ecotypes and tests were made with race 710 and then race 304 to search for useful *Pl* genes. The results of these tests are presented in Table 2. Thirty two progenies out of 129 showed some resistance to race 710. Nine of the 22 origins with some resistance and sufficient seed for further tests showed susceptibility to race 304. The ecotypes concerned came from different parts of the US: California, Arizona, Nebraska, New Mexico and Texas. It was concluded that they probably carried *Pl6*, so it seems that this gene is very widespread in wild *H. annuus* populations.

(ii) Not *Pl6*: At Montpellier, 2 lines developed from wild *H. annuus* ecotypes MPHE-361 (from Wyoming) and MPHE-829 (from Iowa) have been shown to be resistant to all races tested. Their resistance segregates independently of the *Pl6*, *Pl5/8* and *Plarg* clusters (Table 1). These genes are being mapped and the lines are available to breeders. J. Miller developed HA458, also resistant to all known races from a cross with a *H. annuus* ecotype from Idaho. The resistance gene appears independent of all known clusters (Table 1), and is also being mapped. It is not yet known whether these 3 sources carry the same or different genes, but they certainly appear different from *Pl6.*

Among the crosses made more recently at Montpellier and continued in research since resistant to both races 710 ands 304, there are origins from 13 ecotypes, from: Texas, Wyoming, Oklahoma, Utah, Kansas, Colorado and California (Table 2). It may be some time before the resistance genes they contain are identified as many show considerable self-sterility and require further crosses to cultivated sunflower to obtain sufficient seed by selfing to be able to demonstrate homozygous resistance and so be able to make test crosses and prepare material for mapping. Among these origins, it would be interesting, and quite logical, to find a "*Pl6+"*, which has the whole *Pl6* cluster in resistant form, combining the resistances of *Pl1*, *Pl2* and *Pl6*. Or there is something not known about the structure of this cluster which would make such a form non-viable.

Table 1. Origins of sunflower downy mildew resistance genes and results of test crosses to determine whether they segregate independently

Source line/pool	Origin	Resistance to main French races	gene	Test cross segregations* or publication				
				HA335	XRQ	RHA419	QHP1	PMI3
Resistance from cultivated sunflower								
AD66	953-102-1-1	100	*Pl1*	Vrânceanu and Stoenescu, 1970				
RHA265/266	953-102-1-1	100	*Pl1*					
HA60	953-102-1-1	100	*Pl1*					
RHA274	953-102-1-1	100,304,334	*Pl2*	Zimmer and Kinman, 1972				
HA61	953-88	100,304,334	*Pl2*					
Guyacan/HAR5	953-102-1-1	100,304,314, 334,710,714	Pl_{QHP1}					
QHP1	HAR5 x PRS7*(Pl1*)	100,304,314, 334,710,714	Pl_{QHP1}	70/310	79/348	83/329	--	70/270
Charata/HAR4/ Caburé	(Russian pool x wilds)	100,304,314, 334,710,714	Pl_{QHP1}					
Resistance directly from wild *H. annuus* (*Pl6*)								
HA335/336	HA89x*H.ann*. 423/432 (Texas)	100,703,710	*Pl6*	Miller and Gulya, 1991; Roeckel-Drevet et al., 1996				
HA458	HA434x*H.ann.* (Texas)	100,703,710	*Pl6*	0/292	78/254			
"TP5"	HA434x*H.ann.* (California)	100,703,710	*Pl6*	0/430	98/376			
"MPHE-519"	MPHE-519 x 90R19	100,703,710	*Pl6*	0/153	13/98			
not *Pl6*								
HA458	HA434x*H.ann.* (Idaho)	100,304,314, 334,703,704, 710,714	*Pl?*	133/523	74/223	22/82	16/94	
MPHE-361	90R19x*H.ann.* (Wyoming)	100,304,314, 334,703,704, 710,714	*Pl?*	76/348	87/330	48/267	36/167	52/275
MPHE-829	RT1B11x*H.ann.* (Iowa)	100,304,314, 334,703,704, 710,714	*Pl?*	42/198	69/181	51/200	50/217	73/259
Resistance from *H. argophyllus*								
RHA340	HA89x *H.arg*415	100,304,314, 334,703,704, 710,714	*Pl8*	Miller and Gulya, 1991; Vear et al., 2000				
RHA419	RHA373x *H.arg*1575	100,304,314, 334,703,704, 710,714	Pl_{arg}	Miller et al., 2002; Vear et al., 2003				
"79ARGMTP"	MPHE-92 x FS20	100,304,314, 334,703,704, 710,714	Pl_{arg}	83/391	50/304	0/260	59/257	80/373
PAA1/OQP7	PBP1xAR22	100,304,314, 334,703,704, 710,714	Pl_{arg}	--	34/109	0/106		
Resistance from *H. tuberosus*								
Progress/DM3/ Rf5566		100,304,314, 703,710,704, 714	*Pl5*	Vrânceanu et al., 1981; Miller and Gulya, 1987				
XRQ	HA89xProgress	100,304,314, 703,710,704, 714	*Pl5*	Bert et al., 2001; Vear et al., 2000				
Novinka/XPQ		100,304,314, 703,710,704, 714	*Pl5?*	Vear et al., 1998				
DM2/PMI3	Novinka	100,304,703, 704	P_{PMI3}	Vear et al., 1998				
HIR34	Armair9343xH.tubD19-6	100,304,314	*Pl4*	Leclercq et al., 1970; Vear et al., 1998				

*numbers of susceptible plants in resistance tests with race 710 (703 with PMI3) on test cross progenies (susceptible x (known resistance cluster x new source)F1

Table 2. Downy mildew resistance of *H. annuus* introgressions, susceptible or resistant to race 304

Pl6 ?				Not *Pl6*			
Genotype	Origin	Resistance		Genotype	Origin	Resistance	
	Wild *H. annuus*	710	304		Wild *H. annuus*	710	304
HAS9	Arizona	Seg	**S**	HAS1	Texas	**R**	seg
HAS20	California	Seg	**S**	HAS6	Wyoming	**R**	**R**
HAS46	Arizona	Seg	**S**	HAS40	Texas	**R**	seg
HAS101	Kansas	Seg	**S**	HAS42	Oklahoma	seg	seg
HAS147	California	Seg	**S**	HAS32	Texas	seg	seg
HAS164	New Mexico	Seg	**S**	HAS54	Oklahoma	seg	seg
HAS186	Texas	Seg	**S**	HAS62	Utah	seg	seg
HAS210	Wyoming	Seg	**S**	HAS85	Wyoming	seg	seg
HAS238	Nebraska	Seg	**S**	HAS94	Wyoming	seg	seg
				HAS103	Kansas	seg	seg
				HAS122	Colorado	seg	seg
				HAS156	California	seg	seg
				HAS171	Texas	seg	seg

Resistance from H. argophyllus: RHA340 was developed by Miller and Gulya from a cross between *H. argophyllus* 415 and HA89. The gene was identified as *Pl8*, resistant to all known races, but with pronounced sporulation on cotyledons, in seedling tests although perfectly efficient in the field. Miller et al. (2002) developed RHA419 from RHA373 x H. *argophyllus* 1575 and its gene was mapped by Dussle et al. (2004) to a different LG from *Pl6* and *Pl8*. At INRA, Montpellier a resistant line, 79ARG was developed from an interspecific pool obtained from crossing *H. argophyllus* (MPHE-92) with cultivated sunflower. This line is also resistant to all known races, showing no segregation with the resistance of RHA419. It was also found to have the same marker linkages (ORS610 and ORS543). In studies of quantitative resistance, it was found that some INRA inbred lines (PAA1, OQP7, OQP8) developed from a cross with *H. argophyllus* made by Leclercq in about 1975, and considered to be susceptible to downy mildew when the presence of the slightest spore was considered to show susceptibility, are resistant to race 710 and also to all the other French races. A test cross with RHA419 showed no segregation (Table 1), so it was concluded that this origin also contains *Plarg*.

Resistance from H. tuberosus: Pl5 was first reported by Vrânceanu et al. (1981) and resistant lines were also developed by Miller and Gulya (1987) from the Russian open pollinated variety Progress, obtained at Krasnodar apparently from an interspecific cross with *H. tuberosus*. This resistance was selected to obtain resistance to race 710 (race 4). Other lines, such as the INRA line XRQ, were developed independently in France, from a sample of Progress provided to Leclercq by Novi-Sad. This source has been widely used since *Pl5* gives resistance to all French races except 334, (which is only observed very rarely). Like *Pl8*, it gives type II resistance (sporulation on cotyledons). Incomplete forms of *Pl5* occur: whereas XRQ is resistant to a Spanish isolate of race 330, the differential D5, PM17 is susceptible. The open pollinated variety Novinka, apparently from the same origin as Progress gave the INRA line XPQ, with resistance not distinguishable from XRQ, but from this variety were also derived USDA pool DM2 and the INRA line PMI3, which is resistant to race 703 but susceptible to 710. It seemed likely that its gene was an incomplete *Pl5*, but genetical analyses with races 703 and 304, to which it is resistant, showed segregation in test crosses with XRQ. Using bulk segregant analysis, it showed no linkage with markers in the region of *Pl5/Pl8* or with the Pl6 cluster. The gene Pl_{PMI3} has still not been mapped.

The other resistance source obtained from *H. tuberosus* was HIR34, with a gene denoted *Pl4*. It has a similar range of resistance to *Pl2*, except that it is susceptible to races 334, 307 and a US isolate of race 330, it has type II resistance and does not map in the *Pl2/Pl6* cluster.

Resistance from other species: HA337, HA338 and HA339 were all developed from *H. praecox* by Miller and Gulya (1991), with a gene designated *Pl7*, but which has not been distinguished from *Pl6* by its resistance to different races or its map position. At the same time as the interspecific pool from *H. argophyllus* was studied, a number of other interspecific pools developed at INRA Montpellier were also tested for their resistance to race 710. All showed some downy mildew resistance. Progenies apparently homozygous for resistance to 710 were obtained from a *H. neglectus* pool but these were susceptible to

races 304, 714 and 334, suggesting that a *Pl6* type gene was present. Interspecific pools from *H. petiolaris fallax*, *H. resinosus* and *H. debilis*, showed some resistance to 710 but no homozygous lines were obtained. For a pool from *H. occidentalis*, it was concluded more recently (Vear, 2006) that resistance may be under quantitative control rather than *Pl* genes.

In more recent studies, resistance to both races 710 and 304 has been fixed in introgression lines from *H. resinosus, H. strumosus, H. debilis* and *H. tomentosus*. These lines are in the course of study to determine whether they provide new *Pl* genes.

DISCUSSION

Resistance genes all appear to come from quite recent crosses with wild *Helianthus*, and in particular wild *H. annuus*. What is identified depends on the resistance requirement. New sources of *Pl1* and *Pl2* probably exist quite widely but are of little interest in modern breeding and so are not introgressed. In contrast, tests made with races 710 or 730 have shown that *Pl6* is present in many wild *H. annuus* ecotypes, most frequently in southern US but from Texas to California. In addition, the gene *Pl7*, from *H. praecox* and the resistance in a pool from *H. exilis* also appear to be the same. There does appear to be at least a second cluster from wild *H. annuus*, but the results of mapping of resistance derived from MPHE-361, MPHE-829 and HA458 are necessary to conclude whether their resistance genes are indistinguishable. The absence of segregation between *Pl5* (from *H. tuberosus*) and *Pl8* (*H. argophyllus*) was surprising, it was questioned whether these genes, which appear to have the same structure, were the result of natural interspecific crosses, or whether, in the multiple interspecific hybridisation at Krasnodar, the open pollinated variety Progress included a gene from *H. argophyllus*. However, since then, Pl_{arg} has been identified from three completely independent crosses at Fargo, Montpellier and Clermont-Ferrand, over 20 years and with quite different *H. argophyllus* ecotypes. Now it seems that *H. argophyllus* has only one "*Pl* gene" (it may be that new races will show some differences between them). So what is the relation between *Pl8* and Pl_{arg}? It could be that *H. argophyllus* contains the same genes as *H. tubersosus* or that there are sites in the sunflower genome (susceptible alleles) where these resistance genes become integrated so that the cultivated genotype used in the interspecific cross may determine the position of interspecific *Pl* genes.

At present, wild *H. annuus* appears the most fruitful source of *Pl* genes, but *Pl6* is often found, and it is this species with which it has been easiest to work. It is also true that the sources derived from both *H. annuus* and *H. tuberosus* show variation in the numbers of downy mildew races controlled, giving the appearance of more "new" genes than there really are. *Pl1* appears to be a *Pl2-*, having lost resistance to races such as 304, and forms of *Pl6* which had lost resistance to races 100 and 300 were obtained experimentally (Vear et al., 1997). For *Pl5*, there appear to be several sources differing slightly in the races they resist, although no within-cluster recombination has been obtained intentionally. It is also true that, with sporulation on cotyledons, individual plants or single progenies with incomplete forms of *Pl5* and *Pl8* may be difficult to identify. In contrast, so far, there do not appear to be any *Plarg-*. This last resistance may be a different structure from the other clusters, but its appearance from 3 different crosses suggests that *H. argophyllus* is not very rich in different *Pl* genes.

In the last 20 years breeders have spent a lot of effort on introducing new *Pl* genes into their best lines following changes in *P. halstedii* races. Since 2003 studies have been made on quantitative, hopefully non-race specific, resistance with levels that could be sufficient alone but which certainly would be of use in combination with *Pl* genes (Tourvieille de Labrouhe et al., 2008). QTL have been identified (Vear et al., 2008) which appear independent of the known *Pl* gene clusters, but it is important to continue identification and mapping of the other sources of complete resistance to check that quantitative resistance is not controlled by incomplete major genes. Overall, if it is found that, among the new sources of complete resistance, there are some new *Pl* genes and that quantitative resistances are different and not race specific, breeders should have the resources necessary to provide durable resistance to downy mildew quite rapidly. In addition, in the long term, if it becomes possible to introgress genes from the perennial *Helianthus* species, the small successes so far from *H. tubersosus* and the apparent resistance in *H. resinosus, H. tomentosus and H. occidentalis*, suggest that new and perhaps different types of downy mildew resistance could become available.

ACKNOWLEDGEMENTS

We would particularly like to thank J. Miller for his collaboration over many years which enabled much of this work. PROMOSOL and CETIOM have provided support for this research.

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Caracterización de la resistencia genética a podredumbre basal en girasol causada por *Sclerotinia sclerotiorum* en Argentina

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ABSTRACT

Sclerotinia sclerotiorum is an optional parasite fungi causative of stem rot in sunflower and others crops in Central and North Area of Argentina. Planting resistant materials is the most economical and efficient (Fick and Miller, 1997) management strategy. The objectives of this work were to identify differences in behaviour between materials of a wide genetic diversity compared with *S. sclerotiorum* inoculation and to obtain a reliable, simple, repeatable method for an assessment consistent with the genotypes over the years in Manfredi, INTA Experimental Station, Argentina. During 4 years, 384 entries from the INTA collection and improvement program, 21 commercial hybrids and 8 susceptible and tolerant controls were evaluated. The design of the trials was in alpha lattice with two replications. The planting was done manually in an area naturally infested with *S. sclerotiorum* and reinforced with mycelium of the pathogen grown in wheat grains. The evaluation was performed at 30 days of infection, recording the percentage of diseased plants. During the four years, there was a good manifestation of the disease allowing the evaluations. The contrasting behaviour between susceptible and resistant controls over the years was consistent. There were statistical differences between genotypes. Three groups with different levels of resistance to stalk rot between genotypes with a broad genetic variability were identified. High infection stalk rot in sunflower, and consistency in the response of genotypes through the years were achieved. The methodology used in infection and evaluation showed reliability, repeatability and simplicity by allowing the selection of superior genotypes.

Key words: breeding – genetic resistance – genetic resources – *Sclerotinia* – stalk rot – sunflower.

RESUMEN

Sclerotinia sclerotiorum es un hongo polífago agente causal de Podredumbre Basal de tallo en girasol y otros cultivos de importancia económica, principalmente en la zona Central y Norte Argentina. La siembra de materiales resistentes es la estrategia de manejo más económica y eficiente. Los objetivos de este trabajo fueron detectar diferencias de comportamiento entre materiales de amplia diversidad genética frente a inoculación con *S. sclerotiorum* y contar con un método confiable, simple y repetible para una evaluación consistente de genotipos a través de los años en INTA EEA Manfredi, Argentina. Durante 4 años, se evaluaron 384 entradas de la colección y el programa de mejoramiento del INTA, 21 híbridos comerciales y 8 testigos susceptibles y tolerantes. El diseño de los ensayos fue alpha lattice en 2 repeticiones. La siembra se realizó manualmente en infectario de *Sclerotinia sclerotiorum* reforzado con micelio del patógeno vehiculizado en granos de trigo. La evaluación se realizó a los 30 días de infectado determinando la proporción de plantas enfermas. Durante los cuatro años, existió buena manifestación de la enfermedad y el comportamiento entre los testigos susceptibles y resistentes fue contrastante y consistente durante todos los años. Existieron diferencias estadísticas entre los genotipos. Se identificaron tres grupos con diferentes niveles de resistencia a Podredumbre Basal entre genotipos de amplia variabilidad genética. La metodología utilizada en infección y evaluación demostró confiabilidad, repetibilidad y sencillez permitiendo la selección de genotipos superiores.

Palabras clave: girasol - mejoramiento - podredumbre basal - recursos genéticos - resistencia genética – *Sclerotinia*.

INTRODUCCIÓN

Sclerotinia sclerotiorum es un hongo polífago y agente causal de podredumbres en diversos cultivos de importancia económica, entre ellas la Podredumbre Basal del tallo en girasol (Cuk, 1980) y también en soja. En su infección micelial invade desde el suelo la zona radical superior y la base del tallo (Huang y Dueck, 1980).

La Podredumbre Basal puede causar severos daños en el girasol, principalmente en la zona Central y Norte de Argentina, y es una limitante para este cultivo, fundamentalmente en fechas de siembra tardías. En la campaña 2002/03, se reportaron severos daños (9,5 y 32,4% de plantas afectadas) en ensayos de evaluación de híbridos comerciales bajo condiciones de infección natural, fecha de siembra normal, y labranza convencional en la localidad de Serrano, Córdoba, (Alvarez y Guerra, 2005). La creciente expansión de la soja y su inclusión en las rotaciones, constituyen un agravante potencial ante las probabilidades de expansión de esta enfermedad.

La infección natural suplementada con inoculación artificial es considerada un efectivo método para realizar selección entre materiales (Miller, 1996). La siembra de materiales resistentes es la estrategia de manejo más económica y eficiente.

Los objetivos de este trabajo fueron: 1) Detectar diferencias de comportamiento entre materiales de la Colección de Germoplasma de Girasol, del Programa de Mejoramiento del INTA y de Híbridos Comerciales frente a inoculación asistida con micelio de *S. sclerotiorum*. 2) Contar con un método confiable, simple y repetible que permita una evaluación consistente del comportamiento de los genotipos posibilitando la detección de resistencia.

MATERIALES Y MÉTODOS

Durante 4 años (2002/03, 2004/05, 2005/06 y 2006/07), se evaluaron 423 genotipos compuestos por 384 entradas de la colección de Recursos Genéticos del INTA y 21 híbridos comerciales, de los cuales se utilizaron 8 como testigos en base a su comportamiento sanitario diferencial (4 susceptibles y 4 tolerantes) frente a Podredumbre Basal y de capítulo (Trogia et al., 2002).

Los ensayos se dispusieron en diseños Alpha lattice en 2 repeticiones. Las parcelas eran de 1 hilera de 5,10 m de largo, distanciadas a 0,70 m. La siembra se realizó en un infectario natural de *S. sclerotiorum* en forma manual a 0,30 m entre sí. El análisis estadístico mediante ANOVA, consideró Genotipo como fuente de variación. LSMeans se calculó por el procedimiento GLM de SAS (SAS 2002).

Cuando las plantas alcanzaron el estado V4 se realizó el raleo, dejando 1 planta por golpe (Schneiter y Miller, 1980). En el estado de prefloración, se reforzó la presencia de patógeno introduciendo micelio vehiculizado en granos de trigo en la zona de suelo próxima al cuello de la planta a una profundidad de 2 a 3 cm y parcialmente cubierto. El inóculo se preparó cultivando esclerotos de *S. sclerotiorum* en APG (2%) e incubándolos a 25ºC. Posteriormente a los 4 días se vehiculizaron en granos de trigo. Este vehículo se preparó remojando trigo durante 24 horas, posteriormente se escurrió el exceso de agua y fraccionó en frascos de 500 ml de capacidad. Se esterilizaron en autoclave secuencialmente en 3 oportunidades a 1 atm durante 30 min, dejando enfriar. En condiciones de asepsia se inoculó con colonias de *S. sclerotiorum* (aislado a partir de esclerotos recolectados en el campo) y se incubó a 25ºC durante 25 a 30 días, agitando periódicamente para permitir un crecimiento homogéneo. La evaluación se realizó en dos oportunidades: a los 15 y a los 30 días de infectado, considerando planta afectada a toda aquella que presentara lesión en la base del tallo (Pereyra y Escande, 1994). Se determinó la proporción de plantas enfermas como el cociente entre plantas con síntomas y plantas totales x 100. Los genotipos se clasificaron en resistentes, intermedios y susceptibles.

RESULTADOS Y DISCUSIÓN

Durante los cuatro años de evaluación, existió una buena manifestación de la enfermedad que permitió realizar las evaluaciones. En la Fig. 1 se observa un comportamiento contrastante entre los testigos susceptibles (S1, S3, S5 y S7) y resistentes (S2, S4, S6 y S8), el cual fue consistente durante todos los años de evaluación, siendo la proporción de plantas enfermas de los susceptibles siempre mayores a los de los resistentes. Durante los tres primeros años de evaluación (2002/03, 2004/05 y 2005/06), el nivel de enfermedad en todos los testigos fue superior a la de 2006/07, lo cual se debería a las condiciones ambientales con un período de sequía y altas temperaturas posterior a la inoculación, que podrían haber afectado el proceso de infección de las plantas. Sin embargo, esta situación natural no afectó significativamente los parámetros estadísticos ni la discriminación de los genotipos por su grado de resistencia.

De acuerdo a la Tabla 1, durante los 3 primeros años se obtuvo una alta incidencia (superior a 80%) de las plantas infectadas para el promedio de todos los genotipos, mayor a la registrada en el 4º año, (18.2% en 2006/07). Los Coeficientes de Variación (C.V), se encontraron dentro de los valores esperados para este tipo de evaluaciones. Las Diferencias Mínimas Significativas (DMS) permitieron diferenciar estadísticamente los genotipos por su comportamiento frente a la enfermedad.

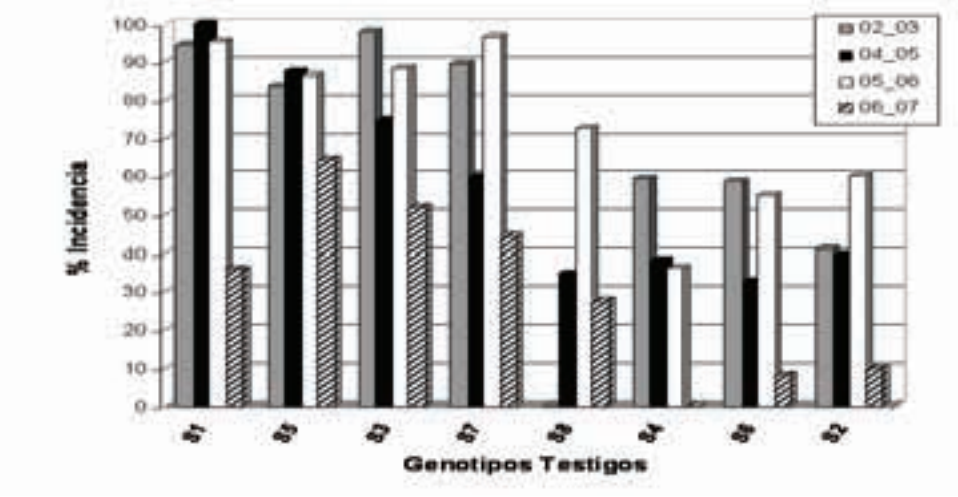

Fig. 1. Incidencia de podredumbre basal en testigos susceptibles (S1, S3, S5 y S7) y resistentes (S2, S4, S6 y S8), en cuatro años de evaluación.

Tabla 1. Número y porcentaje de genotipos con diferente grado de incidencia de Podredumbre Basal. Promedio, Coeficiente de Variación y DMS (α=0,1), durante cuatro años de evaluación.

Incidencia	2002/03		2004/05		2005/06		2006/07	
	Nº	%	Nº	%	Nº	%	Nº	%
Alta	5	4,5	21	20,2	1	1,0	74	71,2
Media	34	30,6	7	6,7	32	30,8	18	17,3
Baja	72	64,9	76	73,1	71	68,3	12	11,5
Promedio (%)	80,3		80,1		86,2		18,2	
C.V. (%)	24,2		20,2		12,6		34,7	
DMS (0,1)	22,8		37,6		18,2		25,7	

En la Fig. 2, la severidad del ataque y la reacción de los genotipos permitieron identificar tres grupos de materiales con diferente comportamiento según grado de resistencia. Resistentes: genotipos con valores menores a la suma del valor mínimo del ensayo y la DMS (Negros); Medios: con valores que difieren del valor mínimo y/o máximo del ensayo (Blancos) y Susceptibles: valores mayores a la diferencia entre el valor máximo del ensayo y la DMS (Grises).

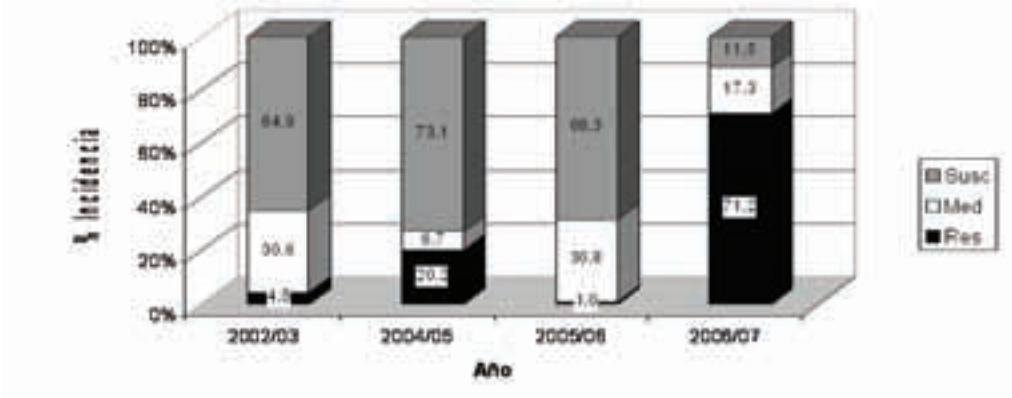

Fig. 2. Proporción de genotipos con diferente grado de resistencia frente a podredumbre basal (susceptibles, medios y resistentes) en cuatro años de evaluación.

Las proporciones entre los tres grupos varió con el año y los materiales evaluados en cada año, de 11.5 (2006/07) a 73.1 % (2004/05) para los genotipos de susceptibles, y de 1.0 (2005/06) a 71.2 % (2006/07) para los genotipos de resistentes. Comparando los diferentes años de evaluación, la alta proporción de entradas con mayor nivel de resistencia en 2006/07, se debería a que un alto número de genotipos participantes fueron seleccionados por su buen comportamiento frente a la enfermedad por esta metodología, en años anteriores.

De acuerdo a la Fig. 3 en el año 2002/03, 4 genotipos se comportaron como resistentes (barras negras), y 1 sola entrada perteneciente a la Colección de Recursos Genéticos de INTA (CGGI 491, PROCISUR-B-C0, datos no publicados) superó al mejor testigo resistente (rayas horizontales).

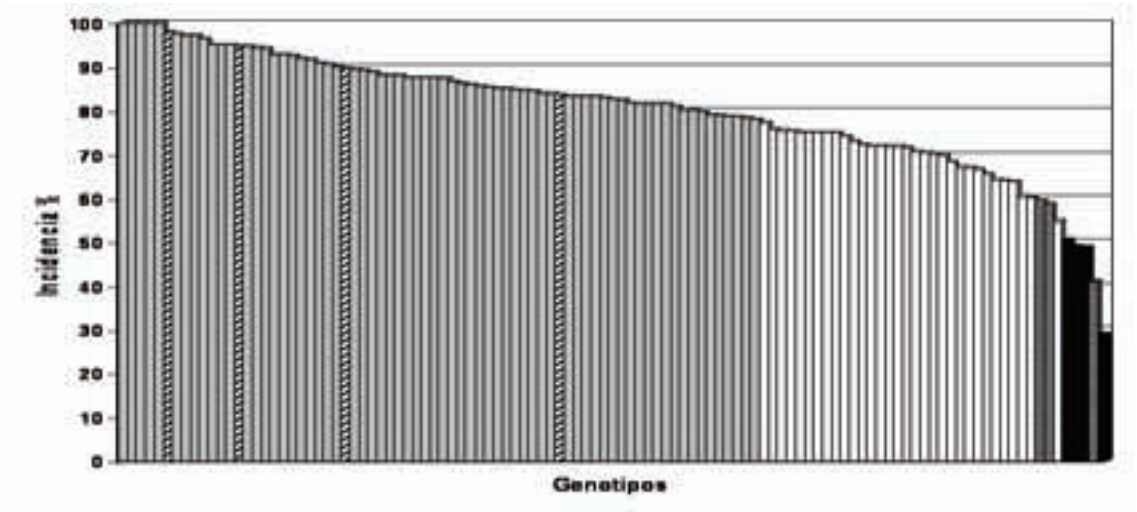

Fig. 3. Respuesta de 111 genotipos frente a *Sclerotinia sclerotiorum*, año 2002/03, INTA Manfredi. Susceptibles: barra color gris, Medios: barra color blanco, Resistentes: barra negra, Testigos Resistentes: rayas horizontales, Testigos Susceptibles: rayas diagonales.

En las evaluaciones correspondientes a los años 2004/05 y 2005/06, de acuerdo a Fig. 4 y 5, ninguna entrada de Colección demostró mejor comportamiento que el mejor testigo resistente (rayas horizontales). 16 entradas (2004/05) y ninguna (2005/06), integraron el grupo resistente (barras negras).

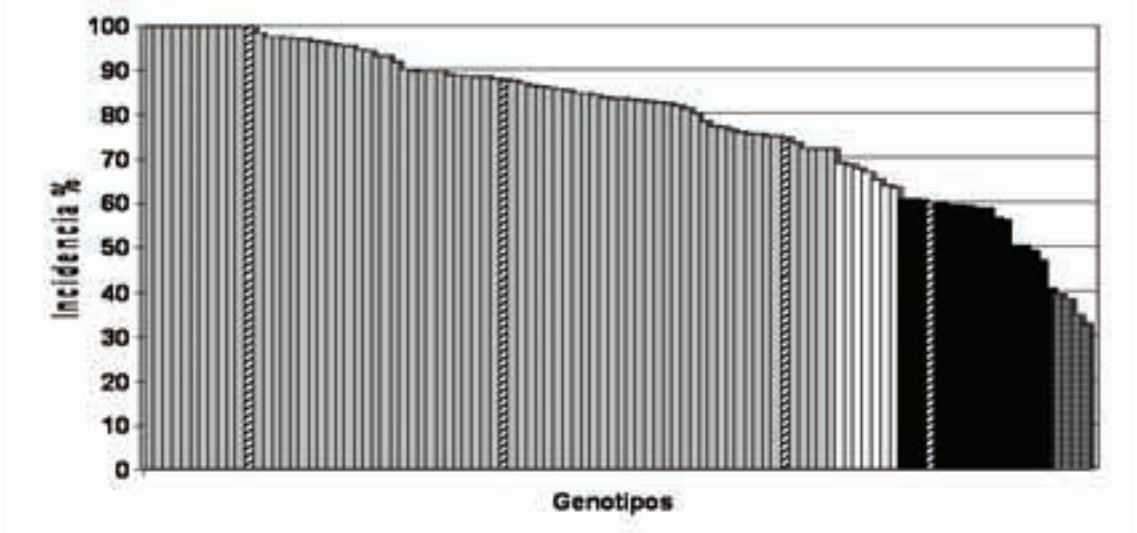

Fig. 4. Respuesta de 104 genotipos frente a *Sclerotinia sclerotiorum*, año 2004/05, INTA Manfredi. Susceptibles: barras color gris, Medios: barras color blanco, Resistentes: barras color negro, Testigos Resistentes: rayas horizontales, Testigos Susceptibles: rayas diagonales.

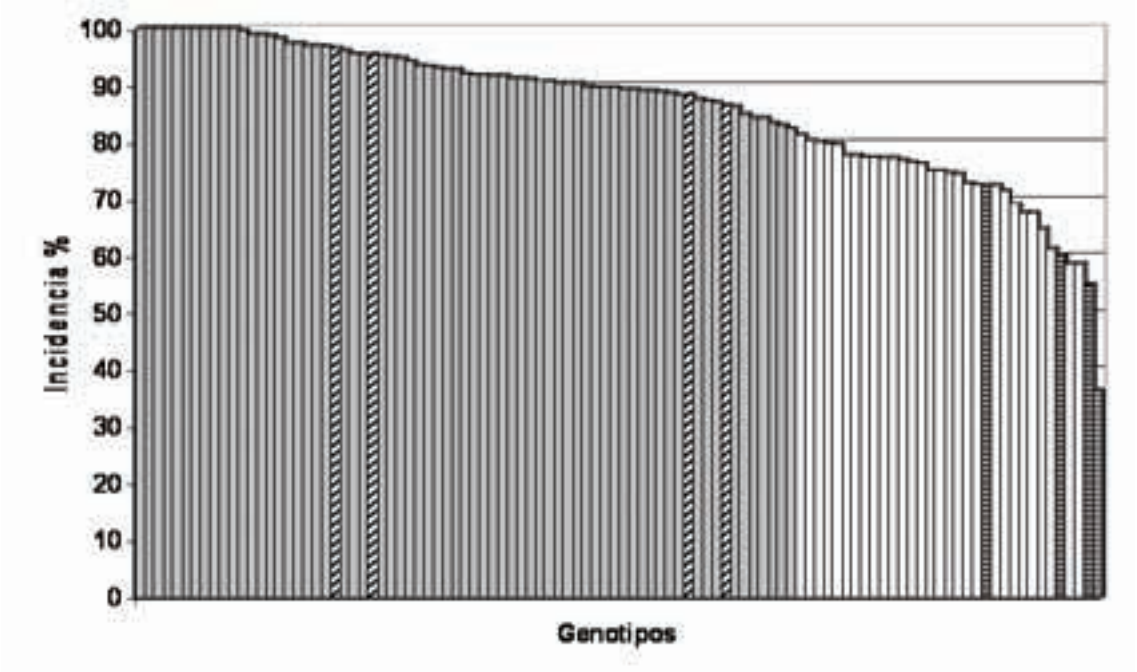

Fig. 5. Respuesta de 104 genotipos frente a *Sclerotinia sclerotiorum*, año 2005/06, INTA Manfredi. Susceptibles: barras color gris, Medios: barras color blanco, Resistentes: barras color negro, Testigos Resistentes: rayas horizontales, Testigos Susceptibles: rayas diagonales.

La Fig. 6, muestra que en el año 2006/07, 61 genotipos integraron el grupo resistente (barras negras), 26 entradas superaron el valor del mejor testigo resistente (rayas horizontales), 25 de ellos corresponden al programa de mejora seleccionados años anteriores por esta metodología y sólo 1 (CGGI 13_3, IDANOV 8281-3), pertenece a la Colección de Recursos Genéticos de INTA (datos no publicados).

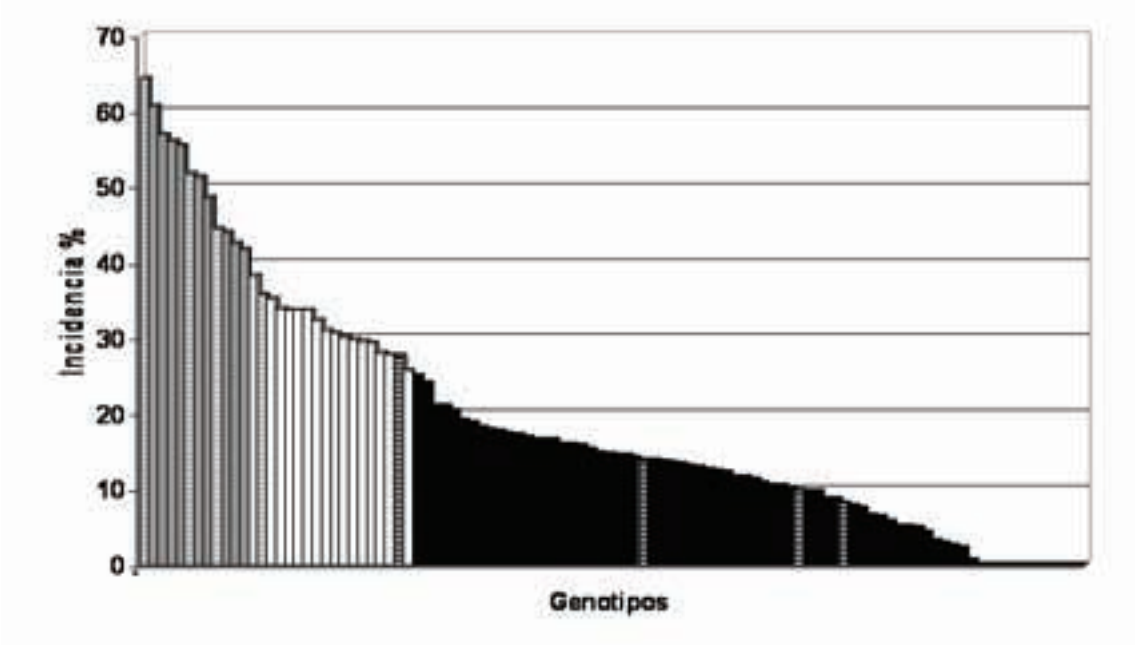

Fig. 6. Respuesta de 104 genotipos frente a *Sclerotinia sclerotiorum*, año 2005/06, INTA Manfredi. Susceptibles: barras color gris, Medios: barras color blanco, Resistentes: barras color negro, Testigos Resistentes: rayas horizontales, Testigos Susceptibles: rayas diagonales.

Como resultado del presente trabajo, se puede concluir que:

1. Es posible lograr elevados niveles de infección y enfermedad de Podredumbre Basal del tallo en girasol, determinando a la vez consistencia en la respuesta de los genotipos a través de diferentes años y distintos niveles de infección general.
2. La metodología utilizada en la infección y evaluación demostró confiabilidad, repetibilidad y una aceptable sencillez permitiendo la utilización de la variabilidad en los procesos de mejora.
3. Existen diferentes niveles de resistencia a Podredumbre Basal en condiciones de infectario con inoculación micelial entre genotipos de amplia variabilidad genética como poblaciones de la Colección de Germoplasma de Girasol del INTA, líneas e híbridos comerciales.
4. La colección de Recursos Genéticos de Girasol de INTA es una fuente valiosa para selección de genotipos por caracteres de resistencia genética a la PodredumbreBasal.
5. Mediante mejoramiento genético, es posible la obtención de genotipos superiores con altos niveles de resistencia frente a *S sclerotiorum*.

AGRADECIMIENTOS

Se agradece la colaboración del personal técnico y auxiliar Agr. L. Ceballos, Agr. J. Nievas, J. Gatti y G. Rojo, en las tareas de laboratorio y campo.

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Molecular characterization of a novel *Sunflower chlorotic mottle virus* (SuCMoV) strain

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ABSTRACT

Sunflower plants showing chlorotic ring spot symptoms were observed during the 2005/2006 crop season in the southeast of the Province of Buenos Aires in Argentina. Preliminary studies, including host range symptoms, serological tests and electron microscopy, had identified this virus isolate as a potyvirus closely related to *Sunflower chlorotic mottle virus* (SuCMoV). The nucleotide sequence of the genomic 3´ terminal region of this potyvirus was determined and characterized. The sequence consisted of 1304 nucleotides (nt) includingthe C-terminal region of the nuclear inclusion b protein gene (NIb), the capsid protein gene (CP) and the 3´non-coding region (3´-NCR). The partial putative NIb gene (240 nt) encoded a protein of 80 amino acids (aa) residues and the CP gene (807 nt) encoded a protein of 269 aa residues. The 3´-NCR was 257 nt in length excluding the poly (A) tract. Sequence comparisons of the predicted CP aa and 3´-NCR were analyzed separately in order to determine the relationship between this potyvirus and SuCMoV, and other reported potyviruses. The CP of this potyvirus isolated from sunflower shared 94.8% aa identity with SuCMoV (Argentina) and 89.2% with SuCMoV-Zi (Brazil). The 3´-NCR shared 94.2% nt sequence identity with SuCMoV. These data indicate that the potyvirus causing chlorotic ring spot (CRS) symptoms in sunflower is closely related to SuCMoV and it is provisionally referred to as SuCMoV strain CRS.

Key words: coat protein sequence – molecular assays – SuCMoV – sunflower – strain.

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the most important oilseed crops in Argentina, with a total planted area of 2,380,000 ha and a total yield of 3,500 million tons in the 2006/2007 growing season. It has been a strategic revenue crop for this country since Argentina is the main exporter of edible sunflower oil in the world. *Sunflower chlorotic mottle virus* (SuCMoV) is one of the most widely distributed potyviruses on cultivated and wild sunflowers in this country and was reported in several provinces. Achene yield was significantly reduced by SuCMoV infections occurring at early ontogenetic stages (Lenardon et al., 2001). Recently, SuCMoV has been recognized as a new PVY strain by the ICTV (Dujovny et al., 2000; Berger et al., 2005), however some inconsistencies of its taxonomic status need to be clarified. During the 2005/2006 growing season, SuCMoV broke out in commercial sunflower hybrids in the southeast of the province of Buenos Aires with an unusual increase in disease incidence. At the same time, a whole commercial sunflower field showing chlorotic ring spot symptoms (CRS) on leaf blades (Fig. 1) was detected in the same geographical area. Preliminary biological, serological experiments, and electron microscopy studies had previously identified the virus as a potyvirus related to SuCMoV (Lenardon et al., 2005).

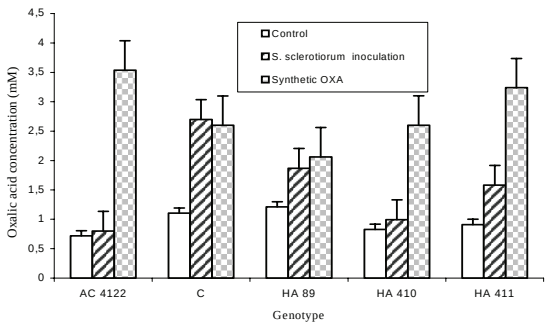


Fig. 1. Sunflower leaves showing chlorotic ringspot symptoms.

The genus Potyvirus, in the plant family Potyviridae, is by far, the largest virus genus known in the plant kingdom, with nearly 200 members, which accounts for almost 25% of known plant viruses. Its members share similar morphology, particle structure, host range, and modes of transmission (Berger et al., 2005). The virions contain a single molecular linear, positive-sense 8.2 to 9.7 kb ssRNA that has a VPg structure at its 5´terminus and a poly (A) tract at its 3´terminus. The coding ORF is translated to one polyprotein, which is subsequently processed into 10 different proteins by virus-encoded proteinases (Allison et al., 1986). The potyvirus genomic RNA is encapsidated by a single type of coat protein (CP). Genomic sequence data have become useful for demarcating virus strains and species (Fauquet et al., 2003; Berger et al., 2005). Sequences within the 3´ proximal portion of the genome are commonly used for species demarcation (Shukla et al., 1994; Adams et al., 2005), including the nucleotide or amino acid sequences of CP and the nucleotide sequence of the 3´ non-coding region (3´-NCR).

This report was undertaken to determine molecular properties (genome organization, amino acid sequence and phylogenetic analyses) of SuCMoV-CRS.

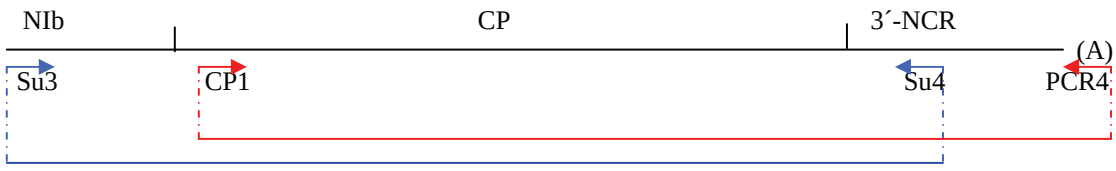
MATERIALS AND METHODS

Virus source and maintenance

Sunflower plants showing leaf blades with isolated and confluent chlorotic ring spots were collected from commercial sunflower fields located in Pieres county, in the southeast of the province of Buenos Aires. Single chlorotic ring spots were mechanically inoculated onto sunflower hybrid CF-7 and *Nicotiana occidentalis* L., in which they were maintained. The mechanical inoculations were conducted according to our standard procedure: leaves from infected plants were ground in 0.01M phosphate buffer, pH 7, containing 0.1% Na_2SO_3 (1:5 w/v) with a mortar and pestle. Extracted juice was mixed with 600 mesh carborundum before being rub-inoculated on the hosts and plants were kept under greenhouse conditions (22ºC + 5ºC) for symptom expression.

RT-PCR

Total RNA was extracted from sunflower fresh leaf tissue (100 mg) using the RNeasy Plant Mini Kit (Qiagen, California, USA) according to the manufacturer's instructions. To clone the 3´ end of the NIb and the CP cistrons, DNA was synthesized using the Access RT-PCR system (Promega, Wisconsin, USA) and specific primers Su3 and Su4 (Fig. 2). To clone the CP cistron and the 3´-NCR a first strand cDNA was made with M-MLVreverse transcriptase (Promega) and Eco/Not as initial primer (Tsuneyoshi et al., 1998). PCR was carried out using Taq DNA Polymerase (Promega) and the primers CP1, and PCR4 (Fig. 2). The amplified products were visualized by electrophoresis on a 1.4% agarose gel stained with ethidium bromide.



Su3: 5´-GAGGCGTGGGGCTATCC-3´
Su4: 5´-AAAAGTAGTACAGGAAAAGCC-3´
CP1: 5´-GGTGACAACATAGATGCAGG-3´
PCR4: (Tsuneyoshi et al., 1998)

Fig. 2. Cloning strategy of the SuCMoV-CRS 3´end. The position of the different PCR-generated cDNA clones is shown below the viral genomic map.

Cloning and sequencing of PCR products
PCR products were cloned using the pGEM T-easy vector system (Promega), following the manufacturer's instructions and subsequently subjected to DNA sequencing at Macrogen Inc. (Seoul, Korea).

Sequence analysis
The nucleotide (nt) and predicted amino acid (aa) sequences of the whole CP coding region and the nt sequence corresponding to the 3´- NCR of the SuCMoV-CRS were compared with 38 potyvirus sequences deposited in GenBank, EMBL, DDBJ and PDB databases using pair-wise Align program (Table 1).

Sequence assembly and analysis were performed utilizing the Lasergene software package, including Editseq, Seq Man and MegAlign programs (DNASTAR, Inc., Madison, Wi, USA). Multiple sequence alignments produced by Clustal W algorithm were used as input data for reconstructing phylogenetic trees by the Neighbor-Joining method using the software MEGA version 4 (Tamura et al., 2007). Statistical significance was estimated by performing 500 replications of bootstrap resampling of the original alignment using the bootstrap option of the phylogenetic tree menu.

RESULTS

Following mechanical inoculation sunflower and *N. occidentalis* plants became infected, developing symptoms similar to those seem in the field collected plants.

A 1304 nt fragment of the 3´ terminal region genome of the SuCMoV-CRS was cloned and sequenced (GenBank accession number EU418771). Sequence analysis of this virus genome portion revealed putative proteins and a 3´- NCR similar in size and arrangement to those of representative potyviruses. This sequence covered part of the NIb coding region (nt 1 to 240), the whole CP coding region (nt 241 to 1047) and the 3´-NCR (nt 1048 to 1304). The first predicted 80 aa belonged to the C-terminus of the NIb and the dipeptide at the putative NIb/CP junction was Q/G. The CP gene encoded 269 aa residues with the Asp-Ala-Gly (DAG) motif presented at the N-terminus of CP (4 aa from the cleavage site). Also, the following consensus motifs have been found in the putative CP: MVWCIENGTSP, AFDF, QMKAAAL at 117, 200 and 220 aa from the cleavage site. The 3´-NCR consisted of 257 nt excluding the polyadenylated tract.

The percentage of identity between the nucleotides of the SuCMoV-CRS CP and selected potyviruses ranged from 39.8% to 87.3%. Comparisons of the predicted CP aa ranged from 47.6% to 94.8% identity (Table 1). SuCMoV-CRS shared 94.8% aa identity with the CP of an Argentinian SuCMoV isolate which caused chlorotic mottling on sunflower, 89.2% aa identity with a Brazilian SuCMoV-Zi isolated reported from zinnia plants and 84.1% with *Bidens mosaic virus* (BiMV) (Table 1). Comparisons of the CP core aa (Lys^{32} to $Prol^{184}$) between this virus and SuCMoV (Argentina) showed identity of 96.1%. Additionally, the identity of the CP of SuCMoV-CRS was less related with other potyviruses-infecting sunflower such as *Sunflower chlorotic spot virus* (syn *Bidens mottle virus*) (72.8%) and *Sunflower mosaic virus* (66.7%) (Table 1).

Nucleotide comparisons of the 3´-NCR between SuCMoV-CRS and several potyvirus species showed the highest degree of sequence identity with SuCMoV (94.2%) and BiMV (76.8%). The other potyviruses showed a lower degree of sequence identity ranging from 34.4% (TEV) to 57.9% (PepMoV) (Table 1).

In the phylogenetic analysis the high bootstrap values confirmed grouping of the SuCMoV-CRS with SuCMoV, SuCMoV-Zi and BiMV, which are clustered with high confidence to PVY isolates and PepSMV (Fig. 3).

Table 1. Percentage of nucleotide and amino acid identities between the coat protein and the 3´NCR of SuCMoV - CRS with those of selected potyviruses, respectively

Virus acronym	Accession number	CP nucleotide % identity	CP amino acid % identity	3´-NCR nucleotide % identity
SuCMoV	AF255677	**87.3**	**94.8**	**94.2**
SuCMoV-Zi	AY344048	82.4	**89.2**	without data
BiMV	AY960151	77.4	**84.1**	**76.8**
PVY-LYE	AJ439545	77.0	80.4	57.7
PVY-H	M95491	76.8	80.1	54.8
PVY-NTN	AJ890347	76.5	80.0	56.2
PVY-T	D12570	77.6	79.6	51.7
PVY-O	EF026074	75.5	79.3	56.2
PVY-US	M81435	76.7	79.3	55.3
PVY-MN	AF463399	76.7	79.2	56.5
PVY	U09509	77.0	78.9	55.3
PVY-N	D00441	77.4	78.9	51.7
PVY-N:O	EF026076	75.9	78.9	56.8
PVY-Wilga	AJ889867	75.6	78.8	56.3
PepSMV	NC_008393	72.5	77.4	40.1
PepMoV	AY748921	70.0	76.6	**57.9**
PepYMV	EF488081	70.0	74.5	56.8
PTV	AJ437280	69.9	73.9	41.8
SuCSV	AF538686	69.3	**72.8**	48.4
WPMV	AJ437279	70.6	72.2	40.4
PVV	AJ243766	68.4	71.1	44.6
SuMV	AF465545	60.9	**66.7**	37.8
LMV	AJ278854	61.9	63.7	45.4
TEV	M11458	63.4	63.2	**34.4**
TuMV	AB105134	61.0	61.1	41.7
ChiVMV	AJ237843	54.3	59.6	40.7
ZYMV	AB369279	59.6	59.6	41.7
CDV	AM113761	59.3	59.5	45.8
PetFMV	AF030689	47.6	59.5	45.8
SMV	D00717	58.7	59.1	42.3
PRSV	AY162218	60.0	59.0	37.6
PVA	Z21670	59.7	58.9	47.3
BCMV	AM258976	59.1	56.6	44.4
MDMV	D00949	58.9	55.3	45.9
TVMV	U38621	**39.8**	54.1	38.3
ClYVV	AB011819	58.0	53.0	40.6
PPV	NC_001445	55.1	51.4	45.0
SCMV	EU196455	**52.1**	**47.6**	45.4

DISCUSSION

Molecular characteristics of the sunflower-potyvirus inducing CRS, such as nucleotides, predicted amino acid sequence identities and the arrangement of the 3´ end of the genome clearly showed that it is closely related to SuCMoV. This research confirms previous findings based on biological and serological properties and virion morphology (Lenardon et al., 2005).

The 3´-NCR of SuCMoV-CRS showed a higher sequence identity with SuCMoV (94.2%) and BiMV (76.8%) and a lower one with other potyvirus members than usually reported for strains (34.4% to 57.9%). According to Frenkel et al. (1989), the nt sequence of the 3´-NCR of potyvirus strains is highly conserved (83-99%), while distinct potyviruses have only 30-53% nt sequence similarity. Furthermore, Shukla et al. (1994) proposed that species of the same virus should have a 3´-NCR sequence identity of >75%. Considering this criterion, SuCMoV-CRS should remain as a SuCMoV strain.

The CP of SuCMoV-CRS showed a sequence identity of 94.8 % with SuCMoV and most of its differences in aa residues between them were confined to the N-terminal part of this protein. This region is known to be highly variable and to contain mayor virus specific epitopes, due to its localization at the surface of the virions (Shukla et al., 1988). The essential DAG triplet for aphid transmission in the potyvirus genus was found in the N-terminal region together with other consensus aa motifs for CP coding regions common in other potyviruses (Shukla et al., 1994; Atreya et al., 1990, 1991).

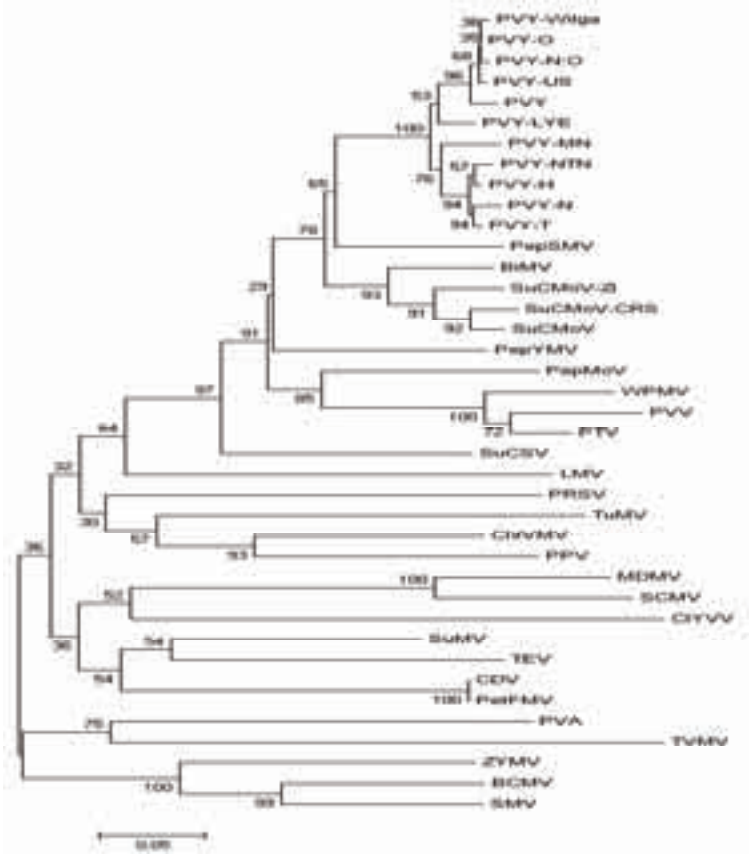


Fig. 3. Phylogenetic tree illustrating the position of the SuCMoV-CRS among the members of the *Potyviridae* family. Neighbor-joining trees were constructed by the program Mega-4 from multiple sequence alignments of CP aa using Clustal W. The bootstrap values of 500 replications are shown in each node.

Comparisons within the SMV/BCMV and the SCMV subgroups demonstrated that the discrimination between strains of the same species and isolates of different virus species occurred at about 83% aa identity (Chen et al., 2004), whereas Adams et al. (2005) consider that a value of 82% aa identity would reliably distinguish between most species except for the PPV, WPMV and PTV group. More recently, species demarcation criteria for the genus *Potyvirus* included CP nt identity of less than 76% and aa identity of less than about 80% (Berger et al., 2005) based on earlier studies of several virus species and strains.

The comparisons between the CP aa among potyviruses have shown that SuCMoV is regarded as a PVY strain (Berger et al., 2005), and BiMV is also considered a strain of PVY (Inoue-Nagata et al., 2006). Nevertheless, other propositions were made recently for species demarcation based on nt and aa identity within ORFs and the CI gene have been proposed as being the best region for diagnosis and taxonomy studies if only a sub portion of the genome is to be sequenced, rather than the CP usually used, because it most accurately reflects the taxonomic status according to the complete ORF (Adams et al., 2005).

The phylogenetic analysis based on the CP aa sequence identities confirmed the taxonomic relatedness of this sunflower-potyvirus inducing chlorotic ring spot symptoms to SuCMoV, SuCMoV-Zi and BiMV, which could be clustered into a new subgroup among PVY isolates.

The denomination of SuCMoV- (strain) CRS is proposed for this potyvirus closely related with SuCMoV in the light of its association with the symptoms on naturally-infecting sunflower and its ability to reproduce the same systemic symptoms on healthy sunflower plants mechanically inoculated under greenhouse conditions. Research about the identification of the viral genomic region involved in symptom expression is under way and may provide insight of the virus-host interaction.

ACKOWLEDGEMENTS

We acknowledge FONCyT (Grant PICT 2004-25982) for financial support of this research.

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Estimation of sunflower breeding material tolerance to *Diaporthe/Phomopsis helianthi*

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ABSTRACT

Phomopsis of sunflower [caused by *Phomopsis helianthi* Munt.-Cvet. et al. (teleomorph *Diaporthe helianthi* Munt.-Cvet. et al.)], is one of the principal sunflower diseases in the Republic of Croatia and Europe, and has a great influence on grain and oil yield. Hence, in the framework of the sunflower breeding program at the Agricultural Institute Osijek, one of the main objectives is to work on the resistance to this and other principal pathogens. Although sunflower (*Helianthus annuus* L.) has a narrow genetic variability, the source of genetic resistance to this pathogen is found among wild *Helianthus* species, and differences among cultivated genotype tolerance are observed as well. This paper presents only one segment of the work on tolerance by artificial infection under field conditions with the aim to investigating the level of tolerance to this pathogen of a wide range of breeding materials (e.g. cms and restorer lines). The most tolerant material will be used in the creation of new commercial sunflower hybrids.

Key words: artificial infection – *Diaporthe/Phomopsis helianthi* – sunflower – tolerance.

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the most important oil crops in world production and the area under sunflower is in constant increase. Same trend is in Europe, where area under sunflower increased in the period 1995-2005 by 2.3 million ha (FAOSTAT, 2005). Major sunflower producers in Europe are: Russia, Ukraine, Romania, France, Bulgaria, Spain, Hungary and Moldova.

In the Republic of Croatia, sunflower production is characterized by significant oscillations in areas, grain and oil yield. These oscillations significantly depend on the occurrence and intensity of diseases, which, in some years, lead to significant decreasing in grain and oil yields. Different diseases are dominant in different production areas and significantly depend on agroecological conditions. It is known that over 30 different pathogens (among them fungi are predominant) attack sunflower and cause diseases which can produce important economic damage (Škorić et al., 2002.).

Phomopsis helianthi Munt.-Cvet. et al. (teleomorph *Diaporthe helianthi* Munt.-Cvet. et al.), is one of the most important sunflower pathogens in Europe. It causes a disease named gray stem spot (stem canker). It was first described in the former Yugoslavia in 1981 (Mihaljčević et al., 1982.) and from then on it spread all over the world and became one of the most prevalent diseases of cultivated sunflower (Degener et al., 1999). In environmental conditions favorable to disease development (Laville, 1986), it could cause significant grain yield losses (10-50%) and oil content decrease.

Growing resistant hybrids is the most effective measure for disease control. However, there are no completely resistant genotypes and the main challenge to the breeders represents searching for sources of resistance and introducing them into genotypes with valuable agronomic traits. Sources of resistance could be found in some wild species, first of all in some populations of *H. tuberosus* (Škorić et al., 2002). According to Deglene et al. (1999), sunflower resistance in breeding programs could be improved by using inbred lines, which have high values of general combining abilities. In sunflower breeding aimed at disease tolerance, artificial infection in controlled (laboratory) or uncontrolled (field) conditions is essential. There are a few methods of artificial infection and some authors use the least aggressive ones, which are closer to natural infection. Also, there are differences regarding a place of infection (Vear et al., 1997). Sunflower breeding programs in Croatia have a long tradition and have been carried out through scientific projects and programs in the framework of The Agricultural Institute Osijek (Vratarić and Sudarić, 2004; Mijić et al., 2004; Krizmanić et al., 2006). The main goal is the creation of new, superior hybrids, with a high grain yield (above 5 t/ha), oil content (above 50%), and high, stable oil yield. Special attention is given to creation lines with an emphasized tolerance to predominant pathogens. Sunflower

breeding with resistance/tolerance to main diseases is the best way to control them and represents the most ecologically acceptable way to do so (Fick and Miller, 1997; Miller and Fick, 1997; Škorić et al., 2002; Vratarić and Sudarić, 2004).

The aim of the investigation was to estimate the tolerance to the pathogen *D. helianthi* of a wide spectrum of inbred lines, including cytoplasmic male sterile (cms, A lines), male fertile (mf, B lines), restorers of fertility (rf, R lines) and two-way sterile hybrids or single cross (SC), by artificial infection method in the field. Inbred lines of good combining abilities for the most important agronomic traits (grain yield, oil content), which show the lowest level of susceptibility, will be considered as potential parents for hybrid development in the framework of the Agricultural Institute Osijek sunflower breeding program.

MATERIALS AND METHODS

The research was conducted during two consecutive years (2006 and 2007) at the experiment field of The Agricultural Institute Osijek (Croatia). Tested breeding material involved 19 different sunflower genotypes, 5 of which were cytoplasmatic male sterile (cms) inbred lines (L-301 A, L-271 A, L-G/04 A, L-205 A, L-101 A), four male fertile lines (L-302 B, L-14 B, L-190 B, L-272 B), 6 sterile single-crosses (female component for three-way hybrids, G/04 A x L-104 B, G/04 A x L-14 B, G/04 A x L-282 B, G/04 A x L-272 B, G/04 A x L-190 B, G/04 A x L-302 B), and four restorer-fertility lines (PI 12/99 R, O3G R, L-Š 89 R, O3 MR). Tested material was developed at the Agricultural Institute Osijek sunflower breeding program. Each genotype was sown in two 5-m long rows, in three replications. One row of each genotype represented the control, while the other was artificially infected. In each replication, 7 plants of each genotype were artificially infected in full button stage (R2, according to Schnieter and Miller, 1981). Sunflower stems were infected on 11th July 2006 and 15th July 2007, with fungal mycelium grown in the laboratory. Previously, during 2004 and 2005, the patogenicity was tested in a considerable number of strains in the location of Osijek, a location of large-scale sunflower production in Croatia. The most aggressive one was used for this investigation. Circular plug of mycelia was placed on a leaf stalk intercept (2-3 cm long) from one of mid-stem leaves. Infection spot was covered with a piece of wet cotton wool and aluminum foil to prevent mycelial dryness and create favorable micro-climate conditions for pathogen development. Susceptibility estimation was performed by weekly measurements of the length of lesions during three weeks after infection. Analysis of variance (ANOVA) and LSD test were processed by Statistical Analysis System for Windows software (SAS Institute, 2003).

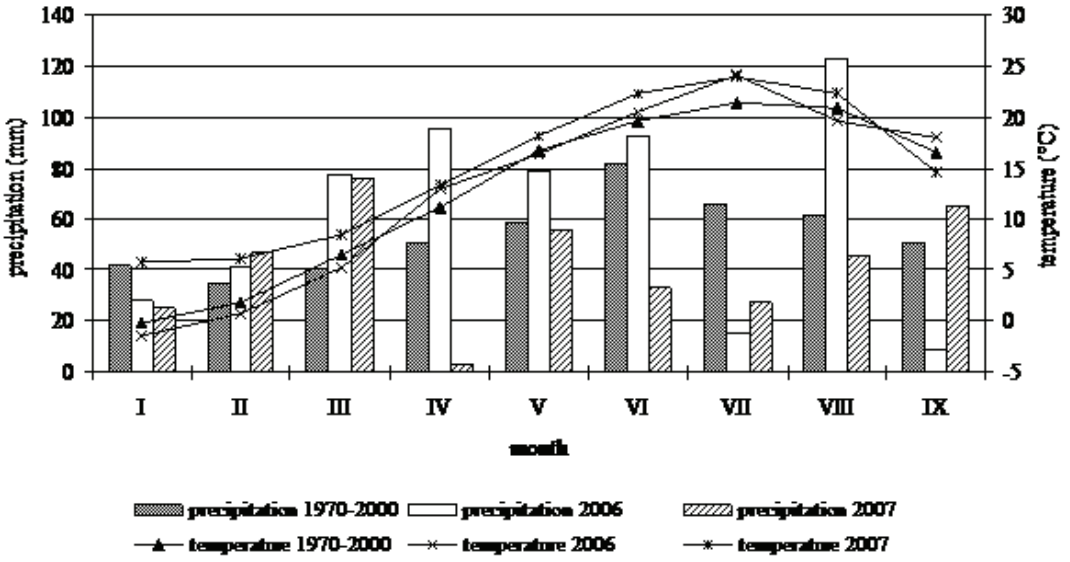

Fig. 1. Monthly air temperatures (°C) and precipitations (mm) for investigated years (2006-2007) and 30-year average (1970-2000), Osijek.

RESULTS AND DISCUSSION

During the first lesion measurement, artificial infection success was clearly visible. Most of the infected plants showed disease symptoms. Occurrence of symptoms was perceived clearer in the second and particularly in the third measurement in both years. Lesion length per measurement as well as the average length of lesions for both years is shown in Table 1. On average, the highest tolerance to the pathogen was shown in SC, then A lines, and B lines, while the lowest resistance recorded was in fertility restorer lines (R). The lowest average value was recorded for the single cross hybrid L-G04 A x L-14 B (2.48), and the highest average value for mf (B) line L-302 B (5.06). The lowest susceptibility to the pathogen corresponded to the cms lines L-101 A and L-205 A. From mf (B) lines, L-272 B and L-190 B were more tolerant. The most tolerant two-way hybrids to artificial infection in this investigation were L-G/04 x L-14 and L-G/04 x L-282. These results should be examined in a further investigation, particularly after developing hybrids from tolerant lines. Although the procedure of creating three-way hybrids is longer and more complex, some authors (Giriraj et al., 1988; Bochkovoy et al., 2000) give these hybrids a certain advantage regarding grain yield stability. Fertility restorers L-O3 M R and L-O3 G R showed the lowest susceptibility to infection (Table 1).

Table 1. Average lesion length (cm) of sunflower inbred lines after infection with *D. helianthi* at Osijek in 2006-2007.

No	Lines	Lesion length (cm)		
		2006	2007	Average
1	L- 271 A	3.94	5.87	4.91
2	L-G/04 A	3.72	5.23	4.48
3	L-301 A	1.84	6.53	4.19
4	L- 205 A	2.58	3.20	2.89
5	L-101 A	2.01	3.39	2.70
	Average	3.88	3.80	3.83
6	L-302 B	4.87	5.25	5.06
7	L-14 B	3.76	4.03	3.90
8	L-190 B	2.33	4.31	3.32
9	L-272 B	2.04	4.23	3.13
	Average	3.41	4.30	3.90
10	L-G/04 x L-104 SC	3.66	5.48	4.57
11	L-G/04 x L-272 SC	1.79	5.29	3.54
12	L-G/04 x L-190 SC	1.51	5.51	3.51
13	L-G/04 x L-302 SC	2.10	4.19	3.14
14	L-G/04 x L-282 SC	0.92	4.43	2.68
15	L-G/04 x L-14 SC	0.97	3.99	2.48
	Average	1.83	4.82	3.32
16	L- 12/99 R	3.52	5.59	4.56
17	L-Š 89 R	1.65	7.07	4.36
18	L-O3 G R	1.44	6.08	3.76
19	L-O3M R	2.22	4.97	3.59
	Average	2.90	5.24	4.10
	LSD 0.05	0.89	1.17	0.72

Legend: A – cytoplasmatic male sterile lines; B – male fertile lines; SC – single cross sterile hybrids; R – restorer of fertility

It is important to emphasize that, besides their genetic potential, the environment has a strong influence on genotype tolerance level. Regarding the fact that these results were obtained in field trials, all data should be observed through climate conditions during investigation (Fig 1). During a two month period (July, August) the amount of precipitation in a 30-year average recorded at the Agricultural Institute Osijek experimental field was 128.2 mm. In 2006, the same period of time recorded a little more than the 30-year average (134.9 mm), while in 2007 this amount was significantly lower (72.4 mm). Observing only July, the month when the artificial infection was carried out, the amount of precipitation was lower in 2006 (15.3 mm) in comparison with 2007 (27.4 mm) and the 30-year average (66.3 mm). In August 2006, this value was 122.6 mm, significantly above the 30-year average (61.9 mm) or the same month in 2007 (45 mm). However, artificial infection and measurements were, in both years investigated,

conducted during the second and the third ten day's period of July, when only 15.3 mm of precipitation (2006) was measured, which makes this period drought and unsuitable for artificial infection and pathogen development. In 2007, the rainfall in July was almost double (27.4 mm), but still under the 30-year average (66.3 mm). Air temperatures for 2006 (21.8 °C) in these two months were on average on the same level as the 30-year average (21.1 °C). In 2007, the two month average was 23.1 °C, which made that period more suitable for pathogen development.

Comparing these meteorological data with the results of lesion length for investigated sunflower lines given in Table 1, it could be concluded that precipitation and air temperatures in July are most important for artificial infection as well as for pathogen development. Regarding that fact, in 2007 all investigated lines have longer lesions in comparison with 2006. Also, it can be concluded that, in this investigation, the precipitation had a stronger influence than temperatures on artificial infection as well as on pathogen and disease development. It is known that years with lower air temperatures and a higher precipitation are extremely suitable for white head rot development (Vratarić and Sudarić, 2004; Jurković and Ćosić, 2004; Duvnjak et al., 2006), while higher temperatures and moisture are suitable for Stem canker development.

Although these results were obtained in two-year trials, they could be a good indicator and guideline in further sunflower breeding work related to disease resistance on *D. helianthi*. The research should be continued in following years, including new genotypes. Additionally, testing important agronomic traits in combination with resistance to this pathogen will give a more objective estimation of selecting material for new sunflower hybrid development.

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Study of the expression of shikimate dehydrogenase activity in sunflower genotypes treated with *Sclerotinia sclerotiorum*

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ABSTRACT

The expression of shikimate dehydrogenase in cotyledons of five sunflower inbred lines treated with *Sclerotinia sclerotiorum* was compared with exogenous application of synthetic oxalic acid. Normally, shikimate dehydrogenase becomes enzymatically active in sunflower at seed germination stage, and it reaches its maximum during the cotyledon stage, gradually decreases and disappears after four leaf stage. We found that shikimate dehydrogenase activity was very faint in control plant protein extract whereas its intensity greatly increased in samples derived from seedlings inoculated with *S. sclerotiorum* as well as with synthetic OXA at the same stage. The expression of shikimate dehydrogenase at the first phase of growth may serve as a tool for rapid screening and selection of resistant genotypes of sunflower to *S. sclerotiorum.* Some agronomy parameters in terms of plant dry and fresh weight and the total chlorophyll concentration were assessed for both treatments compared with their untreated controls. Exogenous oxalic acid treatment caused more deleterious effects in comparison with its endogenous production of the pathogen, considering stem rot and eliciting photosynthesis reduction. The excessive toxicity of exogenous treatment suggests that *S. sclerotiorum* infection triggers a more complex metabolic pathway involvings oxalic acid secreted by the pathogen.

Key words: dehydrogenase activity – *Helianthus annuus* – resistant genotypes – *Sclerotinia sclerotiorum* – screening – shikimate.

INTRODUCTION

Sclerotinia root, stem, and head rot are major diseases of sunflower caused by the pertotrophic fungus *Sclerotinia sclerotiorum* (Lib.) de Bary (Rönicke et al., 2005). The general inability of economically important crops to develop germplasm resistant to this pathogen has focused attention on the need for a more detailed understanding of the pathogenic factors involved in disease development (Cessna et al., 2000). Oxalic acid secretion by *Sclerotinia* appears to be an essential determinant of its pathogenicity (Maxwell and Lumsden, 1970; Noyes and Hancock, 1981; Marciano et al., 1983; Godoy et al., 1990; Dutton and Evans, 1996; Zhou and Boland, 1999). Evidence for such involvement is based on the recovery of millimolar concentrations of oxalate from infected tissues (Bateman and Beer, 1965; Maxwell and Lumsden, 1970; Marciano et al., 1983; Godoy et al., 1990) and from the manual injection of oxalate, or of culture filtrate containing oxalate, into plants and observation of the development of *Sclerotinia* disease-like symptoms independent of the pathogen (Bateman and Beer, 1965; Noyes and Hancock, 1981).

Speculation regarding the mechanism or mechanisms by which oxalate secretion might enhance *Sclerotinia* virulence currently centers on three modes of action (reviewed in Dutton and Evans, 1996). First, because several of the fungal enzymes secreted during invasion of plant tissues (e.g., polygalacturonase) have maximal activities at low pH, various researchers have postulated that oxalate might aid *Sclerotinia* virulence by shifting the apoplastic pH to a value better suited for enzymatic degradation of plant cell walls (Bateman and Beer, 1965). Second, because oxalate may be directly toxic to host plants, presumably because of its acidity, the secretion of oxalate has been suggested to weaken the plant, thereby facilitating invasion (Noyes and Hancock, 1981). Finally, chelation of cell wall Ca^{2+} by the oxalate anion has been proposed both to compromise the function of Ca^{2+}-dependent defense responses and to weaken the plant cell wall (Bateman and Beer, 1965). Although each of these hypotheses has its logical appeal, evidence supporting them is incomplete, and arguments against their validity have also been made (Dutton and Evans, 1996).

Shikimate dehydrogenase (EC 1.1.1.25) (SKDH) is an important biochemical marker produced by plants for investigation of *S. sclerotiorum* infection that catalyzes the fourth step in the shikimate pathway, which is essential for biosynthesis of aromatic amino acids and aromatic compounds. The increase in SKDH activity occurring after *Sclerotinia* infection affects the biosynthesis of shikimic acid, which is involved in the synthesis of lignin for cell walls (Buiatti, 1993; Carrera and Poverene, 1995) and is considered to be the most interesting component in relation to the plant resistance to *S. sclerotiorum* (Quillet, 1990).

The aim of this study was to determine the effects of OXA treatment in *Helianthus annuus* L. either when OXA was endogenously produced by *S. sclerotiorum* or, alternatively, when it was exogenously treated as synthetic moiety. Study of the expression of SKDH activity may help to develop a fast and reliable screening technique in breeding sunflowers for resistance to *S. sclerotiorum*.

MATERIALS AND METHODS

Fungal and plant material

Black sclerotia of *S. sclerotiorum* collected from stems of infected plants were germinated and grown on potato dextrose agar (PDA) at 25 °C. After several passages on PDA and controlling the proper hyphae by observation under optical microscope 400 X, sclerotia were subcultured on PDA (Becton Dickinson, Sparks, MD, USA) under light (24 h/day). After 3 days, 0.2 cm agar plugs were removed with a sterile cork borer from the leading edge of the mycelia and were subcultured on PDA agar plate, 0.5 cm agar plugs were removed from the leading edge of the second two-day old mycelia and used for inoculation.

Five inbred lines of sunflower of different origins were used in the experiments: AC 4122 and C are maintainer inbred lines, developed at University of Udine from an Italian open pollinated population ALA, HA 89 is a maintainer inbred line and HA 410 (Reg. no. GP-227) and HA 411 (Reg.no.GP-228) are inbred lines released by USDA-ARS, Fargo, ND, North Dakota. AC 4122 and HA410 are resistant inbred lines, C and HA 89 are susceptible inbred lines, and HA 411 is an intermediate inbred line. Seeds of five genotypes were surface sterilized as described by Burrus et al. (1991) and germinated in sterile test tubes (130 x 25 mm) on a solid MS medium (Murashige and Skoog, 1962).

Plugs of PDA prepared as described earlier were placed on the leaves which were wounded slightly. Leaves of uninoculated, control plants were treated similarly with PDA agar plugs without the mycelia. The inoculated parts of plants were then washed by sterilized water and transferred to Hoagland solution (H2395, Sigma Chemical Co. prepared according to manufacture's directions, autoclaved and stored at room temperature) and maintained at 20-25°C, relative humidity about 40-50% and light intensity about 500 mM. m^{-2} s^{-1}.

Preparation of synthetic oxalic acid

A stock solution of 1 M oxalic acid (Sigma Chemical Co.) was prepared, then it was diluted to obtain the same toxin concentration of culture filtrate (toxin concentration was estimated using Oxalate kit 591C followed by spectrophotometer assessment).

The recovery of vegetal extraction

Five days after exposure to *S. sclerotiorum* and synthetic OXA, the plant tissue above the cotyledons (also from untreated control plants) was collected and homogenized in a mortar and placed in a sealed tube containing buffer 50 mM Tris-HCl, (pH 7.4), 0.25 M sucrose, 1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM PMSF (phenylmethanesulfonyl fluoride), 2.5% v/v β-mercaptoethanol. After homogenization and centrifugation at 2000 *g for 5 min, the supernatant was used for OXA determination, total protein determination, and SKDH activity assay.

Oxalic acid measurement

Ten µl of extracted plant material as previously described was used for measuring toxin (OXA) concentration by oxalate kit 591 C followed by spectrophotometer assessment at 590 nm wave lengths.

Determination of total soluble protein and Shikimate dehydrogenase activity assays

The extracted plant material was employed to determine the total protein content, using the Bio-Rad protein assay kit with BSA as standard (Bradford, 1976) followed by spectrophotometer assessment at 595 nm wave length.

Native-PAGE

Native Polyacrylamide Gel Electrophoresis was performed using 12% (W/V) polyacrylamide slab gel in 0.2 M Tris, 2 mM EDTA and 0.15 M boric acid (pH 8.5) as electrode buffer (Guries and Ledig, 1978). Staining of SKDH was done by fixing for an hour in buffer solution containing tetrazolium salt as described by Tanksley and Rick (1980).

Experimental design and statistical analysis

The treatments corresponded to five genotypes inoculated with *S. sclerotiorum* culture filtrate, treated with synthetic OXA and the controls, which were grown in hydroponics with Hoagland solution under similar conditions. The analyses of total fresh weight, total dry matter per plant at the end of the experiment, OXA concentration (mM), and chlorophyll concentration (mg m^{-2}) 5 days after treating and the expression of SKDH were carried 48 h after treating with either *S. sclerotiorum* or exogenous OXA.

The experiment was carried out following a bifactorial completely randomized block design with three replicates and four plants for each replication. The first factor, genotype, was constituted by the five inbred lines, and the second factor, toxin treatment, was constituted by endogenous OXA produced by *S. sclerotiorum* and exogenously applied synthetic OXA. Statistical analyses of triplicate determinations of OXA contents and enzymatic activity of SKDH from five genotypes were subjected to Analysis of Variance. Significant differences were expressed as $P<0.01$, and the least significant difference procedure was used to compare means of treatments. Correlation coefficients and regression analysis were calculated between the variables having significant differences between genotype means.

RESULTS AND DISCUSSION

The effects of two treatments, inoculation with *S. sclerotiorum* and exogenous oxalic acid, on plant growth were compared by measuring fresh weight and dry weight (plants were dried at 60 ºC in an oven for 3 days), which were the only growth parameters that could be calculated in early growth phase. Significant differences were recorded between genotypes for dry and fresh weight. In samples treated with culture filtrate, the fresh weights (considered as a percentage of controls) were significantly higher in HA 410, intermediate in HA 411 and AC 4122, and low in C and HA 89. These differences revealed an individual variability of responses to toxin penetrated into the cells, which confirms the polygenic nature of this disease (Mestries et al., 1998). The dry matter of these samples did not show significant differences between genotypes (Table 1). These data indirectly suggest that *S. sclerotiorum* manipulates the metabolism of host-derived carbohydrates and consequently increases in cell water content.

In the cases of samples treated with synthetic OXA, fresh weight of HA 411, AC 4122 and C had higher values whereas other genotypes followed them with lesser significant differences. Concerning dry matter, there were no significant differences except in resistant line AC 4122 with the lowest dry weight. These results provide an alternative explanation for oxalate-induced wilting. It seems that synthetic OXA induces an equal effect of destruction on all resistant or susceptible genotypes. This firstly causes a great reduction in plant growth, then self-reconstruction of the plant happens and it continues its growth.

Table 1. Growth characters of five sunflower genotypes analysed 5 days after inoculation with *S. sclerotiorum* and treated with synthetic OXA

Genotypes	Synthetic OXA		*S. sclerotiorum* inoculation	
	Dry Weight Plant (%)[1]	Fresh Plant Weight (%)[1]	Dry Weight Plant (%)[1]	Fresh Plant Weight (%)[1]
AC 4122	58.6 b[2]	43.91 a	65.6 a	36.8 b
C	76.4 a	39.3 ab	64.9 a	27.7 c
HA 89	71.1 a	30.9 c	64.4 a	28.6 c
HA 410	71.1 a	36.9 bc	71.4 b	47.8 a
HA 411	75.5 a	43.4 a	68.4 ab	36.2 b

[1]Values are reported as percentage of the controls,

[2]Means followed by the same letter are not significantly different at 1% level as indicated by Duncan's Multiple Range Test.

The toxic metabolite of pathogen causes a decrease in chlorophyll (chl) concentration and this reduction is clearly associated with other symptoms of phytopathogenicity, i.e. stem rot. Chl

concentration data will provide information on a plant's photosynthetic potential (Raymond et al., 2004). The effect of both treatments on plant metabolism was revealed as a reduction in Chl concentration (data not shown). This phenomenon for the samples treated with synthetic OXA was not accompanied by any signs of stem rot and basal stalk rot, which implies the different nature and effect of OXA on the plant (Fig 1).

a **b** **c**

Fig. 1. Comparison of growth in plants a) uninoculated control, b) inoculated with *S. sclerotiorum* and c) treated with synthetic OXA.

The intracellular OXA content in sunflower seedlings was determined five days after inoculation with *S. sclerotiorum* or synthetic oxalic acid treatment to verify whether the metabolic response to OXA could be correlated with disease resistance.

As reported in Fig. 2, in uninoculated lines C (1.10 mM) and HA 89 (1.21 mM) a higher OXA content was observed, which demonstrates that they are more susceptible to fungal disease as compared to the other lines. This confirms previous observations by Tahmasebi Enferadi et al. (1998 b) about the different thresholds of OXA concentration between different genotypes.

Concerning samples inoculated with *S. sclerotiorum*, OXA concentration values were the highest in susceptible HA 89 (1.81 mM) and C (2.6 mM) when compared to their untreated controls. On the contrary, it was observed that OXA intracellular concentration in samples treated with synthetic acid was lowest in HA 89 (2 mM) whereas AC 4122 had the highest content.

Since OXA concentration increases in pathogen-infected plants, our data demonstrate that the more resistant the plants, the more they were able to control catabolism of this acid, as shown in HA 410 and AC 4122. This is probably due to an intercellular mechanism which inhibits abnormal increases in their pH. Therefore, specific macromolecules are produced by a pathogen that can be recognized by the plant (Buiatti, 1993) and lead to the activation of a host defense response. These signals were absent after synthetic OXA treatment, causing the plant to be unable to manage OXA.

Other studies showed that phenolic compounds play an important role in plant defense responses against pest and pathogens (Nicholson and Hammerschmidt, 1992). In sunflower the induction and accumulation of phenolic compounds, their deposition on cell walls and lignification is a well-characterized mechanism of disease resistance against *S. sclerotiorum* (Prats et al., 2003). A higher content of phenolic compounds in resistant varieties was observed as compared to susceptible ones (Prats et al., 2003; Rodríguez et al., 2004). Conceivably, resistant plants had higher activity levels of phenylalanine ammonia–lyase (PAL), which provides the biosynthesis of important phenolic derivatives such as lignin.

Similar to phenolic compounds and PAL, shikimic acid and the related enzymatic activity of SKDH are used in order to find out a biochemical paradigm, which provides a clear correlation with disease resistant genotypes. SKDH is an intermediate step in aromatic amino acid biosynthetic pathway, essential to lignin production, and is considered as a resistance mechanism against *S. sclerotiorum* related to its chemical and/or physical cell barriers.

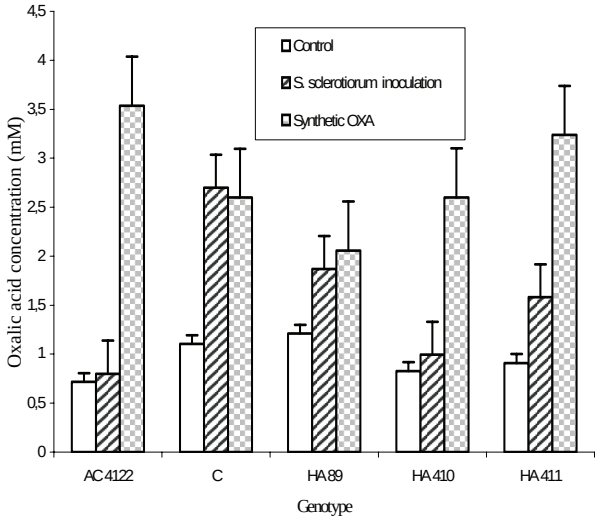

Fig. 2. OXA concentration of different sunflower genotypes 5 days after inoculation with *S. sclerotiorum* and synthetic OXA and untreated plants (control). Bars represent L.S.D. for $P < 0.01$

SKDH that becomes enzymatically active in sunflower at seed germination stage, and it reaches its maximum during the cotyledon stage, gradually decreases and stops after 4-leaf stage as reported by Diaz et al. (1997). SKDH reactivates treating the plant either with pathogen or synthetic OXA. Parental inbred lines have a single band with identical mobility (Carrera and Poverene, 1995), indicating the presence of the same allele in all genotypes, as reported by Ledoux (1992). Enzyme SKDH has a monomeric structure, encoded by a single gene and a single locus with two different co-dominant alleles in heterozygous plants, *skdh*-a and *skdh*-b, with the molecular weight of 64.5 kDa and 58.9 kDa, respectively (Tahmasebi et al., 1998a). The increase in SKDH activity for both homozygous and heterozygous individuals following the attack of *S. sclerotiorum* is accompanied by the expression of only skdh-b. The lack of the expression of skdh-a in homozygous individuals confirms the hypothesis by which skdh-a is considered a null allele, as described by Goodman et al. (1980). Both alleles have most likely the same domains with a few changes in the variable regions, which concerns regions interacting with OXA. In Fig. 3, the domain family of SKDH is shown. It is suggested that the interaction between OXA and reactivation of skdh-b relates to the third domain, Shikomate_DH. This domain involves the biosynthesis of aromatic amino acids and is related to the mechanism of resistance. It seems that other domains have a structural role.

Fig. 3. Domain pattern of SKDH along its polypeptide

A study of the expression of SKDH on Native-PAGE 48 h after treatment of the studied inbred lines demonstrated that only a single allele, *skdh*-b, expressed and its mobility was identified. Conceivably, to data dealing with the enzymatic activity dosage, SKDH was very faint in control plant protein extract whereas its intensity greatly increased in samples derived from seedlings inoculated with *S. sclerotiorum* as well as with synthetic OXA (Fig 4.)

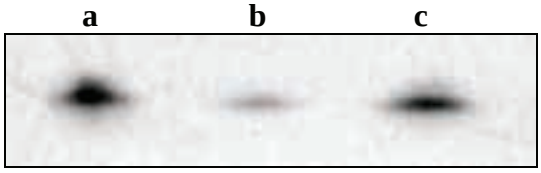

Fig. 4. Expression of skdh-b at the end of cotyledons in homozygous lines of sunflower on Native-PAGE 48 h after treating with a) Synthetic OXA, b) untreated plant, control, and c) *S. sclerotiorum* inoculation.

CONCLUSIONS

In conclusion, the differences observed between symptoms generated by OXA produced by pathogen and OXA originating from a synthetic source can be related to the different nature of biochemical pathway elicited by each treatment, both in resistant and in susceptible inbred lines. Subsequently, this prevents the use of synthetic OXA instead of direct inoculation of plants in rapid screening methods for identification of genotypes resistant to *S. sclerotiorum*. Other advantages of measuring SKDH activity as a rapid and reliable method of screening, are the early discrimination of resistant genotypes in the first growth stage, at the laboratory, on many individuals (since *S. sclerotiorum* is of a polygenic nature, it needs to provide a resistant mass individual) and its cost effectiveness.

Furthermore, our results indicate that SKDH may be a promising biochemical marker that could be used in breeding programs to discriminate between sunflower genotypes resistant and susceptible to *Sclerotinia* infection.

Although different disease resistance mechanisms can be activated simultaneously during defense response, SKDH levels could be directly evaluated to identify resistant lines or, possibly, related to other molecular markers such as total content of phenolic compounds and PAL activity.

ACKNOWLEDGEMENTS

We thank Azienda agraria universitaria “A. Servadei” for providing us with the genetic materials. This work was supported by grants from the Regione Autonome Friuli Venezia Giulia on the improvement of the Project Cargi2.

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Molecular changes in downy mildew-infected sunflower triggered by resistance inducers

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ABSTRACT

Benzothiadiazole (BTH), a plant defense activator, has recently been found to restrict downy mildew development in sunflower. To elucidate the background of this phenomenon, a research programme was started and some of our preliminary results are reported in this paper. The gene expressions of glutation S-tranferase (GST), defensin (PDF) and catalase (CAT) were the subject of investigation using compatible, incompatible and partially resistant sunflower – *Plasmopara halstedii* interactions, respectively. The accumulation of all three gene transcripts were found to be increased in the susceptible sunflower genotype following BTH treatment. Furthermore, in case of the resistant sunflower, HA 335, BTH enhanced GST and PDF accumulation, whereas with the partially resistant RHA 340 the results were ambiguous. It is hoped that our findings may contribute to a better understanding of the plant's own defense system triggered by chemical inducers.

Key words: benzothiadiazole – catalase – defensin – glutathion S-transferase – *Plasmopara halstedii* – sunflower.

INTRODUCTION

Although *Plasmopara halstedii* can be effectively controlled by using genetic resistant plants and seed treatment with fungicides, protection can be hindered by the genetic variability of the fungus (Albourie et al., 1998; Gulya, 2007). Thus, besides the traditional control strategies, there was a need to look for alternative methods to provide effective disease control. One solution can be the use of systemic induced resistance, i.e. the activation of the defense system of plants.

The plant activator BTH (benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester) has already been shown to induce activated resistance in many crops against a broad spectrum of diseases (Cohen et al., 1994; Kogel et al., 1994; Pajot et al., 2001). BTH appears to be able to restrict downy mildew symptoms in sunflower under greenhouse conditions (Bán et al., 2004). Microscopic observations show that BTH treatment significantly decreased the development of fungal structures associated with cell necrosis and H_2O_2 accumulation in the BTH-treated susceptible sunflower hypocotyls.

Glutathione S-transferase (GST) has a well defined role in plant detoxification reactions. It is capable of catalyzing the binding of various xenobiotics, like pathogens. Various abiotic stressors are the inducers of GST activity in plants (Dean et al., 1990). GST is also considered one of the antioxidative enzymes, because it plays an important role in the protection against oxidative membrane damage and necrotic disease symptoms. Enhanced GST activity has been found in plants after pathogen infection, for example in barley plants infected by powdery mildew (El-Zahaby et al., 1995), and tobacco plants infected by TMV (Fodor et al., 1997).

To protect themselves against pathogenic attacks, plants evolve diverse strategies, for example the synthesis of antimicrobial peptides, like defensin. Defensin is a small, cysteine-rich antimicrobial peptide, existing in a wide range of plants and animals. Urdangarin et al. (2000) described full length sunflower cDNA from *Helianthus annuus* flowers encoding for defensin, and the authors supposed there was a relationship between enhanced expression of a defensin gene and decreased susceptibility to *Sclerotinia sclerotiorum*. Solis et al. (2006) isolated a defensin gene from *Lepidium meyenii*, having activity against *Phytophthora infestans*.

Catalase is one of the main antioxidant enzymes; it catalyzes the dismutation of H_2O_2 into water and dioxygen. This enzyme is located in peroxisomes and glyoxisomes. Catalase activity is affected by abiotic stressors, like boron (Karabal et al., 2003), light and chilling (Gechev at al., 2003), and acid rain (Gabara et al., 2003). In sunflower, catalase activity was increased by UV-B radiation (Costa et al., 2002) and cadmium treatment (Azpilicueta et al., 2007). Niebel et al. (1995) demonstrated induction of catalase in potato upon nematode and bacterial infection as well. Several plants have multiple CAT isoenzymes. For

example, in sunflower at least eight isoforms (CAT1-CAT8) have been described (Azpilicueta et al., 2007).

MATERIALS AND METHODS

The USDA sunflower inbred lines RHA 274, RHA 340 and HA 335, as well as *Plasmopara halstedii* pathotype 700 were used to get one compatible, and two incompatible combinations. While HA 335 is characterized by total resistance, RHA 340 exhibits HLI (hypocotyl-limited) resistant type (Virányi and Gulya, 1996).

Pre-germinated seeds were soaked in an aqueous solution of BTH (160 mg/L) for at least 6 hours (first day), followed by their inoculation with *P. halstedii* sporangia (50000 sporangia/ml) using the whole seedling inoculation technique (Cohen and Sackston, 1973). Germlings were subsequently planted into pots filled with a commercial soil mixture and grown in the greenhouse (18/24ºC, 60 % RH, 16h light) for 3 weeks.

Samples were taken 3, 9, 13, 16 days after infection (dpi). The whole seedlings were frozen in liquid nitrogen and ground with mortar and pestle. Total RNAs were extracted using the Qiagen Plant Mini kit, and then the extracted RNA treated with RNase inhibitor to protect the extracted RNA and with DNase I to remove genomic DNA contamination. The extracted RNAs were measured with spectrophotometer and the RNA concentration of 1µg/µl adjusted. One µg of RNA was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad).

Primers for PCR amplifications were applied according to Radwan et al. (2005) and Azpilicueta et al. (2007) as shown in Table 1. Twenty-five µl of the PCR reaction mixture contained 1µl RNA, 1 unit of Taq DNA polymerase (Fermentas), 2.5 µl 10X Taq polymerase buffer, 1 µl 2.5mM dNTP mix, 1.5 µl 25mM $MgCl_2$, 2.5 µl 5 µM primers and 13.8 µl PCR water. PCR reactions were performed using a Gene Amp PCR System 2700 PCR machine. The amplification program included an initial step at 94°C for 3 min and 25-32 cycles (Ha-EF1a: 25; Ha-GST: 26; Ha-PDF: 30; CATA2: 31) of 15 sec at 94 °C, 15 sec at Tm °C (Ha-EF1: 58; Ha-GST, Ha-PDF: 61; CATA2: 50), 20sec at 72°C.

The PCR products were electrophorized through 1% agarose gel, visualized with ethidium bromide and photographed in a Molecular Imager Gel Doc system (Bio-Rad). The signals from gels were quantified using a Quantity One program with molecular mass ruler (Bio-Rad), and normalized over the signals from Ha-EF1α.

Table 1. Primer sequences and accession numbers used in this study

Gene[1]	Primer sequences	Accession number
Ha-EF-1α	Forward 5'-AGGCGAGGTATGATGAAATTGTCA-3' Reverse 5'-GTCTCTTGGGCTCATTGATTTGGT-3'	AAM19764
Ha-GST	Forward 5'-CCTCAGGATGCTTACGAGAAGG-3' Reverse 5'-GCAGAAATATCAACCAGGTTGATG-3'	AY667502
Ha-PDF	Forward 5'-ATGGCCAAAATTTCAGTTGCTTTCA-3' Reverse 5'-AAGACTTGCACTGGTCATCACAG-3'	AF364865
CATA2	Forward 5'-TTCCCGCTTGAATGTGAAG-3' Reverse 5'-CCGATTACATAAACCCATCATC3'	AF243517

[1]Ha-EF-1a: constitutive elongation factor 1a, Ha-GST: glutathione S-transferase, Ha-PDF: defensin, CATA2: catalase isoenzymes.

RESULTS

In general, Ha-GST transcript accumulation was higher in the untreated resistant sunflowers than in the susceptible ones. At 3 and 9 dpi the highest transcript accumulation was detected in the HA 335 plants. At 13 and 16 dpi, however, this accumulation was higher in the 'HLI resistant' RHA 340 plants as compared to HA 335. The BTH treatment increased Ha-GST transcript level in both the susceptible and totally resistant plants throughout the experiment. The effect of BTH treatment on this transcript accumulation in the HLI resistant plant was contradictory, because the treatment increased the transcript accumulation at 3 and 16 dpi, but appeared to reduce it at 13 dpi (Fig1).

HA-PDF transcript accumulation was found to be higher in the resistant sunflower lines than in the susceptible one, similar to the HA-GST transcript accumulation. The effect of BTH treatment on PDF activity was detectable in both the susceptible and totally resistant sunflowers. In case of the 'HLI resistant plants, Ha-PDF transcript accumulation was increased by BTH treatment on the second and the

last sampling days only. Among the untreated plants, the totally resistant plants showed Ha-PDF transcript accumulation at 3 dpi, whereas in all the BTH treated plants this transcript accumulation could be detected on the first sampling day. In the second sampling day the increase in transcript accumulation was observed in all plants examined, and there were no differences between the two resistant genotypes. At 13 and 16 dpi the maximum accumulation was evident in the 'HLI resistant' plants. It is interesting to note that there were no differences detected between the untreated 'HLI resistant' and the BTH-treated susceptible plants at 9, 13 dpi (Fig. 1).

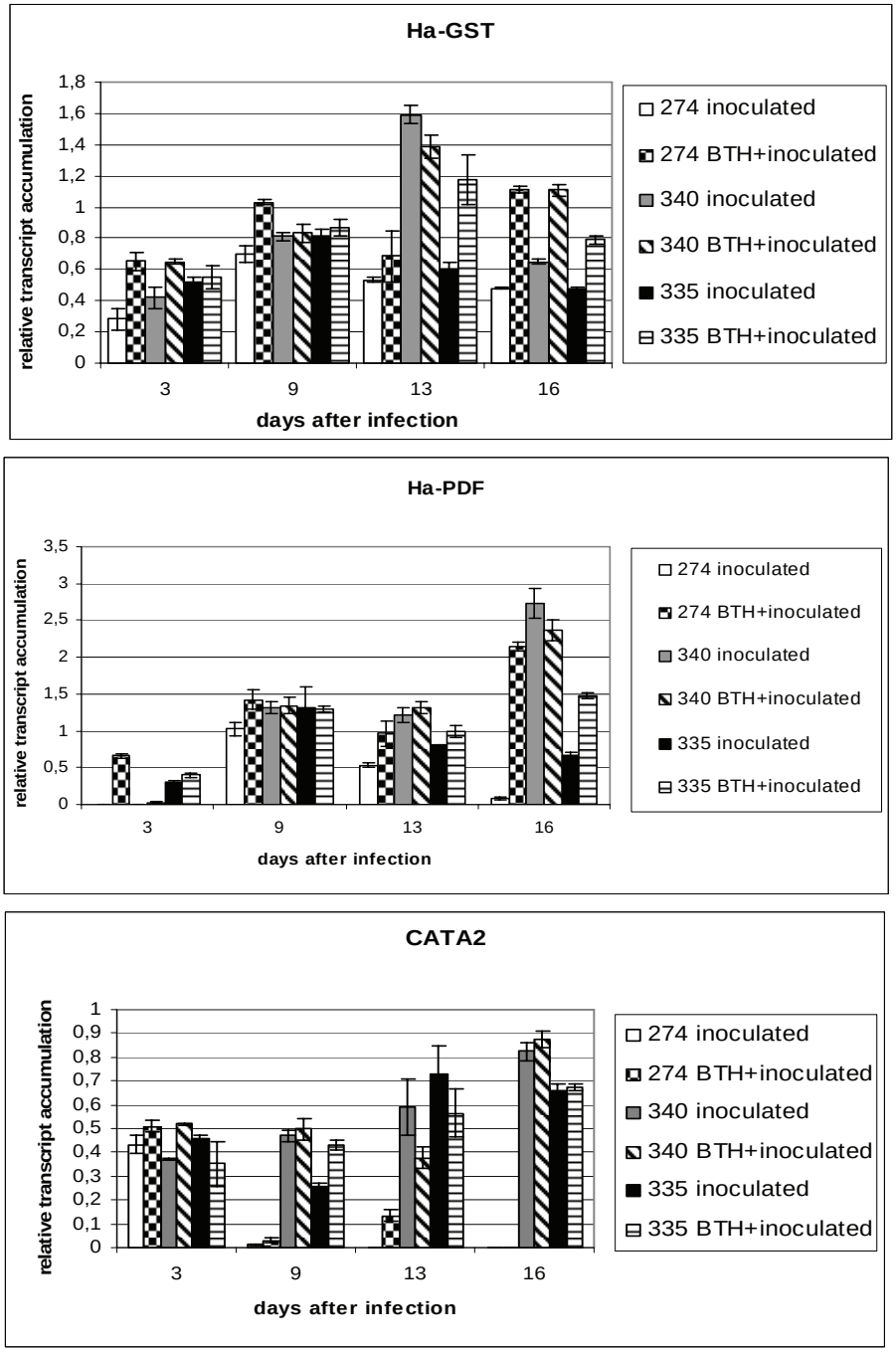

Fig. 1. Accumulation of gene transcripts in sunflower plants after BTH treatment and *Plasmopara halstedii* inoculation. 1. glutathione S-transferase (Ha-GST); 2. defensin (Ha-PDF) and 3. catalase (CATA2) gene expression in a susceptible (RHA 274), partially (HLI) resistant (RHA 340) and totally resistant (HA 335) sunflower line. Each value represents three replicates (±S.D.)

As for catalase activity, both resistant sunflowers exhibited higher CATA2 transcript level, than did the susceptible one, except for 3 dpi. After the first sampling day, catalase activity was not detectable in the untreated susceptible sunflower plants, but BTH-treatment considerably increased the level of CATA2 transcript. In case of untreated 'HLI resistant' plants, a continuous increase in transcript accumulation of CATA2 was found to reach its maximum at 16 dpi. With the exception of the third sampling day, the BTH treatment increased the transcript accumulation in the 'HLI resistant' plants as well. In the totally resistant untreated sunflowers the accumulation of CATA2 transcript reached its maximum at 13 dpi (Fig. 1).

DISCUSSION

In this study molecular changes in BTH-treated sunflowers were the subject of investigations associated with infection by *P. halstedii*. PCR was used in an attempt to describe induced resistance events in different sunflower genotypes.

Glutathione S-transferase usually detoxifies xenobiotics in plant tissues. We found an increased level of GST activity in the BTH-treated, susceptible sunflower and this increased activity resembled that detected in the 'HLI resistant plants'. Fodor et. al (1997) reported similar results with tobacco either treated or non-treated with salicylic acid. In contrast, El-Zahaby et al. (1995) found a significantly higher level of GST activity in susceptible barley plants than in resistant ones after powdery mildew inoculation. They assumed that the fungus itself contained GST enzyme, so that both the host and the pathogen might contribute to this increase in GST activity.

Defensins are a class of antimicrobial peptides found in several plants, including sunflower. In our experimental conditions defensin gene expression was induced by BTH treatment in the susceptible sunflower plants, and this enhanced level was equally found in the 'HLI-resistant' plants. Similar to Radwan et al. (2005), Ha-PDF transcript accumulation was lower in the non-treated susceptible plants, than in the resistant ones.

Catalase is usually considered to be one of the most important antioxidant enzymes. BTH treatment increased CATA2 transcript level in the susceptible sunflower plants but this effect was not evident in the resistant sunflowers.

In conclusion, the plant activator BTH had a positive effect on the natural defense system of sunflower by enhancing the expression of three genes that are considered to be associated with the chemically-induced host resistance to *P. halstedii*.

ACKNOWLEDGEMENTS

This research was supported by the National Research Fund, OTKA no. T0433219.

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Effectiveness of the genetic resistance to *Plasmopara halstedii* under natural conditions and diversity of the pathogen within sunflower fields

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ABSTRACT

Genetic and chemical strategies are effective ways for controlling the downy mildew caused by *Plasmopara halstedii* in sunflower. Although genetic resistance is very frequent in sunflower hybrids, new races of the pathogen that overcome the genes of resistance appear. The chemical treatment with the systemic fungicide metalaxyl-M is mandatory in Spain for sowing susceptible sunflower, but resistance of *P. halstedii* to metalaxyl-M has been reported. Four experiments were conducted under dryland conditions in four locations of Andalusia. Ten resistant hybrids were grown in three of the experiments with the objective of assessing the effectiveness of the genetic resistance against natural infections by *P. halstedii*. Values of downy mildew incidence and also sunflower production depended significantly on the cultivar in the three fields. However, lowest productions did not correspond to the most susceptible hybrids, showing the efficiency of the genetic resistance. Aiming at assessing the frequency of resistance of *P. halstedii* to metalaxyl-M within one sunflower field and the race of the resistant isolates, metalaxyl-M treated seeds of 19 commercial sunflower hybrids were sown in the fourth experiment. Thirty four isolates with resistance to metalaxyl-M were recovered from plants of different hybrids and different positions within the field, and all of them were characterized as race 310. These results show that isolates with resistance to the fungicide can be widely spread within the field and that resistance to the fungicide can happen in races with a high virulence.

Key words: downy mildew – genetic control – *Helianthus annuus* L. – natural infections – resistant sunflower hybrids.

INTRODUCTION

Sunflower downy mildew is a disease caused by the Oomycete *Plasmopara halstedii* Farl. Berl. & de Toni, that was reported for the first time in Spain in the 1970's. Favourable conditions for disease development are temperatures of 14-16ºC and high moisture in the soil during crop emergence (Gulya et al., 1997). Dwarfing of the plants and chlorosis of the leaves are typical symptoms in sunflower. An effective method of controlling the disease is the incorporation of genetic resistance into the host, but more virulent races of the pathogen that overcome the genes of resistance can appear (Molinero-Ruiz et al., 2002). The treatment with the systemic fungicide metalaxyl-M (or mefenoxam) is being widely used in Spain as a chemical way of controlling the pathogen. The fungicide is applied as a seed dressing because of the early infection of sunflower. However, this method of control may not be effective, since resistance of *P. halstedii* to metalaxyl-M has been recently reported in this country (Molinero-Ruiz et al., 2008). In this work, we assessed the efficacy of the incorporation of genetic resistance into sunflower hybrids when natural infections by *P. halstedii* occur and we also studied the diversity of the pathogen in sunflower fields as far as races and reaction to metalaxyl-M are concerned.

MATERIALS AND METHODS

Aiming at assessing the performance of genetically resistant sunflower cultivars and of metalaxyl-M treated sunflower cultivars under natural infections by *P. halstedii*, four experiments were conducted under dryland conditions in different locations of Andalusia: two in Ecija, Sevilla (Casilla Tejada and La Palmera), one in Carmona, Sevilla (Tomejil) and one in Santa Cruz, Córdoba (El Alcaparro). Plants were sown in March, 2007, in fields where there were previous records of infections by downy mildew. In each location the experiment was designed as a randomised complete block with four replications. Experimental unit consisted of four 10-m-long rows 0.7 m apart. In three of the experiments, those in La Palmera, Tomejil and El Alcaparro, 12 sunflower genotypes were sown: the susceptible control (the open

pollinated variety Peredovik), 10 hybrids commercialized by different seed companies as genetically resistant to *P. halstedii*, and two resistant controls (two hybrids whose seed was treated with metalaxyl-M). The fourth experiment was conducted in Casilla Tejada, a field where the existence of populations of *P. halstedii* resistant to metalaxyl-M was suspected. Nineteen commercial varieties from the Andalusian Network of Agricultural Trials (RAEA) and seed treated with metalaxyl-M at the commercial dose of 2 g a.i./kg seed were tested.

Downy mildew symptoms considered were chlorosis of the leaves and/or dwarfing of the plants. Evaluation of symptoms and harvest were performed on the two central rows of each experimental unit. Disease incidence (percentage of symptomatic plants), and yield (kg of seed per hectare) were calculated and analyzed by means of ANOVA and Tukey comparisons ($P = 0.05$).

Samples from diseased plants were collected in the four fields. In each of them, samples were independently collected from different genotypes in order to determine the diversity of *P. halstedii* as far as races and reaction to metalaxyl-M (sensitivity or resistance) were concerned. Sixty-two samples were processed: 7 from Tomejil, 3 from El Alcaparro, 18 from La Palmera and 34 from Casilla Tejada. Also, 62 isolates of *P. halstedii* were recovered after incubation of samples in a humid chamber kept in darkness. The race of each of the isolates was determined with the methodology internationally used for racial characterization of sunflower downy mildew (Gulya et al., 1998; Molinero-Ruiz et al., 2002). Each isolate was inoculated to nine sunflower lines (differentials) that were grown in a chamber under controlled conditions of temperature (15-18ºC) and photoperiod (14 h of light). After two weeks, sporulation of the pathogen in the plants was induced by means of incubation at 100% relative humidity. Resistant or susceptible reactions were noted and considered to determine the race (numeric code) of the isolates. Similarly to race characterization, the reaction of each isolate of *P. halstedii* to the fungicide metalaxyl-M was determined after its inoculation to 40 treated (2 g a.i./kg seed) and 40 non treated Peredovik seeds. After inoculation, growth, and induction of symptoms, as explained, sensitive or resistant reaction of the pathogen to the fungicide was noted as the percentage of sporulated treated plants. When resistance was observed, the inoculation was repeated in order to verify the results.

RESULTS AND DISCUSSION

The incidence of the disease in the susceptible control Peredovik (DMI), ranged between 2.4 and 18.7% in Santa Cruz and Tomejil respectively, and depended significantly on the sunflower cultivar in the three fields ($p \leq 0.005$). Peredovik and Midi were the most susceptible varieties, with DMI values of between 2.5 and 18.7% and 1.8 and 9.9% respectively (Fig. 1). The remaining varieties, with the exception of Leila, showed DMI values not significantly different from zero in the three fields. Leila showed an intermediate DMI (2.1%) in Tomejil (Fig. 1). First downy mildew infections were observed between 6 and 7 weeks after sowing and the incidence of the disease reached its highest values between 9 and 12 weeks after sowing (Fig. 1). All the isolates of *P. halstedii* recovered from Tomejil, Santa Cruz and La Palmera were race 310 (Table 1). Although Molinero-Ruiz et al. (2002) suggested that a diversity of races of *P. halstedii* can exist in one sunflower field, we only found one race.

The disease in the trial of commercial varieties also depended significantly on the sunflower variety ($p = 0.0049$) and the DMI ranged between 0.4% (Kardan) and 17.2% (F-101) (Table 2). Amira was the only variety which did not show symptoms of downy mildew, what could be due to genetic resistance of the hybrid. Since high incidences of disease happened, no records on yield were obtained. Thirty-four populations of *P. halstedii* were recovered from this field. All of them were characterized as race 310 and all of them were resistant to metalaxyl-M at the commercial dose tested (Table 2). The resistance to metalaxyl-M has already been reported in Spain (Molinero-Ruiz et al., 2008), as well as in USA and France.

Yield was only analyzed in the three fields of genetically resistant cultivars and it depended significantly on the cultivar in the three cases ($p \leq 0.0005$) (Fig. 2). Peredovik was the only cultivar with a significantly lower production in Tomejil, with a little more than one third of the average production of the rest of the varieties (Fig. 2). It showed the highest DMI, but it is also an open pollination variety and not a hybrid, and, consequently, its potential of production may be lower. On the other hand, both resistant controls PR64A14 and Olimpia were highly productive, and their yields did not differ from those of the most productive varieties of each of the experiments (Fig. 2). Fig. 2 shows that although the highest DMI was observed in Tomejil, the average yield in this field was twice times higher than those in Santa Cruz and in La Palmera. These differences were due to very high infections by broomrape (*Orobanche cumana* Wallr.) in these two fields compared to those in Tomejil (Table 3). The highest yields not

significantly different to those of the resistant controls in the case of Es Isabella and Leila in La Palmera seem to be due to the good production potential of the hybrids, since incidence of downy mildew was recorded in both of them.

Our results show that the effectiveness of the genetic resistance to downy mildew depends on the race of *P. halstedii* that is present in the field, since races not controlled by the genes of resistance may easily exist. Therefore, a good knowledge of the genetic resistance in sunflower hybrids is advisable. On the other hand, this work also shows that when resistance of *P. halstedii* to metalaxyl-M happens, treatment with the fungicide at the commercial dose is ineffective, and infections can result in a complete loss of sunflower production. As a conclusion, it seems important to analyze the advantages and disadvantages of both the genetic and the chemical strategies for the control of sunflower downy mildew.

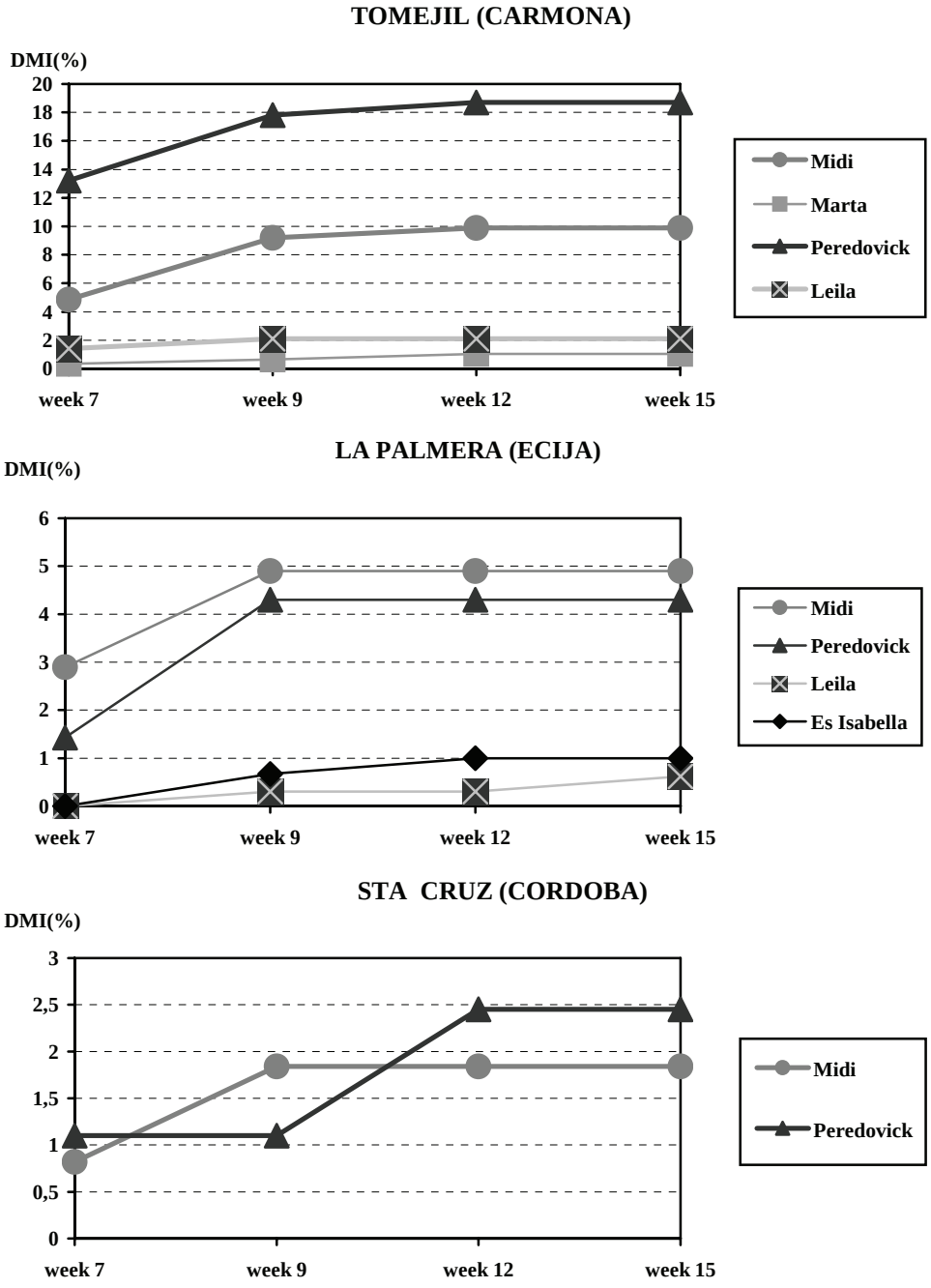

Fig. 1. Downy mildew incidence (DMI) in susceptible sunflower cultivars grown in three different fields of Seville and Córdoba in 2007.

Table 1. Race of 28 isolates of *Plasmopara halstedii* recovered onto different cultivars with resistance to the disease collected in three different sunflower fields

Field	Cultivar	Race
Tomejil	Marta (1) [a]	310
Tomejil	Leila (2)	310
Tomejil	Midi (2)	310
Tomejil	Peredovik (2)	310
Santa Cruz	Midi (1)	310
Santa Cruz	Peredovik (2)	310
La Palmera	Leila (1)	310
La Palmera	Midi (10)	310
La Palmera	Peredovik (5)	310
La Palmera	Es Isabella (2)	310

[a] Parentheses show the number of isolates from each cultivar that were recovered and characterized.

Table 2. Race and reaction to metalaxyl-M showed by 34 isolates of *Plasmopara halstedii* recovered in Casilla Tejada from different commercial sunflower hybrids

Cultivar	Incidence (%)	Race	metalaxyl-M reaction
NX 35607	6.8	310 (1)[a]	R [b]
Quisol	6	310 (1)	R
F-103	4.6	310 (2)	R
PR64A71	9	310 (1)	R
F-104	3.8	310 (1)	--
Voraz	7.5	310 (1)	R
Imigen	5.4	310 (3)	R
F-101	17.2	310 (2)	R
Es AMIRA	0	-- [c]	--
PR64A14	4.1	310 (2)	R
Masoli	3.2	310 (1)	R
PR63A76	10.5	310 (2)	R
Solnet	7.6	310 (2)	R
Transol	6.2	310 (5)	R
Kardan	0.4	310 (6)	R
Olimpia	6	310 (4)	R

[a] Parentheses show the number of isolates from each cultivar that were recovered and characterized.
[b] R= resistant.
[c] -- Not characterized.

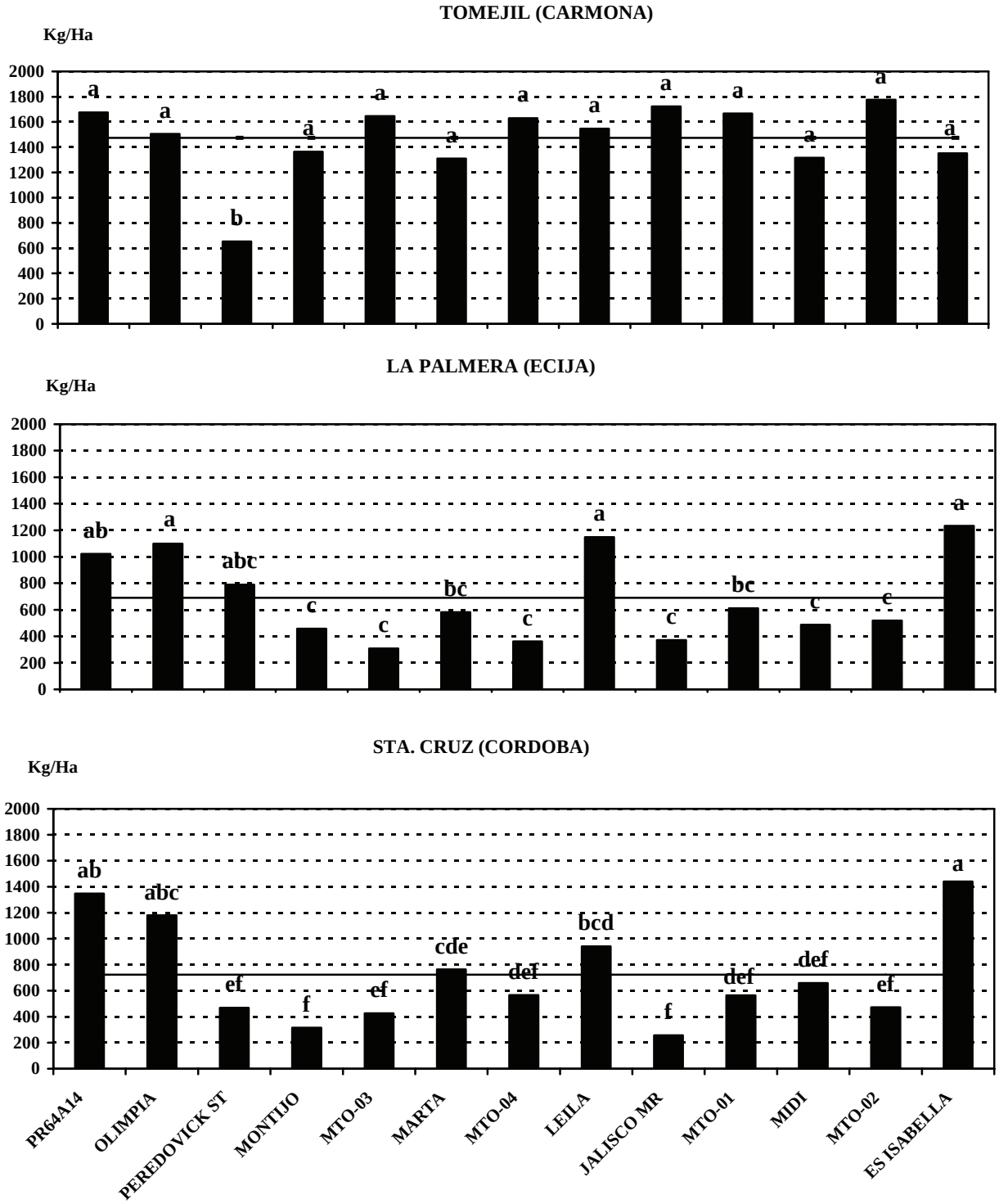

Fig. 2. Yields of 12 sunflower hybrids genetically resistant to sunflower downy mildew and one susceptible control (Peredovik) obtained in three different fields of Córdoba and Seville.

Table 3. Incidence of broomrape in downy mildew experiments in three different locations of Andalusia in 2007

	Incidence of broomrape (%)		
Cultivar	La Palmera	Santa Cruz	Tomejil
Montijo	100	98.7	13.5
MTO-03	100	100	32.25
Marta	100	93	4.25
MTO-04	100	100	25.75
Leila	100	46.6	1
Jalisco MR	35.25	100	20.5
MTO-01	100	100	22.25
Midi	100	96.3	3.75
MTO-02	100	95	24.25
Peredovik	93.75	89.2	3.5
Es Isabella	9.5	11.2	0
PR64A14	71.5	66.4	1
Olimpia	18.75	20.4	0.75

ACKNOWLEDGEMENTS

This work was supported by ERDF through the Research Project RTA05-131 (National Institute for Agricultural Research-INIA) and the IFAPA (Andalusian Institute for Research and Training in Agriculture, Fishing and Food). Third author was supported by a research contract, and last author was supported by a post-doctoral contract, both from the I3P Program financed by the Spanish National Research Council (CSIC) and the European Social Fund.

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Determining the sunflower downy mildew risk by soil analysis

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ABSTRACT

A bioassay using a soil sample was developed for assessing downy mildew risk at the field level. The results were correlated with the rate of infected plants when no other limiting factors were observed. The first tests carried out in fields in 2007 allowed the evaluation of soil infestation and seemed to confirm that inoculum could usefully be reduced by crop rotation. Moreover, the bioassay was used to follow the evolution of soil infestation during the spring. It reached a maximum around April 15th under the French conditions. The test conditions had very little effect on the results, so, hopefully. a large-scale use could easily be developed. For less infested soil, the direct characterization of the pathogen population's virulence profiles was not reliable. However, this protocol allows us to obtain fresh inoculum even when susceptible species are not present in the field, which makes it possible to achieve the characterization of the races after isolate multiplication. The interest of this protocol for the management of control methods is discussed.

Key words: bioassay – downy mildew – epidemiology – *Helianthus annuus* – *Plasmopara halstedii* – risk analysis – soil infestation.

RESUME

Afin d'évaluer le risque «mildiou du tournesol» en parcelles agricoles, un biotest réalisé sur un échantillon de terre a été mis au point. Les résultats obtenus sont corrélés avec les taux de plantes malades observés en absence d'autres facteurs limitants. Les premiers essais de ce protocole en parcelles agricoles en 2007 ont permis d'évaluer le potentiel infectieux du sol et semblent confirmer l'intérêt d'un allongement des rotations pour limiter l'inoculum. Ce biotest a également montré son utilité pour suivre l'évolution du potentiel infectieux durant le printemps. Ce potentiel passe par un maximum qui se situe, dans les conditions françaises, autour du 15 avril. Les résultats obtenus sont relativement peu influencés par les conditions de réalisation du test, ce qui laisse espérer une généralisation aisée. Pour les terres peu contaminées, la caractérisation directe du profil de virulence de la population parasitaire n'est pas fiable. Cependant, ce protocole permet l'obtention d'inoculum frais, même en absence d'espèce sensible sur la parcelle, ce qui permet ensuite de mettre en œuvre la caractérisation des races présentes après multiplication de l'isolat. L'intérêt de ce protocole pour la gestion des méthodes de lutte est discuté.

Mots clés: analyse de risque – biotest – épidémiologie – *Helianthus annuus* – mildiou –*Plasmopara halstedii* – potentiel infectieux

INTRODUCTION

Plasmopara halstedii is mainly a soilborne plant pathogen which can survive as oospores from one year to the next (Tourvieille de Labrouhe et al., 2000). This kind of conservation, which results from the sexual reproduction, allows the survival of the pathogen for several years waiting for a susceptible culture. Among arable crops, only sunflower is susceptible to *P. halstedii*. However, some *Asteraceae* known as weeds could harbor the pathogen and enhance the inoculum reservoir. Under favourable conditions, oospores in the soil can germinate and give rise to a zoosporange which releases mobile zoospores in free water. These zoospores are responsible for the primary infection, which is the most harmful form of the disease. If the level of risk depends on the weather conditions, in parallel, quantitative and qualitative (pathotypes) aspects of the inoculum are essential to explain the severity of attacks. In order to understand the evolution of downy mildew risk and also be able to make a diagnosis

of fields before sowing, we have developed a bioassay based on soil sampling. The principle has already been published (Tourvieille and Walser, 2005) and it has served to show the relationship between the presence of downy mildew in a field and the risk for the next sunflower crops. Moreover, this device seems to be of interest for predicting the behaviour of various sunflower hybrids against the endogenous pathogen population. The article presents experiments using this protocol and whose aims were to specify: i) the link between level of soil infestation and downy mildew risk; ii) the evolution of the infestation level of the soil during spring and iii) the possibility of using this protocol in a regional management of the downy mildew risk. It is not certain that downy mildew finds favourable conditions for its expression because of environmental conditions and/or absence of susceptible plants. For this reason, with a large scale study, on the level of a pilot site, we wanted to know if the protocol of soil bioassay could be a decision-making aid in the management of control methods.

MATERIALS AND METHODS

Plant material: The open-pollinated line Peredovik, without any known resistance gene, was used to quantify the infestation level of the soil or to estimate the disease incidence in fields. The virulence profiles of *P. halstedii* populations were determined using a set of nine international differential host lines (D1 to D9) (Gulya et al., 1998).

Test in culture: Experiments were carried out in plots of calcareous clayey loam soil located in Limagne (Centre of France) under a continental moderate climate. To assess the downy mildew risk independently of the climatic conditions, a contamination of plants before emergence was performed ensuring a very important irrigation (≈ 100 mm) when the root of the seedlings reached a size ranging between 0.5 and 1.0 cm length (Vear et al., 2007). The number of infected plants was observed at the stage "appearance of the second pair of leaves". Plants with systemic symptoms of downy mildew resulted from a telluric primary infection.

Soil bioassay: Experimental soils were collected in the seed bed at the sowing period in each field by focusing on low ground locations or headlands. The soil samples were directly placed in pots (30 cm x 30 cm x 6 cm). Two hundred seeds of a trap genotype Peredovik or 10 seeds for each of the nine differentials were sown in each pot, covered by 1 cm of soil and grown at 18°C. After 48 hours, which was the time required for obtaining germs from 0.5 to 1.0 cm in length, each pot was separately immersed in water during 8 to 12 hours. Then the pots were maintained at 18°C with a 16 h photoperiod (12 000 Lux) per day. After 12 days, sporulation was induced by covering the infected seedlings with a plexiglass cap or a transparent plastic bag (PEBD 50µm) for 48 hours to provide a saturated humidity (Tourvieille de Labrouhe and Walser, 2005).

Choice of the fields for the study in a pilot site: Fields were chosen according to 3 factors: i) downy mildew history: the whole of the fields had already expressed downy mildew during the last 3 years or were located in an area where downy mildew was usually observed, ii) there was a delay between two sunflower crops (1 year, 2 years, 3 years and more) and iii) the type of soil (calcareous clay, clayey silt, silt like “boulbène”). Ten fields located in the departments of Gers and Tarn-et-Garonne (South-western France) were finally selected. A soil sampling was performed in each field. In 3 field plots, the same bioassay was carried out in a ring-test between 3 laboratories under variable conditions (Table 1), and in 4 field plots, two methods of sampling were studied: 4 independent samples of 4 liters taken from 4 points in the field were compared with a pooled sample made with 1 liter of soil taken from the same 4 points.

Table 1. Experimental conditions in the different laboratories carrying out the soil bioassay.

	Lab 1	Lab 2	Lab 3
Delay between soil sampling and seed sowing	72 h	48 h	120 h
Light	12 000 lux (neon)	day light	day light
Temperature	18°C ± 1°C	uncontrolled (-)	uncontrolled (-)
Time of immersion	17 h	8 h	8 h
Time of saturated humidity	48 h	66 h	45 h

In addition, we tried to characterize the virulence of the pathogen population by carrying out the bioassay directly with the 9 differential host lines in 4 fields. Results were confronted with the

characterization of the virulence profile according to the classical method using an infected plant collected in the field as the inoculum source (Tourvieille de Labrouhe et al., 2000).

RESULTS

Relationship between the response of the soil bioassay and the disease incidence in the field: On small plots (≈ 70 m²) known to be infested by *P. halstedii*, soil samples were analyzed and downy mildew incidence was observed *in situ* in 2006 and 2007. Observations showed a close relationship between the number of seedlings with symptoms of downy mildew from the soil bioassay and the number of infected plants observed in the year of sampling (Fig. 1).

The relationship between the disease incidence in the field and the rate of infected seedlings as a result of the soil bioassay was quite similar in spite of the differences in the disease pressure, which was 54.5% in 2006 and 16.3% in 2007. The correlation coefficient of the 12 data pairs was highly significant (r=0.917).

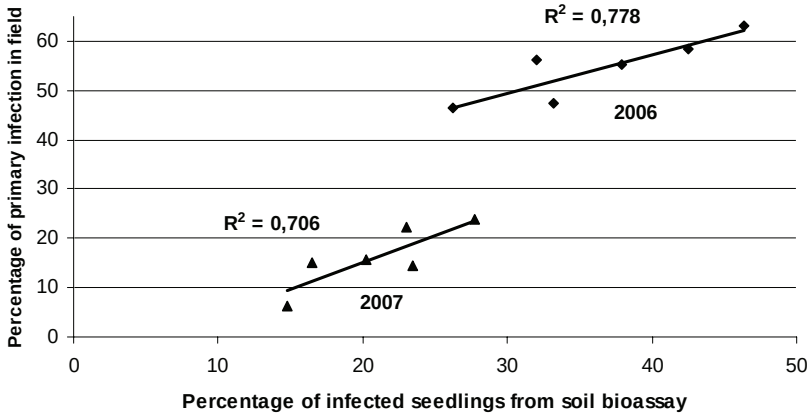

Fig. 1. Relationship between primary infection of downy mildew in the field and infection of seedlings from the soil bioassay.

Infestation of a field plot according to the farming past: On small plots followed for many years, the rate of infected seedlings given by the bioassay was correlated with the number of infected plants observed the previous years. The best correlation was obtained when infected plants were grown the previous year (y-1) or 2-years before (y-2) (Fig. 2).

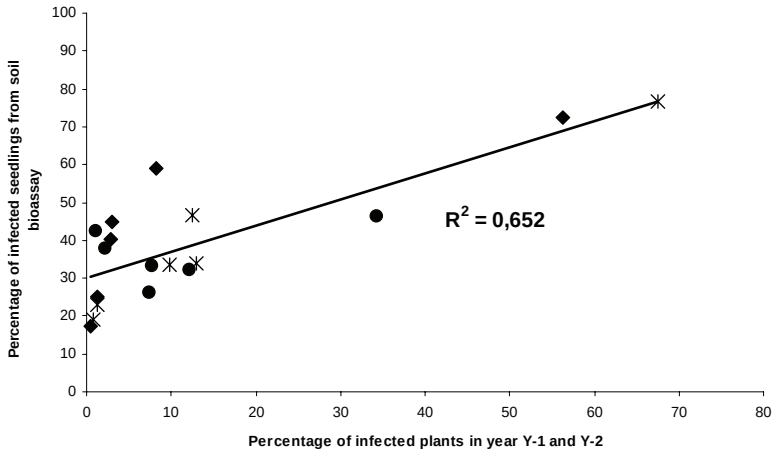

Fig. 2. Relationship between downy mildew incidence in the field observed 1 and 2 years before the soil bioassay and the rate of infected seedlings from the soil bioassay (◆ 2004, ❄ 2005, ● 2006**).**

The rates of infected seedlings assessed by the soil bioassay varied from 14.8% to 76.8% and were on average 43.0% in 2004, 38.7% in 2005 and 36.4% in 2006. These rates were highly correlated with the

downy mildew incidence observed in the field the two previous years (y-1 and y-2). The downy mildew incidences varied from 0.4% to 67.5% and were on average 12.0% in 2004, 17.5% in 2005 and 10.8% in 2006. So the relationship between the soil infestation measured by the soil bioassay and the presence of infected plants the previous years is confirmed in this experiment.

Use of the soil bioassay for measuring the evolution of the soil infestation: To appreciate the evolution of the soil infestation during the whole period of sunflower sowing, soil samples were collected once per week, from March to May. This experiment was carried out in the site of Clermont-Ferrand in 2006 (April-May: mild and humid conditions with soil average temperature ~15°C and sum of precipitations = 178 mm) and in 2007 (April-May: warm and dry conditions). Ten micro-plots were analyzed weekly. Results and the adjusted curves are presented in Fig. 3.

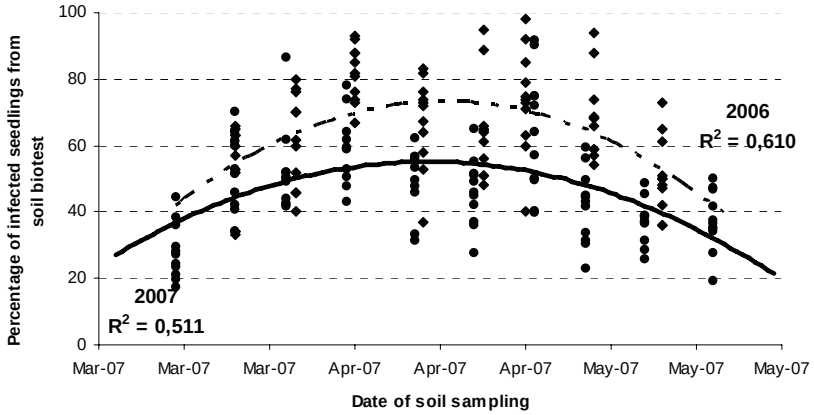

Fig. 3. Evolution of the proportion of infected seedlings given by the soil bioassay according to the date of soil sampling (10 samples per date) in 2006 (◆) and 2007 (●).

Under the environmental conditions of the Centre of France, the best sampling date for assessing the soil infestation appeared to be in mid-April. If the levels of infection seemed to be dependent on the weather conditions of the year (65.1% on average in 2006 and 46.7% on average in 2007), the data corresponding to the maximum of primary infection appeared to be fairly constant in both years.

Application of methodology in a pilot site: The 10 field plots of the pilot site can be classified in 3 classes (Table 2):

- slightly infested: Les Mariettes, Le Carascau and Sarrault with less than 10% of infected seedlings.
- moderately infested: Utaut and Janicaut with less than 25% of infected seedlings.
- strongly infested: La Poëte, Le Rauy, Bordeneuve and La Plèche with more than 30% of infected seedlings.

The comparison of the results from different laboratories showed a very good repeatability for 2 field plots, “La poëte” and ‘Utaut”. In contrast, the two analyses of the plot "Le Rauy" appeared to be rather contrary (Table 2). Moreover, information on the soil of the field plot "Les Barbès" could not be given due to the absence of the emergence of sunflower during the bioassay.

In the 4 field plots where two methods of sampling were tested, the levels of response varied from slightly infested (Le Rauy) to very strongly infested (La Poëte). Differences between the individual samples suggest variability in the soil infestation of the field plot (Table 3). It must be noted that the pooled sample did not correspond to the mean of the 4 independent samples and the mean rate was always the weakest.

Table 2. Percentage of seedlings of a susceptible genotype presenting symptoms of downy mildew in soil bioassay according to the location of sampling and analysis

Location	Type of soil	Laboratories		
		Lab 1	Lab 2	Lab 3
Les Barbes	Silt «Boulbène»	?	-	-
Les Mariettes	Silt clay	6.8%	-	-
La Poëte	Calcareous clay	59.4%	65.6%	62.6%
Utaut	Calcareous clay	21.4%	21.6%	22.2%
Le Rauy	Silt clay	37.7%	8.5%	-
Bordeneuve	Calcareous clay	31.3%	-	-
Le Carascau	Calcareous clay	6.3%	-	-
La Plèche	Calcareous clay	36.4%	-	-
Sarraut	Calcareous clay	3.6%	-	-
Janicot	Calcareous clay	14.3%	-	-

Table 3. Percentage of infected seedlings from soil bioassay according to the method of sampling

Location	Pooled sample	Point 1	Point 2	Point3	Point 4
Les Mariettes	2.4%	2.4%	13.8%	12.1%	3.1%
La Poëte	33.3%	67.7%	80.1%	50.0%	82.1%
Utaut	16.7%	18.8%	22.2%	14.3%	39.3%
Le Rauy	3.8%	12.8%	5.6%	15.4%	5.1%

When the virulence profile was determined by using the soil bioassay, rates of infected seedlings were very low, although the results were not in contradiction with results given by the classic test (Table 4)

Table 4. Characterization of virulence profile of the population of *P. halstedii* in the soil (soil bioassay = a) and of a sample taken on an infected plant (classic test = b).

Location	Test	For each differential: Number of infected seedlings / Number of emerged seedlings									Profile
		D1	D2	D3	D4	D5	D6	D7	D8	D9	
Les Barbes	a [(1)]	*0/2*	*0/3*	*0/2*	*0/1*	*0/0*	*0/8*	*0/2*	*0/0*	*0/1*	?
	b	**7/10**	**7/10**	**8/10**	*0/10*	*0/10*	*0/10*	**5/10**	**7/10**	*0/10*	703
Utaut	a	**8/19**	**16/29**	**1/19**	*0/16*	*0/22*	*0/18*	**2/20**	**1/19**	**14/29**	707 [(2)]
	b	**3/10**	**7/10**	1/10	*0/10*	*0/10*	*0/10*	*0/10*	*0/10*	**2/10**	304 ?
La Poëte	a	**6/18**	**26/30**	**4/21**	*0/22*	*0/19*	*0/20*	**10/31**	**9/29**	**11/20**	707 [(2)]
	b	**1/9**	**6/10**	*0/10*	*0/10*	*0/10*	*0/10*	*0/10*	*0/10*	**4/10**	304
Janicot	a	**3/12**	**2/11**	**2/13**	*0/11*	*0/10*	*0/12*	**1/12**	**3/10**	*0/10*	703
	b	**9/9**	**10/12**	**6/13**	*0/10*	*0/8*	*0/2*	**5/7**	**3/6**	*0/5*	703

[(1)] asphyxia. [(2)] or mixture of pathotypes (304 + 703).

DISCUSSION

Results obtained on small plots clearly showed the interest of analysing the soil infestation using a soil bioassay since its response was well correlated with downy mildew risk observed in the absence of limiting weather factors. The soil bioassay also allowed us to confirm the close relationship between the mildew history of a field plot and the level of infestation. This is easily explained by the fact that the pathogen is maintained from one year to the next by oospores, which are produced in infected tissues of sunflower (Sackston, 1981). The quantity of oospores is therefore directly related to the number of infected plants. Consequently, short crop rotations are prohibited by recommended measures for control of downy mildew, especially when the presence of the disease is detected (Moinard et al., 2006).

The soil bioassay also allowed us to follow the evolution of soil infestation during the sunflower sowing period in spring. It was demonstrated that soil infestation reached a maximum in mid-April under French conditions. This evolution has to be connected with the weather conditions. These become favourable to the pathogen at the end of winter, inducing a break in soil infestation due to the short lifetime of the zoospores after germination (Goossen and Sackston, 1968). These results could lead to two interesting prospects: i) sowing as soon as possible so that sunflower emergence can escape the favourable periods to downy mildew infection; but this would mean selecting hybrids resistant to cold temperatures and ii) carrying out all experiments of selection (Vear et al., 2007) or screening of molecules (Délos et al., 2000) in April under conditions favourable to the pathogen.

It is always difficult to assess soil infestation of a field plot. When it was possible to analyze either several samples of the same plot or a pooled sample representative of the plot, the pooled sample was always less infected than independent samples. This could be explained by the quantity of sampled soil.

Indeed, this quantity from each sampling point for a pooled sample is less important than the soil quantity which is necessary for an independent analysis. Also, in the first case, soil is not always taken from the whole horizon corresponding to the seed bed. But it is also possible that the lifetime of inoculum could be very low in the upper layer of the soil where more drastic climatic conditions could occur. This leads to recommending sampling soil from the -2cm to -8cm horizon for a more effective soil analysis.

In 2007, the use of resistant sunflower hybrids in the field plots did not enable us to confirm the relation between soil infestation and disease incidence, despite quite favourable weather conditions for downy mildew. Neither did we notice links between type of soil and level of infestation. Nevertheless, it was demonstrated that the protocol was not adapted in the case of silt loam like "Boulbène" because immersion caused a packing of soil and a lack of seed germination. In this case, it would be possible to recover inoculum by percolation and to use this more or less infested water to perform the watering of seeds in uninfested substrate. For the other types of soil, bioassay results indicated that the different test conditions in the three laboratories had little influence, but this should be confirmed under less favourable conditions (e.g. higher temperatures).

Direct characterization of the virulence profile has been seen to have its limits. Its sensitivity depends on the level of soil infestation, which must be high enough to guarantee the infection of susceptible differential hosts. Its specificity is also limited because it uses only nine differentials, which is not enough to determine the virulence profile of a mixture of pathotypes. Moreover, variability in a field plot could only be measured by numerous independent analyses. However, the soil bioassay could potentially be used to investigate the downy mildew risk of the variety to be sown in the field plot.

In the framework of risk management, the soil bioassay shows potential interests:

- determining soil infestation of a field plot in a given year,
- achieving virulence profiles present in the field plot, even in the absence of susceptible sunflower,
- following the evolution of pathogen population in quantitative and qualitative terms in comparison with the means of disease control,
- finally, adapting the means of genetics and chemical control according to the risk in the field plot.

ACKNOWLEDGEMENTS

This work was supported in part by MAP (Ministry for Agriculture and Fishing) within the framework of the program CAS DAR. and by ANR-(Agence Nationale de la Recherche) under the "Programme Agriculture et Développement Durable" (project ANR-05-PADD-0705, Cedre).

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Large scale field evaluations for Sclerotinia stalk rot resistance in cultivated sunflower

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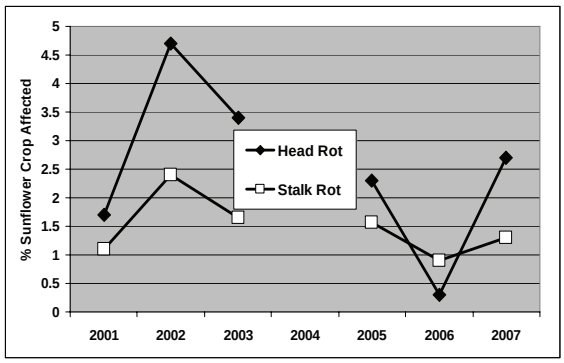
ABSTRACT

An artificial inoculation procedure to incite Sclerotinia basal stalk rot of sunflower was developed that is appropriate for large scale field evaluations. The procedure employs a dried, millet-based mycelial inoculum, without sclerotia. A measured amount of inoculum is deposited in a continuous furrow alongside each row, with ~ 60 g used per 7 m row. Thus, for each 1000 rows in a nursery we use ~ 60 to 70 kg of inoculum, allowing for spillage and inoculum deposited between rows. Preliminary results demonstrated that mycelium produced on oats and millet are equally infective, but the spherical shape of millet seeds facilitates the use of mechanized inoculation equipment. For large scale field inoculations, we modified a granular chemical applicator mounted on a tractor-driven cultivator to deposit uniform amounts of inoculum. The application needs to be made when the sunflower plants are at the V-6 stage or earlier, when the plants are shorter than the tool bar upon which the applicator is mounted. Since the inoculum is deposited in a furrow 20 to 25 cm away from the young plants, the initial symptoms of infection do not appear until 4 to 5 weeks after inoculation, at which time their root systems have contacted the inoculum. Based upon four years of field trials with commercial hybrids, this method has proven capable of producing sufficient and uniform levels of stalk rot, allowing statistical identification of the most resistant hybrids.

Key words: disease testing – *Helianthus* – inoculation methods – *Sclerotinia sclerotiorum* – sunflower.

INTRODUCTION

Basal stalk rot and wilt of sunflower caused by *Sclerotinia sclerotiorum* continues to be one of the major diseases affecting sunflower in North America, along with Sclerotinia head rot (Berglund, 2007; Gulya, 2003, 2004a; Lamey et al., 2002). During the period from 2001 to 2007, the incidence of stalk rot affected fields has ranged from 16 to 35% while head rot in the same period has ranged from 9% to 51% fields affected (Fig. 1). The severity, or percentage of the crop affected, during the same period has ranged from 0.9 to 2.4% for stalk rot and from 0.3 to 4.7% for head rot (Fig. 2). In an effort to develop resistant germplasm and to assess hybrid resistance to both Sclerotinia diseases, we have made an effort to develop artificial inoculation procedures to generate consistent and statistically sound data. While many papers have been published on inoculation procedures for head rot, there is a dearth of information on stalk rot inoculation techniques. We began a study in 2002 to develop a field inoculation method which would produce a reliable level of stalk rot with which to identify sunflower germplasm with improved levels of resistance. This initial study demonstrated the superiority of mycelial inoculum compared to sclerotia (Gulya, 2004b). The next objectives were (1) to adapt this to large scale evaluations encompassing thousands of rows at multiple locations, and (2) verify the method with commercial hybrids tested over several years.



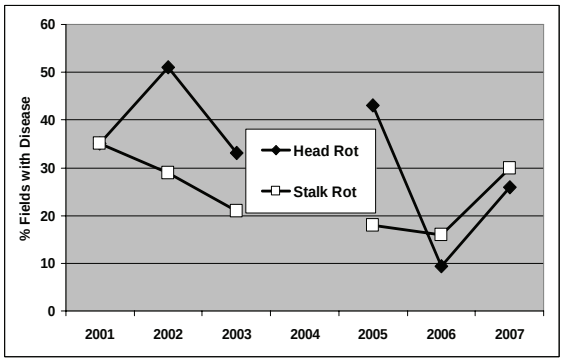


Fig. 1 and 2. Changes in disease severity (% of crop affected, left graph) and disease incidence (% of fields with disease, right graph) for Sclerotinia stalk rot and head rot in North Dakota fields surveyed between 2001 and 2007, using data obtained from the National Sunflower Association annual survey. The survey was not conducted in 2004.

MATERIALS AND METHODS

The inoculation procedure we developed is a version of that initially developed by Mancl and Shein (1982). *Sclerotinia sclerotiorum* inoculum was produced by growing the fungus on autoclaved white proso millet for 7 to 9 days (before any sclerotia developed), drying the inoculum to 10% moisture, and storing it at 4ºC until needed. Using a granular chemical applicator (Gulya et. al, 2005), the inoculum was placed in a furrow ~ 25 cm from each row, about 8 to 10 cm deep. Each 7 m row received ~ 50 to 60 g of inoculum. For each 1000 rows, we used approximately 60 kg. or 135 pounds of millet-based inoculum. Each location was inoculated 5 to 6 wk after planting when the plants were approximately at the V-6 stage, or no more than 45 cm tall. This permitted the use of a tractor-drawn inoculator with minimal damage to the plants. Plots were evaluated for disease incidence at least twice, with the first evaluation in late August (12 to 14 wk after planting and 7 to 9 wk after inoculation), and the second evaluation two weeks later. A plant showing wilt and/or a basal stalk rot lesion was recorded as diseased, and the percent of diseased plants was calculated. Statistical analysis was done using SAS software.

Each year, starting in 2004, U.S. seed companies were asked to submit experimental or commercial hybrids for inclusion in both a stalk rot trial and a head rot trial, the latter which was conducted by personnel from North Dakota State University. Since both diseases are of major concern to U.S. producers, it was felt that information on a hybrid's performance against both diseases was essential. The stalk rot trials have been planted at five locations in eastern North Dakota and northwestern Minnesota each year. Four replications of single row plots, each 7 m long and on 75 cm centers, were planted, starting in late May, with the last location usually planted within three weeks of the earliest planting. A widely grown oilseed hybrid, Cargill 270, was chosen as the long-term susceptible check variety, while the resistant check was a hybrid produced using two USDA inbreds specifically developed for stalk rot resistance (HA 412 x RHA 409). There were six to eight rows of both the resistant and susceptible varieties per replication.

RESULTS

The use of a granular chemical applicator, driven by an electric motor, and mounted on a tractor driven cultivator, allowed us to uniformly deposit *Sclerotinia* mycelial inoculum (grown on millet) beside rows of young sunflowers. Initial symptoms of Sclerotinia wilt do not appear until 5 to 6 wk following inoculation, by which time the roots of the plants had grown and reached the inoculum. By having clear plastic tubes attached to the granular chemical applicator, a person could observe if the millet inoculum was flowing freely and thus minimize the possibility of rows not receiving a uniform dosage.

Each year from 2004 to 2007 some field trials had unforeseen problems, such as flooding, hail storms, extended drought, and downy mildew infestation, that either ruined the plot for Sclerotinia evaluations or made the results statistically insignificant. Thus, the number of stalk rot trials yielding usable information varied from two to four in any given year. In consultation with seed company researchers, a minimum of three statistically sound data sets were considered necessary for the data to be

published. In addition to presenting the information annually at the Sunflower Research Workshop (Gulya and Henson, 2006), the stalk rot and head rot ratings are submitted to North Dakota State University which annually publishes a bulletin on sunflower hybrid performance (containing agronomic data as well as disease ratings), available in hard copies and on-line (Berglund and Grady, 2006).

Starting in 2004, when 75 hybrids were tested, we observed that the gradation in disease incidence from the most resistant to the most susceptible hybrids was continuous, as would be expected from a polygenically controlled quantitative trait, which precludes categorizing the entries into discrete groupings such as "resistant" or "susceptible" (Fig. 3). In 2004, there were four hybrids with less infection than the resistant USDA check (21% infection), but there was no statistical difference between them and the check. While confection hybrids are generally considered to have less resistance to most diseases than oilseed hybrids, there were some confection hybrids with reasonably high levels of resistance, as shown by the banded bars in Fig. 3. For complete information on the performance of the hybrids tested, please consult NDSU publication A-652 (Berglund and Grady, 2006) which is revised annually with new data posted in January.

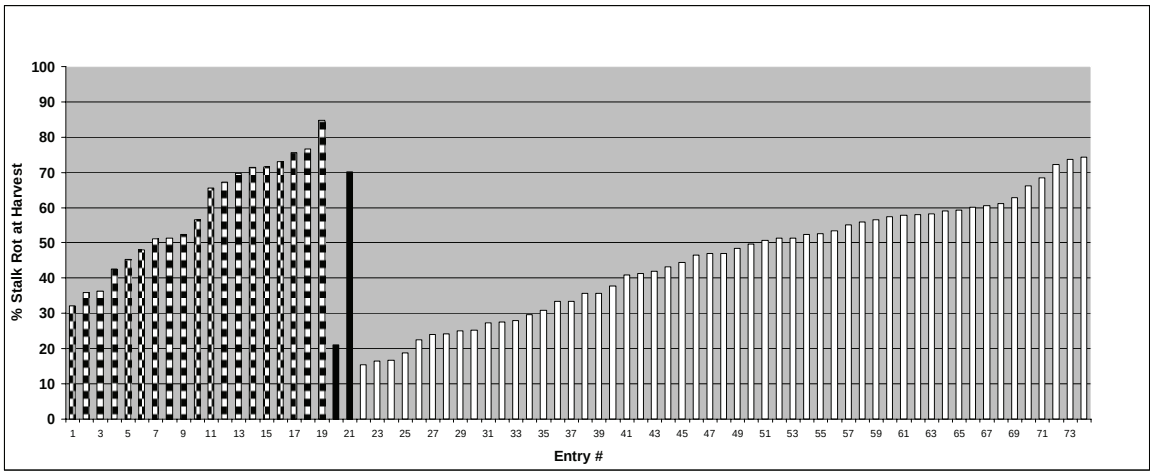


Fig. 3. Histogram of the stalk rot ratings (% diseased plants at physiological maturity) of 75 hybrid entries averaged over two locations in the 2004 tests. The banded bars on the left represent the confection hybrids while the remainder of the entries were oilseed hybrids. The black bars are the resistant check, with 21% infected plants, and the susceptible check, with 70% infected plants.

The stalk rot ratings of hybrids tested in subsequent years followed the pattern observed in the first year, with a continuous range of reaction and no discernible categories. While the range of infection varied from location to location and between years (Table 1), we were able to separate the most susceptible and the most resistant hybrids in each year. In 2007, for example, there were 18 hybrids which had stalk rot levels less than the resistant USDA check, but none of them were statistically different from each other (Fig. 4). Hybrids performing better than the resistant check were tested a second year, at up to five locations. Thus, under ideal conditions a hybrid may have been evaluated at up to 10 locations over two years.

Table 1. Summary statistics for stalk rot evaluations (% diseased plants at maturity) of commercial hybrids in inoculated field trials during 2004 to 2007.

	2004	2005	2006	2007
Number of Hybrids	75	89	97	97
Average % Stalk Rot	48	37	14	27
Minimum	15	10	2	3
Maximum	85	71	42	58
Number of Locations	2	3	4	3
Susceptible Check	70	54	23	35

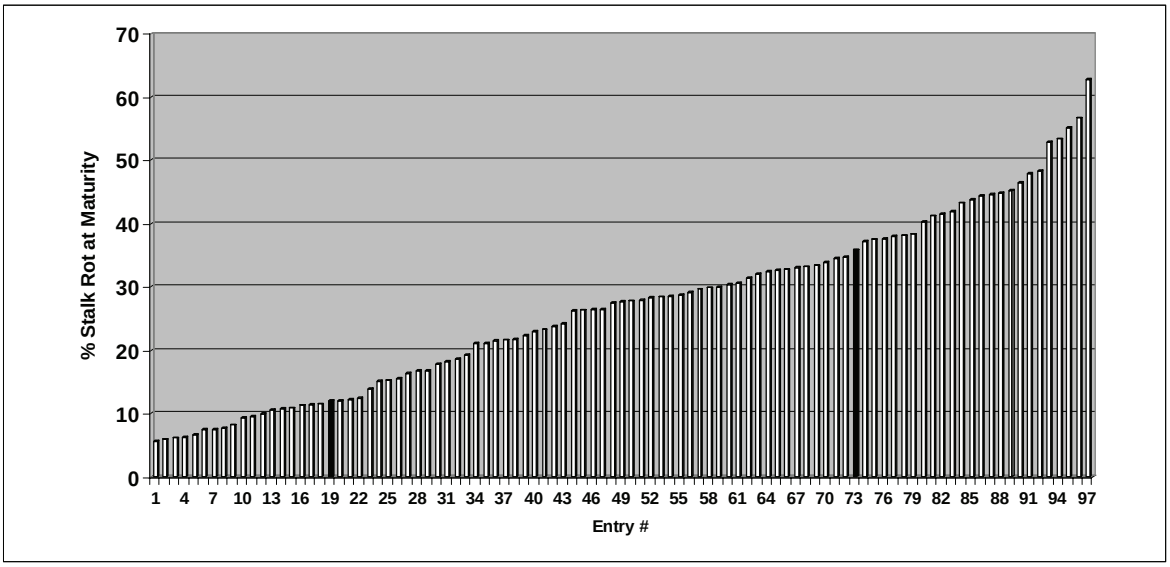


Fig. 4. Histogram of the stalk rot ratings of 97 commercial hybrids entered in the 2007 field trials, averaged over three locations. The black bar at position 19 is the resistant check, with 12% infected plants, and the black bar at position #73 is the susceptible check with 36% infected plants.

DISCUSSION

During the period 1970 to 2000, our USDA Sunflower Unit relied on fields naturally infested with *S. sclerotiorum* to screen sunflower for resistance to stalk rot. The uniformity of disease was often spotty and after repeated crops we often observed declines in disease incidence due to naturally occurring biological control. The field plots were also often located at long distances from our laboratory, all of which contributed to our decision to look for an artificial inoculation technique. Our initial method of placing a measured amount of inoculum beside each plant with a "corn jab planter" was satisfactory, but not practical with large numbers of rows requiring large numbers of people. Thus, the mechanization of the inoculation process not only produced a uniform amount of disease, but allowed us to greatly expand our efforts and test at multiple locations.

The current method of field testing for Sclerotinia stalk rot resistance is also used to evaluate USDA breeding material and other germplasm of interest. For example, the USDA Plant Introduction Collection of cultivated sunflower currently has ~ 800 accessions recently added which do not have stalk rot data listed in the USDA's Germplasm Resource Information Network (GRIN) database (http://www.ars-grin.gov/cgi-bin/npgs/html/crop.pl?7), so these are currently being evaluated, initially for stalk rot and subsequently for head rot resistance. While this method could be used for evaluating wild *Helianthus*, with the variable phenotype of each species, it may be more appropriate to either inoculate wild species by hand in the field, or in the greenhouse with a technique modeled after Grezes-Besset et al. (1994) and modified by Block et al. (2007, 2008).

We have made minor modifications to our stalk rot evaluation methods over the past four years. For example, to minimize the loss of plants due to downy mildew infection, we treat all seeds with a fungicide mix, regardless if they already have a commercial coating. We use fenamidone and zoximide (Gulya, 2002), at rates of 125 g and 250 g, respectively, which effectively protects against downy mildew with no effect on *Sclerotinia*. We have noted that field plots which do not receive any precipitation for several weeks following inoculation often develop little or no stalk rot. On small plots, this problem could be partially prevented by applying some water at the time of inoculation, or alternately, if drip or furrow irrigation were available, this also would minimize the negative impact of dry soils. We have yet to observe any decline in disease incidence at locations which are reused annually, but we do follow a three-year rotation at these locations. In an attempt to make statistical separation of entries more precise, we are continuing to study ways to improve our evaluations, including increasing the number of replications, modifying the experimental design, and increasing the amount of inoculum.

ACKNOWLEDGEMENTS

The author wished to credit and thank Scott Radi and Nikolay Balbyshev for many years of conscientious work that enabled this project to be a success.

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Study on an *in vitro* screening test for resistance to *Sclerotinia sclerotiorum* in sunflower

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ABSTRACT

An *in vitro* method that assayed callus induction on a medium amended with culture filtrate of *Sclerotinia sclerotiorum* was evaluated. Four double haploid R-lines obtained through the method of gamma-induced parthenogenesis at Dobroudja Agricultural Institute were involved (DH-R-128, DH-R-116, DH-R-7 and DH-R-2). The experiment was carried out at two levels, under field and laboratory conditions. After field infection, lines DH-R-128 and DH-R-116 demonstrated high to moderate resistance. Under laboratory conditions, *S. sclerotiorum* filtrate was added to the nutrition medium for callus induction from sunflower hypocotyl explants. Three variants of filtrate concentration in the nutrition medium were tested. The callus induction reaction of *Helianthus annuus* L. explants cultivated on a medium amended with *S. sclerotiorum* filtrate was evaluated. It was established that the higher filtrate concentrations suppressed the reaction of the explants to various degrees for the different lines. In lines DH-R-116 and DH-R-128 a better callus induction reaction was observed in comparison to the other two lines. The results showed that the test for resistance to *S. sclerotiorum* based on the callus induction allowed to identify materials with high to moderate resistance to the pathogen. The test cannot distinguish the differences between high and moderate levels in resistance and in susceptibility.

Key words: callus induction – *in vitro* test – resistance – *Sclerotinia sclerotiorum* – sunflower

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is a very important oil seed crop worldwide and is a main source of vegetable oil in Bulgaria. Multiple factors determine the productivity of sunflower hybrids and varieties. In this respect, a significant effect is brought about by the causal agents of different diseases such as sclerotinia (*Sclerotinia sclerotiorum)*, downy mildew (*Plasmopara helianthi)*, phoma (*Phoma macdonaldii)*, and phomopsis (*Phomopsis helianthi*). Each of these diseases can significantly decrease sunflower productivity, but *S. sclertiorum* (Lib.) de Bary is considered to be one of the most devastating pathogens distributed in almost all production regions. The losses may be up to 100% under suitable conditions (20 ^{o}C and 70% air humidity) in fields where the fungus is spread (Maširević and Gulya, 1992; Rashid, 1993). In spite of the efforts of many researchers, no chemical control on the spreading of the pathogen has been found yet (Nelson and Lamey, 2000). At this stage, the only way to reduce damage from a sclerotinia attack is by the development of forms with genetic resistance; this is a priority in a number of research projects and investigations (Ronicke et al., 2004, 2005). Simultaneously, alternative approaches have been sought for fast selection and screening of the new forms developed which have demonstrated some degree of resistance to the pathogen (Grezes-Besset et al., 1994; Verzea et al., 2004).

The aim of this investigation was to study the effect of the cultural filtrate from *S. sclertiorum* on the callus induction reaction of cultivated sunflower (*Helianthus annuus* L.) and to find out if there is a correlation between *in vitro* and *in vivo* reaction of the plant material to the pathogen.

MATERIALS AND METHODS

The investigation was carried out at two levels – under laboratory and field conditions. We worked with four doubled haploid R-lines developed by the method of gamma-induced parthenogenesis at Dobroudja Agricultural Institute, Bulgaria. The lines were of different origin: lines DH-R-116 and DH-R-128 were produced from hybrid materials with parental forms obtained as a result of interspecific hybridization; lines DH-R-2 and DH-R-7 were obtained from *H. annuus* L. hybrids.

Preparation of inoculum and inoculation of lines under field conditions

Ten plants from each of the studied lines were inoculated by the Straw-method (Encheva and Kiryakov, 2002) at stage 5-6th pair of leaves. A petiole of the fourth pair of leaves from each plant was cut, so that 3 cm of it was left on the stem. A plastic straw (30 x 6 mm) with one end closed was inserted in the place of

incision. The straw contained an agar disc from the periphery of a 3 day old culture of isolate Ss-1 on nutrient medium PDA at 22±1°C. The reaction of the plant was rated three times every 7 days according to a 6-degree scale as follows: 0 – no symptoms at the place of inoculation; 1 - a whitish spot on the petiole (high resistance); 2 – a spot at the base of the petiole reaching to the stem (resistance); 3 – a spot spreading on a part of the stem (intermediate resistance), 4 – a spot spreading on the entire stem (susceptibility); 5 – breaking of the stem (high susceptibility). The last rating of the plant's reaction determined the final evaluation of their resistance to the pathogen.

Laboratory methods

S. sclerotiorum isolate SsPh1 was cultivated on a liquid medium (PDB) for seven days at room temperature (22-25°C), then it was filtered by the method of Miklas et al. (1992) applied to bean. Following cold sterilization in a laminar box, the obtained filtrate was stored at +4° C within 7 days.

Twenty seeds from each line were sterilized in 2.5% solution of potassium hypochlorite for 20 minutes; after the seed coat was removed, the seeds were then plated on medium MS (Murashige and Skoog, 1962) for formation of young plantlets. After 7-9 days cultivation, hypocotyl explants were removed and transferred onto medium for callus induction. The medium consisted of MS + 1.5 mg/l NAA + 0.5 mg/l BAP amended with *S. sclerotiorum* cultural filtrate. Four variants were tested depending on the amount of added filtrate: variant 1 (control, without added filtrate) and variants with added filtrate in the following concentrations: 5 ml/l (variant 2); 10 ml/l (variant 3); and 20 ml/l (variant 4). The number of calli induced was evaluated after one month of cultivation.

The experiment was designed in 5 replications (5 Petri dishes per variant for each line). Cultivation of explants was done under controlled conditions: 25±1ºC, light 6000 lux and photoperiod 16/8 hr.

RESULTS AND DISCUSSION

Field evaluation

After infection under field conditions best results were observed in line DH-R-128, where the reaction of infected plants was within the range 0-3 (Table 1).

Table 1. Field evaluation of the reaction of double haploid R lines to artificial infection with *S. sclerotiorum* inoculum

Genotype	**Plant reaction to *Sclerotinia sclerotiorum***									
plant №	***1***	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***	***10***
DH-R-128	3	1	1	0	1	0	0	2	0	1
DH-R-116	1	1	2	3	3	3	1	1	2	2
DH-R-2	2	4	4	3	2	4	3	4	3	4
DH-R-7	5	5	5	5	5	5	5	5	5	5

In contrast to line DH-R-128, all plants of line DH-R-7 were completely damaged by the pathogen. A moderate reaction was observed in the other two lines, inclining towards the moderate resistance margin in line DH-R-116 and towards the susceptibility margin in line DH-R-2. Taking the origin of the lines as a starting point, no definite conclusion about their reaction to the disease can be reached; however, the expression of this reaction can be analyzed generally. Emphasis is mainly placed on the wild species of genus *Helianthus* as a main source of resistance genes to both *S. sclerotiorum* and all diseases of sunflower of economic importance (Škorić, 1992; Cerboncini et al., 2002). This, to a great extent, confirmed the result we obtained in line DH-R-128, and partially, in line DH-R-116. Parallel to this, forms having a lower susceptibility to sclerotinia attack have been found in cultivated sunflower as well (Castano et al., 1993; Degener et al., 1999 a,b). In the present study, the line DH-R-2 can be referred to as being in this category.

Callus induction reaction

All DH-R-lines had normal processes of callus induction in the control (variant 1); line DH-R-128 showed a slightly weaker reaction than the rest of the lines (Table 2).

The higher concentrations of the filtrate prevented the callus induction reaction of the plants, the degree of suppression being different for the different lines. Regardless of the differences, the general trend of the reaction of the lines is expressed as a progressive decrease in callus initiation with the increase in the *S. sclerotiorum* filtrate concentration (Fig. 1).

Table 2. Callus induction in a culture of hypocotyls explants of *Helianthus annuus* L. on a medium amended with *Sclerotinia sclerotiorum* filtrate

Genotype		Number of plated explants							Number of calli obtained						
DH-R-116	**Replicates**	**1**	**2**	**3**	**4**	**5**	**Σ**	**X mean**	**1**	**2**	**3**	**4**	**5**	**Σ**	**X mean**
	variant 1	28	32	31	30	31	**152**	**30.4**	28	32	31	30	31	**152**	**30.4**
	variant 2	30	29	20	40	34	**153**	**30.6**	30	29	20	37	30	**146**	**29.2**
	variant 3	32	31	29	29	33	**154**	**30.8**	32	31	26	18	30	**137**	**27.4**
	variant 4	32	16	43	32	41	**164**	**32.8**	22	7	9	15	11	**64**	**12.8**
DH-R-128	**Replicates**	**1**	**2**	**3**	**4**	**5**	**Σ**	**X mean**	**1**	**2**	**3**	**4**	**5**	**Σ**	**X mean**
	variant 1	25	33	29	30	29	**146**	**29.2**	25	33	29	25	21	**133**	**26.6**
	variant 2	25	37	32	29	32	**155**	**31.0**	19	33	30	19	32	**133**	**26.6**
	variant 3	33	28	23	25	25	**133**	**26.6**	12	10	17	7	12	**58**	**11.6**
	variant 4	31	28	19	27	28	**133**	**26.6**	11	0	4	17	7	**39**	**7.8**
DH-R-2	**Replicates**	**1**	**2**	**3**	**4**	**5**	**Σ**	**X mean**	**1**	**2**	**3**	**4**	**5**	**Σ**	**X mean**
	variant 1	24	28	16	20	25	**113**	**22.6**	24	26	14	20	23	**107**	**21.4**
	variant 2	20	22	25	21	24	**112**	**22.4**	2	9	11	6	7	**35**	**7.0**
	variant 3	29	21	25	30	26	**131**	**26.2**	0	0	0	0	15	**15**	**3.0**
	variant 4	30	25	22	20	33	**130**	**26.0**	0	0	0	0	0	**0**	**0**
DH-R-7	**Replicates**	**1**	**2**	**3**	**4**	**5**	**Σ**	**X mean**	**1**	**2**	**3**	**4**	**5**	**Σ**	**X mean**
	variant 1	20	25	28	22	23	**118**	**23.6**	15	25	28	22	23	**113**	**22.6**
	variant 2	23	17	17	20	22	**99**	**19.8**	11	6	3	7	5	**32**	**6.4**
	variant 3	17	21	17	23	20	**98**	**19.6**	0	3	3	4	0	**10**	**2.0**
	variant 4	24	26	22	20	22	**114**	**22.8**	1	0	0	1	0	**2**	**0.4**

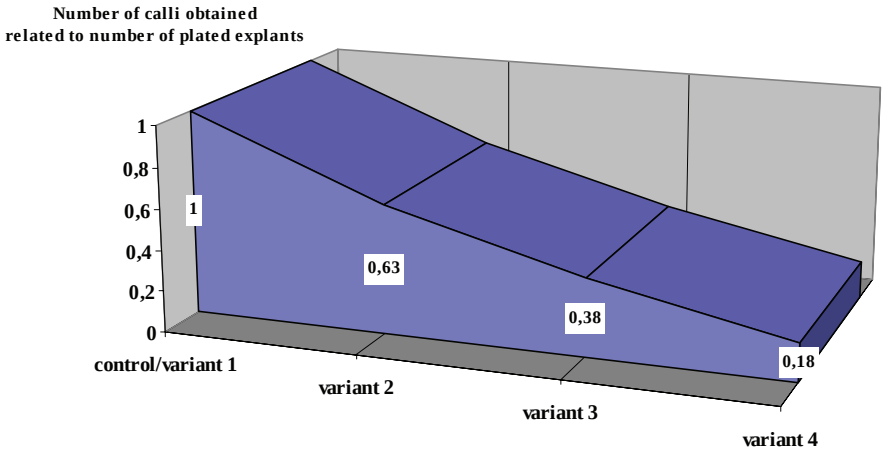

Fig. 1. General evaluation of the callus induction reaction on the investigated lines according to *S. sclerotiorum* filtrate concentration.

This trend was expressed to a higher degree in lines DH-R-2 and DH-R-7; in variants 3 and 4 their callus induction intensity was zero (Fig. 2).

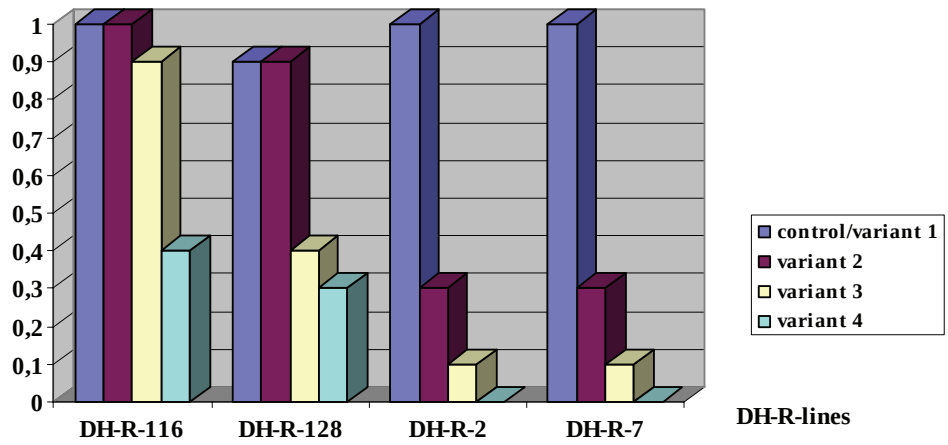

Fig. 2. Callus induction reaction of sunflower DH-R-lines according to the genotype and *S. sclerotiorum* filtrate concentration in the nutrient medium.

Differences were not observed in lines DH-R-116 and DH-R-128 between the control and variant 2. The callus induction in these lines was impeded by the higher concentrations of the filtrate in variants 3 and 4, but, in contrast to the other two investigated lines, callus initiation was not completely blocked.

The results from the *in vitro* investigation showed that line DH-R-116 had the best callus induction reaction in all four variants. Some similarity in the reactions was established for lines DH-R-116 and DH-R-128, with the exception of variant 3, where a sharp decrease in the callus genesis of line DH-R-128 was observed. This decrease was observed in variant 4 of line DH-R-116. In variant 4, the differences between the two lines were insignificant.

The comparison between the other two lines (DH-R-2 and DH-R-7) did not show any significant differences in the callus initiation of all four variants.

The comparison of the results from the field evaluation of the resistance to the *in vitro* reaction of the investigated lines revealed a certain correspondence expressed in the generalization that lines DH-R-128 and DH-R-116 showed resistance under field conditions and the best callus induction reaction, and lines DH-R-2 and DH-R-7 showed a susceptibility to *S. sclerotiorum* under field conditions and weaker in the *in vitro* reaction. By considering the results in detail, the fact comes out that although line DH-R-128 had the best evaluation in the field trial, it ranked second after line DH-R-116 under laboratory conditions. The lack of differences in the *in vitro* reaction of line DH-R-2, which had a relatively lower level of susceptibility to *S. sclerotiorum* under field conditions than line DH-R-7, also indicated that the laboratory test should most probably be more precise involving additional steps/variants between variant 3 and variant 4, so that the differences between the lines could be expressed.

CONCLUSIONS

The *in vitro* test for resistance to *S. sclerotiorum* based on the callus induction reaction is good enough to differentiate high to moderate resistant materials from materials with high to moderate susceptibility to the pathogen. The test cannot detect the differences between high and moderate levels in resistance and in susceptibility.

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EST-derived markers highlight genetic relationships among *Plasmopara halstedii* French races

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ABSTRACT

Twelve EST-derived markers were isolated from *Plasmopara halstedii* (Oomycetes), the causal agent of sunflower downy mildew. A total of 25 single nucleotide polymorphisms (SNPs) and five indels were detected by single-strand conformation polymorphism (SSCP) analysis and developed for high-throughput genotyping of 24 isolates. These markers reveal a good level of genetic diversity and were useful in detecting genotypic variability of French populations. A deficit in heterozygotes indicated that *P. halstedii* is a selfing species. For the first time, these markers allowed to reveal genetic relatedness among 14 races characterized over a 19-year period of *P. halstedii* monitoring in France.

Keywords: evolution – pathotype – SNP – sunflower downy mildew – virulence profile.

INTRODUCTION

Sunflower downy mildew due to *Plasmopara halstedii* (Berlese & de Toni) is potentially one of the most damaging diseases in sunflower. *P. halstedii* is a homothallic oomycete that alternates one sexual generation with several asexual generations. It is an obligate endoparasite that cannot be cultivated independently from its plant host. *P. halstedii* develops gene-for-gene interactions with its host *Helianthus annuus* and presents several physiological races known as pathotypes. Genetic resistance in cultivated sunflower varieties is the most efficient control method against the disease but the efficiency of major resistance genes is regularly challenged. To date, at least 20 different pathotypes have been described in different parts of the world (Tourvieille de Labrouhe et al., 2000).

Our understanding of the recurrent breakdown of sunflower major resistance genes can be improved based on new findings concerning the key processes governing the evolution of *P. halstedii* populations. Indeed, knowledge of the evolutionary potential of plant pathogen species has improved the management of resistance genes and maximized their durability in space and time (McDonald and Linde, 2002). The population genetics approach can be used to evaluate the major forces driving pathogen evolution, i.e. selection, mutation, recombination, genetic drift and gene flow. Previous genetic studies of *P. halstedii* in Europe have reported low levels of genetic and genotypic diversity in this species, with no clear genetic structure revealed with RAPD markers (Komjati et al., 2004; Roeckel-Drevet et al., 1997, 2003), ISSR (Intelmann and Spring, 2002) or ITS sequences (Spring et al., 2006). This precluded any reliable conclusions on the mode of reproduction, genetic structuring or the extent to which pathotypes are related in this species. Single nucleotide polymorphisms (SNPs) are promising molecular markers for population genetics as they are widespread throughout the genome, co-dominant, specific and have a high resolving power. The development of new methods for screening for DNA polymorphism has rendered possible the extensive development of such makers for plant pathogen species. With a total of 174 nucleotide sequences available in the international nucleotide sequence database, *P. halstedii* is a typical example of a non-model organism for which genomic resources are very scarce. We used the 145 cDNA sequences available to design a set of EST-derived markers that may be used for future population genetic studies. Here we report the characterization of 12 polymorphic markers based on SNPs and size variations (insertion-deletion) in Expressed Sequence Tags (ESTs) of *P. halstedii* and the development of high-throughput genotyping methods for 10 of these markers. We used these 12 EST-derived markers to perform a genetic analysis of the "reference races" of *P. halstedii* characterized over a 19-year period of monitoring in France (1988-2006).

MATERIALS AND METHODS

Sampling. We analyzed 24 isolates of *P. halstedii* collected in France between 1966 and 2006. Fourteen of these isolates are "references isolates", corresponding to the first description of the pathotype concerned in France (Table 1). The other 10 isolates (Table 1) were obtained from the French Plant Protection Service monitoring program (Moinard et al., 2006).

Virulence profile determination. Downy mildew pathotypes are defined on the basis of virulence profiles on a set of differential hosts carrying different *Pl* resistance genes. Resistance tests were performed as described by Cohen and Sakston (1974), with the modifications proposed by Mouzeyar et al. (1993): 15 days after inoculation, plants were incubated for 48 h in a saturated atmosphere. Plants were scored as susceptible if sporulation was observed in cotyledons and leaves, and as resistant if no sporulation or only light sporulation was seen on cotyledons. Pathotypes were named according to the international nomenclature of *P. halstedii* pathotypes proposed by Gulya et al. (1998) (Table 2).

Table 1. Race (pathotype), collection site and date of isolation in France for the 24 isolates of *Plasmopara halstedii*. The star (*) indicates isolates corresponding to the “reference pathotype”.

Race	Collection site ("*département*")	Year of collection
100*	Unknown	1966
100	Charente-Maritime	1993
100	Cher	1993
710*	Indre	1988
710	Cher	1993
710	Unknown	2000
710	Maine-et-Loire	2004
710	Deux-Sèvres	2006
703*	Lot-et-Garonne	1989
703	Tarn	1993
703	Lot-et-Garonne	2001
703	Haute-Garonne	2004
703	Gers	2006
300*	Aude	1995
700*	Haute-Garonne	1995
304*	Aude	2000
314*	Manche	2001
307*	Haute-Garonne	2002
704*	Deux-Sèvres	2002
714*	Gers	2002
334*	Charente	2004
707*	Lot-et-Garonne	2004
717*	Gers	2004
730*	Tarn	2005

Table 2. Name of race (pathotype), date of first isolation in France and virulence profiles for the 14 French reference isolates of *Plasmopara halstedii*.

Race name[1]	Isolation year	Virulence profiles according to differential hosts[2]								
		D1	D2	D3	D4	D5	D6	D7	D8	D9
100	1966	S	R	R	R	R	R	R	R	R
710	1988	S	S	S	S	R	R	R	R	R
703	1989	S	S	S	R	R	R	S	S	R
300	1995	S	S	R	R	R	R	R	R	R
700	1995	S	S	S	R	R	R	R	R	R
304	2000	S	S	R	R	R	R	R	R	S
314	2001	S	S	R	S	R	R	R	R	S
307	2002	S	S	R	R	R	R	S	S	S
704	2002	S	S	S	R	R	R	R	R	S
714	2002	S	S	S	S	R	R	R	R	S
334	2004	S	S	R	S	S	R	R	R	S
707	2004	S	S	S	R	R	R	S	S	S
717	2004	S	S	S	S	R	R	S	S	S
730	2005	S	S	S	S	S	R	R	R	R

[1] according to Gulya et al. (1998)
[2] S: susceptible (compatible interaction); R: resistant (incompatible interaction)

Molecular markers. A total of 124 ESTs of *P. halstedii* were screened for their polymorphism by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP). DNA extraction was performed on infected plant tissue as previously described for *Plasmopara viticola* by Delmotte et al. (2006). Marker amplification reactions were carried out in a final volume of 25 µl containing 10 ng of genomic DNA, 2 mM of MgCl2, 150 µM of each dNTP, 4 pmol of each primer and 0.2 U *Taq* Silverstar DNA polymerase (Eurogentec) in reaction buffer. Reactions were performed with the following program: an initial denaturation step of 4 min at 96°C, followed by 38 cycles of 40 s denaturation at 96°C, 50 s annealing at 57°C, 90 s elongation at 72°C, and a final elongation step of 10 min at 72°C. Sequence polymorphism was revealed on a 6% non-denaturing polyacrylamide gel with migration at 4°C at 10 W overnight. The gel was silver-stained. For each of the different profiles of polymorphic EST markers, five alleles were sequenced in order to determine the mutations responsible for the polymorphism.

Statistical analyses. Genepop version 3.2a was used to calculate expected and observed heterozygosities, unbiased estimates of F_{IS} and F_{ST} and to perform exact tests for departure from Hardy-Weinberg equilibrium. The phylogenetic relationships between French pathotypes were investigated by building a neighbor-joining (NJ) tree based on allele shared distance (D_{AS}), using Populations 1.2.28 software. Bootstrap support for the nodes was calculated over 10,000 replications, using the same program. To describe the genetic structure of *P. halstedii* pathotypes, we also applied a Bayesian approach of genetic mixture analysis using the software Structure v2.2.

RESULTS

Molecular markers. Among the 124 ESTs tested by PCR-SSCP, only 12 were found to be polymorphic (9.6%). A total of five indels and 25 SNPs were revealed, one locus (Pha79) presenting 18 SNPs among the 25 (Table 3). The frequency of SNPs in coding regions was 0.52 SNP per kb and was 0.15 when the most polymorphic locus Pha79 was excluded. Five markers presented size polymorphism, with the

number of inserted or deleted nucleotides varying in a range from 2 to 63. For the marker Pha42, the deletion and SNP were linked so there were only two alleles at this locus.

Five SNPs were transformed into Cleaved Amplified Polymorphism Sequence (CAPS) markers. Four indel polymorphisms were automated on a Beckman Coulter Ceq8000 capillary sequencer using the manufacturer's recommendations and one was directly visualized on agarose gel. Finally, two markers were screened by PCR-SSCP since no enzyme discriminating the alleles could be found. The following protocol was used for CAPS markers: 1 µl of PCR product digested by 0.1 U restriction enzyme in 10X enzyme buffer for 1 hour at the appropriate temperature.

Table 3. Characterization and description of 12 EST-derived markers for *Plasmopara halstedii*: locus name, Genbank accession number, homology of sequences, primer sequences, polymorphism type, annealing temperature of primers and genotyping method used are shown.

Locus name	Genbank acc. no.	Homology	Primer sequences (5'-3')	Polymorphism	T_a (°C)	Genotyping method
Pha6	CB174585	Transportin	F: GTCGCTGATTTTATGTTTATGTGC R: TACTACCTCAGTCACATCATCACC	SNP	57	CAPS (T*sp*45I)
Pha39	CB174648	Hypothetical protein	F: GATTGGGTTCCTTGTTTGGA R: ATCTTCGCTGCCAGCTTCT	Indel	57	Sequencer
Pha42	CB174650	Hypothetical protein	F: GGATGTTGCTCGTCAAGTAGC R: ACGCATCCTACGCATTCAAC	indel	57	Sequencer
Pha43	CB174680	Hypothetical protein	F: ACTCAGGACTGGGCAACAAT R: CGACATCCTTGTGAGCTTGT	indel	57	Sequencer
Pha54	CB174708	Hypothetical protein	F: ATTTGGCAACGTCTCAGAGC R: CCATCGTAATAACATTCTTTAAAGTCC	SNP	57	CAPS (F*au*I)
Pha56	CB174714	40S ribosomal protein S2	F: GCGGTACTGGTCTATGTGCTG R: TTCAAGAAGTTTGATTTTTCATGC	SNP	57	CAPS (O*li*I)
Pha74	CB174642	Hsp 90	F: ACCTCGCATGGTTGCTTTAC R: TTGCTATTTCGGCCTACTGG	indel	57	Agarose
Pha79	CB174692	Hypothetical protein	F: GACGCCCCACTTAGCTTTC R: TTCGGGAGTAAGTGATTGAGC	SNP	57	SSCP
Pha82	CB174573	MMSDH[1]	F: ACTCGATCCATGCAGTAAGTAAG R: AGGAGGCTTTGCAGATTGAA	SNP	57	CAPS (B*sp*MI)
Pha99	CB174703	Hypothetical protein	F: CTCGCATTCAAACGGAAAAT R: CAAGCCAACTGTGCATGAAT	SNP	57	CAPS (B*sr*DI)
Pha106	CB174676	Hypothetical protein	F: TTGACGTTTATGCGAAGTGC R: CAAAGGAAGTTGTGATGGTGAG	Indel	57	Sequencer
Pha120	CB174660	Hypothetical protein	F: CTATTTAAAGGGGCCCGAAC R: CGGGTTTCCTCCATTAATCC	SNP	57	SSCP

[1]MMSDH: methylmalonic semialdehyde dehydrogenase.

Genotypic structure. Based on combinations of the 12 genomic markers, we identified 11 different multilocus genotypes among the 24 isolates analyzed. Three multilocus genotypes were found in multiple copies. A combination of eleven EST markers was sufficient to discriminate all the multilocus genotypes in the dataset, demonstrating the high resolving power of the markers (Fig. 1). Three pathotypes were represented by more than one isolate (race 100 represented by 3 isolates, races 703 and 710 each represented by 5 isolates). Isolates of the same pathotype presented an identical multilocus genotype, indicating that the three pathotypes may correspond to three clonal lineages. Conversely, two multilocus genotypes included more than one pathotype: the first multilocus genotype comprised pathotypes 100, 300 and 304 and the second comprised pathotypes 307 and 703.

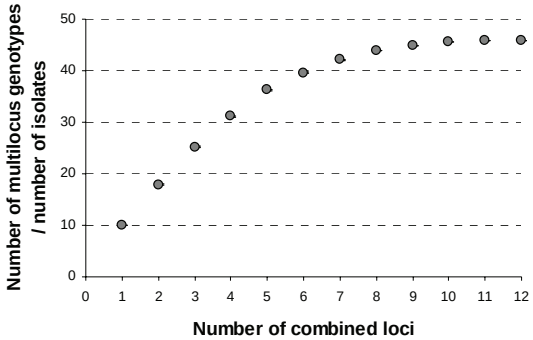

Fig. 1. Number of genotypes discriminated (number of multilocus genotypes/total number of isolates) as a function of the number of loci combined to discriminate isolates of *Plasmopara halstedii*.

Population genetic structure. The expected heterozygosity level was consistent with high levels of genetic diversity across loci ($0.349<H_E<0.677$). However, observed heterozygosity levels were much lower, with a mean H_O of 0.026. Only three of the 11 distinct multilocus genotypes were heterozygous: two at two loci and one at one locus. Tests of deviation from Hardy-Weinberg equilibrium revealed significant strong heterozygote deficits with respect to expectations under the assumption of random mating. All loci presented significant and positive F_{IS} values, with an overall F_{IS} of 0.948.

Bayesian assignment analyses showed three genetic groups of isolates: the first cluster was constituted by a single multilocus genotype including 3 pathotypes: 100, 300 and 304. The second cluster included 4 multilocus genotypes and 5 pathotypes: 703, 307, 700, 730, 707. The third cluster included 6 pathotypes with different multilocus genotypes: 710, 334, 314, 714, 717 and 704. Pathotypes 334, 707 and 730 were clearly 'mixed' genotypes that presented alleles belonging to two different clusters (Fig. 2).

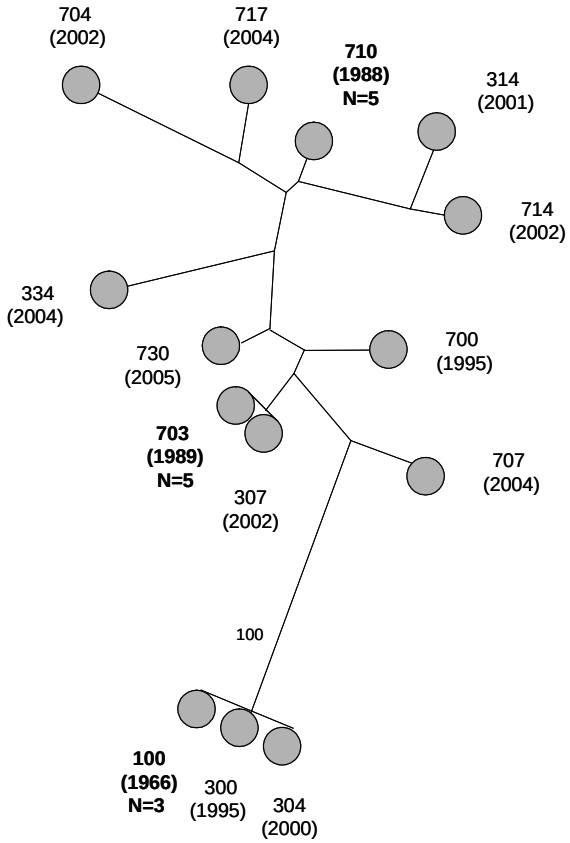

Fig. 2. NJ clustering from the allele shared distance matrix calculated among the 14 pathotypes of *Plasmopara halstedii* based on 12 EST-derived markers. Date of the first description in France of each pathotype is indicated in brackets. Pathotypes 100, 703 and 710 were represented by three to five isolates (N) and isolates belonging to one same pathotype presented an identical multiclocus genotype. Numbers above branches of the tree are bootstrap percentages after 10,000 re-samplings.

DISCUSSION

The EST-derived markers used in this study displayed high levels of intraspecific polymorphism which made it possible to infer the reproduction mode of *P. halstedii* and to assess the relationships among French pathotypes. These markers are specific and could therefore be used for the high-throughput genotyping of isolates directly from sporulating lesions collected from host leaves, avoiding the need for labor-intensive isolate subculture. *P. halstedii* is a homothallic species, and is therefore able both to

outcross and to self. However, our results suggest that *P. halstedii* is mainly a selfing species, with only limited outcrossing.

The finding that *P. halstedii* pathotypes cluster into three genetically differentiated groups, each including one of the first races described in France (i.e. 100, 703, 710), sheds new light on sunflower downy mildew evolution. Races 100, 703 and 710 correspond to three clonal lineages that not only present a strong genetic differentiation but also have very distinct virulence profiles. Given the strong geographic structuration of race distribution in France, we hypothesize that these results reflect (at least) three different introductions of this pathogen in France: the first introduction corresponds to race 100 (before 1966) that is now widely distributed in France, the second to race 710 in the North of France (before 1988) and the third to race 703 in South-West of France (before 1989). From then on, the triple introduction of *P. hasltedii* might have provided the raw genetic materials for more complex evolutionary processes of race emergence such as recombination between pathotypes or accumulation of mutation in clonal lineages (further referred as clonal evolution). These three introductions of *P. halstedii* could have provided the raw genetic materials for more complex evolutionary processes, such as recombination between pathotypes or the accumulation of mutations in clonal lineages (clonal evolution), in the emergence of new races.

ACKNOWLEDGEMENTS

This work was supported by the CETIOM (*Centre Technique Interprofessionnel des Oléagineux Métropolitains*) and partly by MAP (Ministry for Agriculture and Fishing) within the framework of the program CASDAR.

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Effect of sowing date and initial inoculum of *Alternaria helianthi* on sunflower in the south region of Brazil

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ABSTRACT

Three field experiments were carried out in Londrina, PR, south region of Brazil, in 1997/1998, 1998/1999 and 1999/2000 growing seasons, to investigate Alternaria leaf spot severity in sunflower sown at different dates and the effect of inoculum of the first sowing dates on subsequent ones. Four sowing dates (October, November, December and January) and two sowing types (contiguous and isolated) were used to simulate different levels of initial inoculum. Disease severity, under natural conditions in the field, was evaluated weekly, with reference to a diagrammatic scale of this disease, used to calculate the value of the area under disease progress curve (*AUDPC*). Marked plants were harvested individually, for evaluation of grain yield. Disease severity was highest when plants were sown in December regardless of the year. Sowing sunflower in October resulted in high yield and low disease severity. Sanitation measures to reduce initial inoculum concentration delayed the onset of the disease by 11 days.

Key words: *Alternaria* leaf spot – epidemiology – *Helianthus annuus* – primary inoculum.

INTRODUCTION

The potential for increasing sunflower (*Helianthus annuus* L.) cultivated area in Brazil can be limited by leaf blight and stem spot diseases, caused by *Alternaria helianthi* (Hansf.) Tubaki and Nishihara. The disease is reported to affect leaves, stem and sunflower heads, causing necrotic brown to black lesions, with a round or angular shape. Due to the frequent occurrence of climate conditions favorable for disease epidemics, i.e. high relative humidity and temperatures between 25°C and 30°C, Alternaria leaf blight is the most important sunflower disease in Brazil, occurring in all regions and sowing dates (Leite, 2005).

For many foliar diseases, once an epidemic has been initiated, infectious lesions within the crop are the predominant source of initial inoculum for newly planted tissues (Vanderplank, 1963; Jeger, 1982). Two main factors can influence disease severity: sowing date and presence of fungal inoculum in the production area. Preliminary studies about the development of Alternaria leaf spot during one growing season showed that lowest disease severities were observed in sunflower sown in October and November in the south region of Brazil and the fungal inoculum from the first sowing plots was important for pathogen dissemination to the last sowing plants (Carvalho et al., 1995).

Methods to reduce initial inoculum, i.e. Vanderplank's sanitation measures, can be used to decrease disease severity. Vanderplank (1963) used simple exponential equations to describe how sanitation measures could delay the onset of the disease, by reducing the amount of initial inoculum from which an epidemic starts. He argued that, assuming that the disease progress rate (r) is not affected by sanitation, if initial inoculum was reduced by sanitation from x_0 to x_{0s}, then the epidemic would be delayed by the time taken for disease to increase from x_{0s} to x_0. The relationship between the inoculum ratio (x_0 / x_{0s}) and the time delay in the epidemic (Δt) could be described by $\Delta t = \ln (x_0 / x_{0s}) / r$.

The main objective of this study was to investigate Alternaria leaf spot severity in sunflower sown on different dates, in the south region of Brazil, and the effect of inoculum from the first sowing plots on the last ones.

MATERIALS AND METHODS

Field experiments

Three field experiments were carried out in Londrina, State of Paraná, in the south region of Brazil (latitude 23º11'50" south; altitude 585 m), in three growing seasons: 1997/1998, 1998/1999 and 1999/2000. The experimental sunflower hybrid SE02, developed by the Embrapa Soja genetic breeding program, was used. All experiments followed the randomised complete block design, with four sowing

dates, two sowing types and four replications. Each plot consisted of four 5m rows, with between-row spacing of 0.90 m; three sunflower plants were left per linear meter. The trials received the conventional cultural practices of commercial fields, including sowing and top-dressing fertilisation, spraying against insects, and sprinkle irrigation, when necessary (Castro et al., 1996). The trials were planted in an area intensively used for sunflower experimentation.

To establish several levels of disease severity, sowing was carried out in the months of October, November, December and January of each year, in two sowing types: contiguous and isolated plots. The first type had all the plots of the different sowing dates located side by side, while, in the other type, plots of the different sowing dates were separated by six rows of corn, a non-host barrier to the fungus. No artificial inoculation of *A. helianthi* was performed; disease occurred by natural infection of the plants. The pathogen was identified by isolation in laboratory and plant inoculation in glasshouse.

Assessments of disease severity, area under disease progress curve and yield

The evaluation of disease severity and yield was made on the two central rows of each plot, disregarding 0.5 m at each row end. The single plant approach was adopted (Kranz and Jörg, 1989), in which 10 or 8 plants of each plot were marked, thus a total of 320 plants for the first trial and 256 for the second and third trials were obtained. Plants were marked after the appearance of the fourth true leaf (V4 growth stage) (Schneiter and Miller, 1981), and an attempt was made to select individuals of the same development stage, height and vigour.

The leaf areas (*LA*) (cm^2) of all leaves of each marked plant were estimated weekly, starting from the appearance of the fourth true leaf (V4 growth stage). For this, the maximum width (cm) of each leaf (*L*) was measured with a ruler. Leaf area was calculated using the equation $LA = -155.86 + 22.40\ L$ (R^2=0.90) (Leite and Amorim, 2002). Assessment of the severity of Alternaria leaf spot was simultaneous with the evaluation of leaf area, with the aid of a diagrammatic scale, which was previously elaborated and validated (Leite and Amorim, 2002). Marked plants affected by other diseases or showing any problems in their development were discarded.

The area under disease progress curve (*AUDPC*) for each plant was calculated by trapezoidal integration using the formula (Bergamin Filho et al., 1997):

$$AUDPC = \sum_{i=1}^{n-1}((S_i + S_{i+1})/2)(t_{i+1} - t_i)$$

where S_i=$S(t_i)$, n was the number of assessments, S was disease severity (in percentage) and $(t_{i+1} - t_i)$ was the interval (days) between two consecutive assessments.

Marked plants were harvested individually, after physiological maturity (R9) (Schneiter and Miller, 1981), for evaluation of grain yield (kg ha^{-1}).

Data analysis

Data of *AUDPC* and grain yield were submitted to ANOVA, using the factorial design (sowing dates x sowing types), with four replications. Duncan's multiple range test (p=0.05) was performed to detect the significant differences for *AUDPC* and yield means among sowing dates and sowing types, using SAS software (SAS Institute, USA).

Data were also analysed by non-linear regression, using the software STATISTICA 5.0 (Statsoft, Tulsa, USA). Data were fitted individually by negative exponential model, $Y=B_1 \exp(-B_2 X)$, where Y represents the yield component, X represents *AUDPC*, B_1 represents the intercept and B_2 represents the slope.

To calculate the initial inoculum, Alternaria leaf spot severity data for each sowing date and sowing type were individually fitted by logistic model (Berger, 1981), $Y=1/(1+B_1 \exp(-B_2 X))$, where Y is the disease severity, X is the number of days after sowing, B_1 is the parameter related to initial inoculum and B_2 is the disease progress rate, for the three consecutive years (1997/1998, 1998/1999 and 1999/2000).

The time delay in the epidemic was calculated based on the theory of Vanderplank (1963), which assumes that disease progress rate (r) is not affected by any measure of reducing initial inoculum. In the present work, the sanitation measure used for reducing initial inoculum was the isolated sowing type, where the different sowing dates were separated by six rows of corn, compared with the contiguous sowing type, where the different sowing-date plots were located side by side.

Using the logistic model, a constant disease progress rate (r) for each sowing date was calculated with the disease severity means of contiguous and isolated plots, for the three consecutive years. The initial inoculum for both contiguous (x_{0c}) and isolated (x_{0i}) sowing types was calculated keeping the rate

constant. The time delay in the epidemic (Δt) (days) as a function of reducing initial inoculum by the sanitation method was calculated by:

$$\Delta t = \ln (x_{0c} / x_{0i}) / r$$

where: x_{0c} was the initial inoculum present in contiguous sowing plots, x_{0i} was the initial inoculum present in isolated sowing plot and r was the constant disease progress rate for each sowing date.

RESULTS AND DISCUSSION

Sowing sunflower in four different months each year proved to be effective for obtaining a wide range of Alternaria leaf spot severity. A significant higher *AUDPC* mean was observed in plants sown in December of the three years, for both sowing types (Table 1). This variable decreased for plants sown in January, since many leaves were senescent due to the disease and were not considered for disease severity assessment. Healthy plants (*AUDPC*=0) were only observed in plants sown in October and November 1999; for the first month, considering both sowing types (contiguous and isolated plots), all plants remained disease-free. Comparing sowing types, *AUDPC* was significantly lower in isolated plots sown in January 1998 and in December 1999 (Table 1). Sunflower plants sown in January 1999 did not produce grains, as well as plants sown in contiguous plots in December 1998 (Table 1).

Table 1. Area under disease progress curve (*AUDPC*) of Alternaria leaf spot and yield of sunflower, sown on four months and two sowing types, in three consecutive years

Sowing date	*AUDPC*[1]				Yield (kg/ha)[1]			
	Sowing type				Sowing type			
	Contiguous		Isolated		Contiguous		Isolated	
1997/1998								
Oct	336.75	dB	504.19	cA	2450.54	aA	1395.04	aB
Nov	572.94	CA	485.13	cA	649.34	bA	622.3	bA
Dec	907.35	aA	867.59	aA	5.14	cA	137.76	cA
Jan	778.25	bA	630.68	bB	100.11	cA	348.65	cA
Mean	625.44				713.61			
CV (%)	10.41				24.83			
1998/1999								
Oct	163.67	dA	285.51	cA	2898.44	aA	2182.82	aB
Nov	305.26	cA	308.01	cA	920.41	bB	1214.78	bA
Dec	844.32	aA	811.82	aA	0	cB	304.82	cA
Jan	505.93	bB	667.67	bA	0	cA	0	dA
Mean	485.52				940.15			
CV (%)	17.47				15.22			
1999/2000								
Oct	0	cA	0	bA	2062.23	aA	2428.96	aA
Nov	45.92	cA	71.69	bA	1900.83	aB	2462.43	aA
Dec	771.99	aA	452.05	aB	673	bB	1886.73	bA
Jan	481.42	bA	409.77	aA	892.22	bB	1459.84	cA
Mean	279.10				1673.60			
CV (%)	19.38				19.28			

[1]For each variable and growing season, means followed by the same letter (capital letters in columns and minuscule letters in line) are not different by Duncan´s multiple range test (5%).

Data of yield and disease severity observed in this work indicate that sowing sunflower in October resulted in high grain yields and low or no disease severity. This corroborates the fact that the recommended sowing date for sunflower in the State of Paraná is from August to October (Castro et al., 1996). Silveira et al. (1993), studying sowing dates for sunflower in this State, also observed higher yields for sunflower sown in August and lower yields for sunflower sown in December.

Vanderplank´s sanitation ratio theory was used to account for the time delay in the epidemic (Δt) as a function of reducing initial inoculum by a sanitation measure. This theory was also used by Young et al. (2003), for predicting epidemics of yellow rust (*Puccinia striiformis*) on the upper canopy of wheat,

compared to disease severity on lower leaves. Vanderplank (1963) considered that the effectiveness of sanitation should be linearly related to the delay in reaching any given level of the disease. This delay (Δt) is the additional time required to reach a given severity in a crop with sanitation measures, as compared to the crop without sanitation measures (Plaut and Berger, 1981).

In this study, the time delay in the epidemic in terms of reducing the initial inoculum by the sanitation method varied from 0.75 day, on November 1998 sowing date, to 11.56 days, on December 1998 sowing date (Table 2). This confirms that sowing sunflower separated by rows of corn was enough to decrease the primary inoculum, compared with the contiguous sowing, and cause a delay in disease epidemics. The delay of 11 days on the onset of the disease is important considering the early crop cycle of 100 days. As disease is delayed, the damage to sunflower yield becomes lower.

Vanderplank (1963) considered some factors that could limit plant disease development when he first discussed the sanitation theory. He was cautious in recommending sanitation measures as a disease control strategy, particularly for diseases with high infection rates and for epidemics of a long duration. Management of Alternaria leaf spot of sunflower should not be seen as an isolated measure. The reduction in initial inoculum, which was used to simulate the effect of sanitation, may not be identical to benefits derived from actual sanitation measures (Plaut and Berger, 1981). Farmers of the same region should concentrate sunflower sowing on the same date, in order to decrease pathogen dissemination from one area to another. Low initial disease was apparently compensated for by accelerated rates of disease increase. Thus, sanitation measures in the management of compound interest diseases may be less effective than previously theorized (Plaut and Berger, 1981).

We concluded that sowing sunflower in October resulted in a high yield and low Alternaria leaf spot severity in the State of Paraná, Brazil. Sanitation measures to reduce initial inoculum concentration delayed the onset of the disease by 11 days.

Table 2. Time delay in the epidemic (Δt) and parameters of logistic function, $Y=1/(1+(1/x_0)-1)\exp(-rX)$), where Y is disease severity, X is days after sowing, x_0 is initial inoculum and r is constant disease progress rate for each sowing date, for Alternaria leaf spot of sunflower, in three consecutive years. Plants sown on four different dates and two sowing types (contiguous and isolated) were used for regression analysis.

Sowing date	r	Sowing type		Δt (days)
		Contiguous	Isolated	
		x_0	x_0	
1997/1998				
Oct	0.0601	0.0019	0.0025	-
Nov	0.0797	0.0010	0.0009	1.14
Dec	0.0620	0.0077	0.0061	3.77
Jan	0.0492	0.0105	0.0080	5.58
1998/1999				
Oct	0.1467	5.39E-07	1.02E-06	-
Nov	0.1128	5.36E-05	5.83E-05	0.75
Dec	0.0756	0.0061	0.0026	11.56
Jan	0.0563	0.0037	0.0030	3.73
1999/2000				
Oct	-	-	-	-
Nov	0.2474	9.9E-12	1.61E-11	1.97
Dec	0.0555	0.0036	0.0022	9.32
Jan	0.0226	0.0214	0.0175	8.78

ACKNOWLEDGEMENTS

We thank E. da Silva, R. A. Fagundes and V. Saldeira, from Embrapa Soja, for their technical assistance with this project.

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Effects of nitrogen and water on premature ripening caused by *Phoma macdonaldii*, a fungal pathogen of sunflower

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ABSTRACT

Premature ripening (PR) caused by *Phoma macdonaldii* results in yield damage for sunflower, mainly in the South-West of France, a major production area. The aim of the study was to characterize and identify the effect of crop management systems on PR incidence and severity, in 2006 and 2007. This field study used artificial and natural inoculation to investigate the role of host resistance, N-fertilization and water regime in the Phoma epidemics and aimed to reveal the most critical factors responsible for the disease progress and plant injury. On both years, the susceptibility of the cultivar appeared as a main factor influencing PR. However, the most severe attacks were observed in conditions of high nitrogen nutrition, especially when it was associated with water stress after flowering.

Key words: crop management – disease assessment – *Phoma macdonaldii* – premature ripening – sunflower.

INTRODUCTION

Premature ripening (PR), induced by *Phoma macdonaldii* Boerema, is one of the most severe sunflower (*Helianthus annuus* L.) diseases. The disease increased in the early 1990s and the entire French sunflower cropping area is now affected (Penaud and Pérès, 1995). The term "premature ripening" was first used for sunflower by Sackston (1949) to describe wilt and stalk rot. Evidence suggests that collar girdling canker caused by *P. macdonaldii* is the primary cause of PR. Sunflower premature death is most often characterized by loss of plant vigor during mid- to late summer followed by senescence and death of the plant a few weeks before normal maturity (Gulya et al., 1984). Generally, PR plants have small heads, reduced seed yield, low seed weight, and low oil content (Donald et al., 1987).

The Phoma symptoms generally appear on the petiole, the stem and the collar of the plant (Maric and Schneider, 1979; Gulya et al., 1997). The spot may girdle the stem or the collar and the black to brown lesions may only affect the epidermal layer or penetrate into the pith of the plant. If *P. macdonaldii* is not organ specific throughout the stages of sunflower development (Penaud and Pérés, 1995), sunflower resistance depends on the organ infected and the aggressiveness of the pathogen isolates. Research carried out by CETIOM, INRA and ENSAT since 1998 has highlighted that collar infection is the best way to reproduce prematurely dead plants in the field at flowering stage (Pérès and Poisson, 2000).

Recent investigations revealed an impact of crop management on PR, as N-fertilization and water regime. However, few studies have reported on the effects of crop on the incidence of the disease. The aims of this study are to confirm the role of *P. macdonaldii* in causing PR of sunflower by artificial collar inoculation and evaluate the effects of sunflower crop management on the severity of fungus attacks at collar levels. Both of the factors under investigation in thus study may be of importance in explaining the irregular occurrence of this disease, with a special emphasis on the difference of sensitivity of two cultivars, N fertilization and water regime on PR induced by *P. macdonaldii*.

MATERIALS AND METHODS

Climate and soil: Two field experiments were carried out in Auzeville, near Toulouse (Haute-Garonne, South-West of France) over two years (2006-2007) on the experimental INRA Station. The soil was deep, silty-clay to clay with a pH of 7.8 to 8.2. From the inoculation time to the end of the experiment, the mean relative humidity was 63% and 67% and the temperature between min. 9.7-10 °C and max. 38-36°C in 2006 and 2007, respectively. Seasonal precipitation was 115 mm in 2006 and 307 mm in 2007.

Experiment design and crop management systems: The experimentation was done in a split-plot design with inoculation either artificial (AI) or natural (NI) as the main plot (800 m^2 each). Each main plot was subdivided into 2 water regimes (no irrigation vs irrigated), then 2 levels of nitrogen (0 vs 150 kg /ha) and finally 2 cultivars (cv. Heliasol RM (Semences de France) vs cv. Melody (NK Semences)). This resulted in 24 subplots of 22 m^2 in 2006 and 36 in 2007. The plant population was similar (6.7 plants/m^2). In 2006, because of a dry season, 6 irrigations were applied up to 220 mm while only 2 irrigations were applied in 2007 (80 mm). N-fertilization was applied at sowing and at early flower bud stage. The two cultivars differed by their susceptibility to Phoma black stem, cv. Heliasol being the one most affected by premature ripening.

Phoma isolates and plant inoculation: Single pycnidiospore isolates of *P. macdonaldii* derived from sunflower fragments with severe black collar lesions were used in the experiment. The isolation and conservation of *P. macdonaldii* monopycnidiospore strains (MP6 and MPH2) was done following the method described by Roustaee et al. (2000). Artificial inoculation in the field was carried out using mycelium of the fungus (vegetative part). To allow mycelium growth, single pycnidiospore isolates were transferred to Petri dishes containing potato dextrose agar (Difco) (39 g/l, pH 6) and incubated for 10 days at 25°C in the dark. The inoculation on the AI plot was carried out at star bud stage on 25 homogenous plants on the 2 central rows of 6-row plots. A disc of mycelium (6 mm diameter) was placed for 5 days at the plant collar using MP6 as single pycnidiospore in 2006 and MPH2 in 2007. Previous tests had suggested that the latter was more aggressive. Desiccation of the disc was avoided by applying a damp cotton wool plug and aluminum around the collar.

A*ssessments of Phoma macdonaldii incidence and severity:* A disease assessment method was used to evaluate *Phoma macdonaldii* incidence (proportion of necrotic areas of infected collars) and final severity (proportion of early ripened plants). The first evaluation was performed 5 days after inoculation. Severity ratings were assessed weekly on the 25 tagged plants for all treatments from 59 DAE (days after emergence) and 52 DAE until harvest in 2006 and 2007, respectively, at least 15 assessments were performed during the experiment. The disease rating scale used was proposed by Cetiom: (0) healthy plant, (1) black collar in less than ¾ of the stem diameter (2) coalescent spots on collar, (3) all the leaves are wilted but the stem is still green, (4) the plant is totally dry.

The weekly monitoring of disease progress was used to calculate the area by a disease progress curve (AUDPC) and the value was standardized by dividing it by the total number of epidemic days. The AUDPC was calculated according to the equation of Campbell and Madden (1990):

$$AUDPC = \sum_{i}^{n-1} (yi + yi+1)/2 * (t\,i+1 - ti)$$

where n is the number of evaluations, y the severity or incidence of the disease and t the thermal time of each evaluation. $(t,y) = (441, 0)$ is included as the first evaluation date, approximately 1 week before AI. Daily thermal time (DTT) was calculated using daily mean air temperature. from emergence to 120 DAE (equivalent to 1930 DTT) and 126 DAE (1776 DTT) in 2006 and 2007, respectively, the last observation date where disease rating scale might be attributed to PR and not to natural senescence. The threshold temperature was taken at 4.8 °C (Granier and Tardieu, 1998).

Statistical analysis: Final severity ratings and AUDPC values were subjected to one-way and multifactor analysis of variance (ANOVA) both in 2006 and 2007. ANOVA was performed by comparing the impact of water regime*N-fertilisation*cultivar for each inoculation treatment (AI, NI) on AUDPC values. Where significant differences were found at $P \leq 0.05$, means were compared using Fisher's protected least significant difference test (95% LSD). Analysis was performed using Statgraphics Plus 5.1 statistical software (Rockville, MA, USA).

RESULTS

Disease incidence measured by AUDPC values between the two experiments (2006 and 2007) did not differed significantly on AI plots (P = 0.08), whereas AUDPC on NI was significantly higher in 2007 (P=0.00*). According to the mode of inoculation (AI and NI) for each year, disease severity was measured by the final fraction of PR plants and the AUDPC did not vary significantly, except in 2006 when it was higher in AI than in NI plots (Table 1). This lack of significant effects on AUDPC and PR suggests that AI reproduces NI correctly.

Table 1. AUDPC values and percentage of premature ripened sunflower plants for artificial (AI) and natural (NI) inoculation with *Phoma macdonaldii* in 2006 and 2007 as a function of the cultivar, N-fertilisation and water regime.

	AUDPC[1]				PR plants (%)			
	2006		2007		2006		2007	
Treatment	AI	NI	AI	NI	AI	NI	AI	NI
	3860 a	2177 b	3755 a	3792 a	38 a	34 a	49 a	40 a
Heliasol	4141 a	2325 a	3843 a	3927 a	69 a	43 a	60 a	48 a
Melody	3787 b	2028 b	3665 b	3657 b	39 b	26 b	37 b	33 b
0-N	3812 b	2101 b	3627 b	3617 b	34 b	23 b	15 b	11 b
150-N	4116 a	2253 a	3919 a	3882 a	74 a	45 a	83 a	69 a
Unirrigated	4125 a	2184 a	3800 a	3968 a	83 a	52 a	55 a	50 a
Irrigated	3804 b	2169 a	3708 b	3616 b	25 b	17 b	42 b	31 b

[1]Calculated according to Campbell and Madden (1990).
Means were calculated using Fisher's protected LSD test a $P \leq 0.05$. The effect of cultivar, nitrogen and water regime was tested separately for AI and NI. Letters to the right of each value refer to differences between values. Means with the same letter do not differ significantly.

In AI and NI plots, sunflower crop management through cultivar, N amount and water regime increased the proportion of plants infected by *P. macdonaldii* ($P < 0.01$). All the tagged plants had a score of 2 (black coalescent spots on collar), but the evolution into PR plants (score 4) was only induced by the crop management. Cv. Heliasol was systematically more susceptible than cv. Melody to Phoma attacks whatever the season and the mode of inoculation ($P < 0.01$).

AUDPC values and PR (%) differed significantly between 150-N and 0-N and between rainfed and irrigated management for AI and NI ($P< 0.01$ for N and water) both in 2006 and 2007. High N-fertilisation and water stress (resulting from rainfed management) increased the proportion of plants infected by *Phoma macdonaldii* (at stem and collar level) and especially the percent of PR plants.

Two examples of disease progress curves for AI in 2007 are shown in Fig. 1.

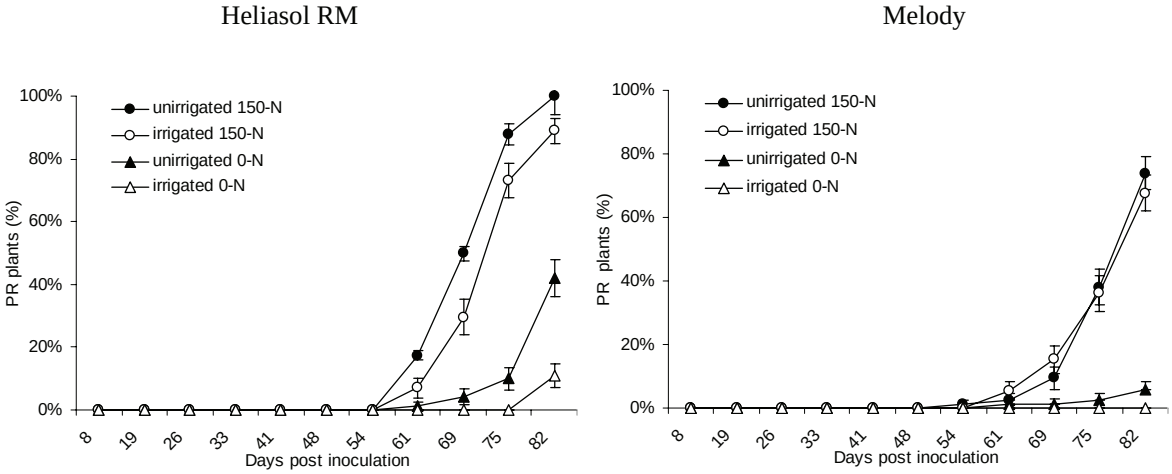

Fig. 1. Disease progress curves of premature ripening plants after artificial inoculation in 2007 for cv. Heliasol (susceptible) and cv. Melody (tolerant) for contrasted crop management systems combining nitrogen and water applications.

The susceptibility of the cultivar was the main factor influencing the final rate of PR plants, cv. Heliasol being more attacked than cv. Melody. Nitrogen fertilisation appeared as the second factor affecting disease progress for both cultivars. Water stress seems to amplify nitrogen effect and may be considered as the third factor stimulating the disease progress. High N fertilization combined with rainfed management resulted in the highest plant injury: up to 100% of PR plants were observed for cv.Heliasol (in AI and NI treatments) on 150-N and no irrigation. Effects of cultivar x nitrogen interaction were observed in 2006 for NI and for AI in 2007 ($P<0.01$).

DISCUSSION

This study intended to point out the most influencing factors of sunflower premature ripening attributed to *Phoma macdonaldii*. A preliminary investigation into the possible role of a different fitness among the fungus isolates did not clearly reveal differences in disease severity. In 2006 and 2007, no significant differences in AUDPC were found in AI plots with two different monopycnidiospores. This suggests that differences in disease severity observed in the field could not be only attributed to isolate aggressiveness but more to environment and crop management.

As expected, the proportion of PR plants was higher in 2007 than in 2006. Higher precipitation and air relative humidity in 2007 resulted in a favourable environment for natural Phoma infection. Weeraratne and Priyantha (2003) observed that HR > 80%, temperature ranging from 25 to 30°C and cloudy weather favour the development of the disease under tropical conditions. Stem injury resulting from *Phoma macdonaldii* infections at collar level probably increased because the microclimate in the lower part of the sunflower stand was more favourable to fungus development and activity. Similar conclusions were drawn by Debaeke and Pérès (2003) on stem and collar attacks. However, if disease injury is influenced by the climatic and microclimatic environment, the PR syndrome relies strongly on cropping system management.

The susceptibility of the cultivar was shown throughout both experiments in AI and NI plots. The response of cv. Heliasol and cv. Melody differed significantly towards the progression of the disease and the final incidence of the inoculation by *P. macdonaldii*. The significantly lower AUDPC values and percent of PR plants for cv. Melody indicate possible differential genotypic susceptibility to premature ripening. Such differences were already noted by Penaud (1994) and Dechamp-Guillaume et al. (2000) on stem attacks. If the susceptibility of the cultivar is one of the main factors inducing PR, host nutrition was responsible for different patterns of epidemics in the experimentation.

As suggested in the literature, mineral nutrition can exert a profound effect on disease development, with fertiliser application increasing development of the disease. The mechanisms leading to these nutrient-induced changes in disease development are complex and multifarious. These mechanisms include the effects of the mineral nutrients directly on the pathogen, on plant growth and development, and on resistance mechanisms. According to Gulya et al. (1997), high N fertilisation increased the proportion of collars infected by *P. macdonaldii*. Conversely, nitrogen deficiency did not predispose plants to PR. The role of N-fertilisation clearly demonstrated in this study should be better explained at a process level. Our investigations did not reveal if the nitrogen had an impact on the sunflower culture that could favour pathogen development or was trophic for the pathogen. Large nitrogen supplies influence the size of the canopy. Dense canopy, observed in the experimentation for a given stand density, especially in 2007, induced a microclimate which might promote inoculum production and create conditions conducive to successful infections. Spore germination of *P. macdonaldii* might be sensitive to this high relative humidity microclimate, constituting a major climatic parameter in disease epidemiology and field infection (Roustaee et al., 2000). The other approaches to the effect of N-fertilisation on the percent of PR plants may concern the possible N sources available by plant pathogenic fungi. Assimilation of N sources, depending on the tissue being colonised, would include nitrate, ammonium, amino acids, amine and protein (Snoeijers et al., 2000). In addition, available N sources may also depend on the mode of nutrition of the pathogen. *P. macdonaldii*, which is a necrotrophic fungus, could have access to a wide range of N sources. The present study did not allow us to determine clearly the role of N-fertilisation. Both nitrogen effects may act on the disease progression, and one probably more than the other. Further investigations will be set up to better understand the N-fertilisation impact on the microclimate by varying crop density of sunflower. The difficulty is that in most plant-pathogen interactions, very little is known about the N content and subsequent colonisation by fungi. This is an area that requires further investigation (Walters and Bingham, 2007).

Previous data showed that infested plants which did not get irrigation presented a higher AUDPC and final percent of PR plants compared to those which were irrigated. If its effect seems to be less influential compared to the susceptibility of the cultivar and high nitrogen supply, rainfed plots receive a significant impact from the severity of the disease. This impact is not clearly demonstrated but this effect might be more linked to the physiological status of the plants. A predisposition to disease is often observed in host plants during water deficiencies. According to the literature, there are no studies in which the biochemical and biophysical causes of predisposition to disease during water deficiencies are known with any certainty. When the plant is infected by the pathogen, changes in host plants may alter their interactions with other organisms and suggest possible mechanisms of susceptibility. Boyer (1995) proposed two mechanisms to explain how water stress increases the susceptibility of plants to attacks from pathogens: (1) reduced photosynthate production induced by drought eliminates the plants' ability to defend

themselves against pathogens and/or (2) plant growth is reduced without reducing the pathogen's ability to reproduce, thus allowing further progression and increased symptom severity in the host. These two mechanisms may be an approach to a better understanding of the water deficiency effect on disease progression in the plant leading to PR as a nitrogen effect.

This study has attempted to identify the most crucial elements of PR induced by P. macdonaldii infestation. Our data revealed that sunflower epidemiology efficiency is mainly influence by Phoma and crop management. The susceptibility of the cultivar, high N-fertilization and rainfed management have a strong impact on the disease. Nitrogen input and water stress enhance PR. The interaction of both may act to favour pathogen infection in the plant and reduce expression in host defence mechanisms. Deployment of resistant lines in combination with an integrated disease management framework is suggested as suitable tools for reducing inoculum pressure and PR plants induced by *P. macdonaldii***.**

ACKNOWLEDGEMENTS

This study was supported by CETIOM, Midi-Pyrénées government and PROMOSOL association.

We are grateful to the technical staff of INRA (D. Raffaillac, M. Labarrère and C. Quinquiry) for patiently and carefully helping in the experimental set up and survey. Several other people participated actively in the disease notations (M. Moreto, P. Joumas, G. Beaure, C. Dujardin, N. Seguin, A. Sirvent and A. Pauvert). We thank them all once more for their kind and useful help.

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Pathological and morphological evaluation of sunflower isohybrids carrying or not the *Rcm-1* gene for *Sunflower chlorotic mottle virus* resistance

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ABSTRACT

Sunflower (*Helianthus annuus L.*) crops are affected by *Sunflower chlorotic mottle virus* (SuCMoV) which reduces yield parameters in commercial hybrids infected at early ontogenic stages. Sunflower isohybrids (between two near isogenic males differing in the *Rcm-1* resistance gene and three different females) were mechanically inoculated with SuCMoV under field conditions in two locations (Balcarce and Venado Tuerto) and evaluated for symptom expression and agronomic characteristics. Symptoms were scarce chlorotic pinpoint in all resistant hybrid combinations and severe chlorotic mottling in all susceptible hybrids, independently of the female parents. Nevertheless, higher symptoms intensity was detected in Venado Tuerto. Morphological parameters were more affected in Venado Tuerto than in Balcarce and differed among hybrid combinations.

Key words: *Helianthus annuus* – isohybrids – *sunflower chlorotic mottle virus* – virus.

INTRODUCTION

Sunflower chlorotic mottle virus (SuCMoV) is a potyvirus, which seemed to be restricted to the Americas. In Argentina, it has been associated with chlorotic mottling and plant stunting symptoms and it has been reported in several provinces including; Entre Ríos, Santa Fé, Buenos Aires and Córdoba. The virus is a member of the *Potyvirus* genus within the Potyviridae family (Dujovny et al., 1998, 2000) and it has been classified as a strain of *Potato virus Y* (Berger, 2005).

Recently, a sunflower line tolerant to SuCMoV infection has been reported (L33) and the resistance gene *Rcm-1*gene has been mapped (Lenardon et al., 2005). Breeding for virus resistance is one of the best ways to manage virus epidemics since no additional agricultural practices are required to reduce disease incidence and severity. Using molecular marker-assisted selection, the *Rcm-1*gene was incorporated to a susceptible L37S male in order to obtain a near-isogenic resistant version L37R. The objective of this work was to study the level of resistance obtained by the presence of this gene in different hybrid combinations, both in terms of symptom expression and agronomic characteristics.

MATERIALS AND METHODS

Plant Material and Experimental design

Crosses between a pair of near-isogenic male lines with (R) and without (S) the *Rcmo-1* gene and 3 susceptible female lines were performed in order to obtain 3 pairs of isohybrids. Hybrids obtained from the cross between the three females and the resistant donor L33 (source of *Rcm-1* gene) (Advanta Semillas S.A.I.C) were employed as controls.

Split plot design experiments with three replications were sown in two locations: Venado Tuerto (Santa Fe Province, 33º45´S, 61º 58´ W, November 20, 2006) and Balcarce (Buenos Aires Province, 37° 45' S, 58° 18' W, November 27, 2007). Each replication consisted of the hybrids as the main plot, and two treatments (SuCMoV inoculated versus non-inoculated) as the subplot. Each subplot was represented by three rows (20 plants per row). The middle row was used for treatment application and evaluation.

Plant inoculations
A SuCMoV isolate maintained on sunflower commercial hybrid CF 7 under greenhouse conditions was used as inoculum source for the whole experiment. Sunflower plants were mechanically inoculated at vegetative stage V 12 (Venado Tuerto) and at R1 (Balcarce) (Schneiter and Miller, 1981) with a high pressure airbrush apparatus, using a slurry prepared from infected leaves ground with phosphate buffer and abrasive (Lenardon et al., 2005).

Evaluation
Inoculation in Venado Tuerto and Balcarce were performed on December 20, 2006 and on January 15, 2007 respectively. . Symptom expression was evaluated 25 days after the inoculation and agronomic parameters (flowering date, plant height, capitulum diameter, length and width of a totally expanded leaf from middle portion of the stem) at proper times.

Statistical analysis
Results were analyzed by ANOVA. Orthogonal contrasts were planned in order to test the effects on the morphological characters of: a) the treatment (inoculated vs non-inoculated) for each hybrid and 2) the effect of the incorporated gene (R vs S) within each treatment (inoculated or non- inoculated).

RESULTS AND DISCUSSION

Inoculation was successful and all inoculated plants expressed virus symptoms. A heavy storm occurred in Venado Tuerto and some plants suffered mechanical stress and lodging before flowering so one pair of isohybrids was eliminated from the analysis.

Qualitative differences between symptoms in all isohybrid pairs were detected as expected. In all resistant hybrids symptoms were scarce chlorotic pinpoints, similar to those observed in the controls when the resistant L33 donor was employed. Nevertheless, the intensity of the symptoms was higher in Venado Tuerto than in Balcarce even in the crosses where L33 was a parent. In the first location, the chlorotic pinpoint was intense and also chlorotic ringpots were detected. In Balcarce, typical scarce chlorotic pinpoint symptoms (SCP) were observed on resistant genotypes. The susceptible counterpart of the isohybrids exhibited severe chlorotic mottling (SCM) symptoms independently of hybrid combination (Table 1).

Table 1. Symptom expression in isohybrid pairs differing in *Rcm-1* gene artificially inoculated in two locations[1].

Pedigree	Venado Tuerto	Balcarce
L16xL37S	SCM	SCM
L16xL37R	ICP + CR	SCP
L16xL33	ACP + CR	ACP + CR
L348xL37S	SCM	SCM
L348xL37R	ICP + CR	SCP
L348xL33	ACP + CR	SCP
L351xL37S	NR	SCM
L351xL37R	NR	SCP
L351xL33	NR	SCP

[1]SCM: severe chlorotic mottle; SCP: scarce chlorotic pinpoints; ACP: intense chlorotic pinpoint; ICP: intermediate chlorotic pinpoint; CR: chlorotic ringspots; NR: no results

One-two day differences in flowering date between treatments were observed according to the isohybrid (data not shown).

ANOVA analysis for both locations detected significant differences between hybrids and treatments and an interaction between hybrid x treatment ($P<0.05$) for the morphological characters with exception of head diameter in Balcarce. The means of morphological traits are presented in Table 2.

Table 2. Morphological traits in isohybrid pairs differing in the *Rcm-1* gene inoculated and non-inoculated with SuCMoV in two locations. Hybrids with L33 (resistant source) are used as controls.

Venado Tuerto

Pedigree	Plant height, cm		Head diameter, cm		Leaf length, cm		Leaf width, cm	
	Inoc.	Non inoc.	Inoc.	Non inoc.	Inoc.	Non inoc.	Inoc.	Non inoc.
L16xL37S	171	193	9	21	17	23	17	23
L16xL37R	196	203	11	18	21	24	21	24
L16xL33	177	181	13	17	23	25	21	23
L348xL37S	179	216	8	18	17	23	16	22
L348xL37R	217	222	15	19	22	23	22	23
L348xL33	195	200	12	18	19	24	17	22

Balcarce

Pedigree	Plant height, cm		Head diameter, cm		Leaf length, cm		Leaf width, cm	
	Inoc.	Non inoc.	Inoc.	Non inoc.	Inoc.	Non inoc.	Inoc.	Non inoc.
L16xL37S	173	186	12	11	20	25	19	23
L16xL37R	185	190	12	13	25	24	25	22
L16xL33	156	152	17	16	30	31	30	31
L348xL37S	180	187	12	14	17	25	15	24
L348xL37R	191	193	13	13	22	24	20	23
L348xL33	154	160	19	20	26	28	27	28
L351xL37S	171	172	17	16	20	25	19	23
L351xL37R	172	176	17	16	26	27	25	27
L351xL33	152	152	22	20	27	26	28	26

Table 3. Orthogonal contrast for morphological traits between inoculated and non-inoculated hybrids, which differ in *Rcm-1* gene in two locations.

Venado Tuerto

Inoculated vs. non-inoc.	Plant height			**Leaf length**			**Leaf width**			**Head diameter**		
	MS	F Value	p-value	MS	F Value	p-value	MS	F Value	p-value	MS	F Value	p-value
L16 xL37S	506.25	16.99	0.0026	62.08	72.40	<0.0001	65.34	80.47	<0.0001	108.37	65.49	<0.0001
L16xL37R	73.50	2.47	0.1507	16.67	19.44	0.0009	17.68	21.78	0.0005	83.10	50.22	0.0001
L348 xL37S	2041.31	68.51	<0.0001	54.00	62.97	<0.0001	51.04	62.86	<0.0001	155.14	93.75	<0.0001
L348xL37R.	48.17	1.62	0.2354	0.74	0.86	0.3728	2.04	2.51	0.1388	28.17	17.02	0.0026

Balcarce

Inoculated vs. non-inoc.	Plant height			**Leaf length**			**Leaf width**		
	MS	F Value	p-value	MS	F Value	p-value	MS	F	p-value
L16 xL37S	261.36	20.25	0.0003	36.51	9.79	0.0058	20.91	3.94	0.0626
L16 xL37R	44.61	3.46	0.0794	5.61	1.50	0.2359	9.63	1.81	0.1946
L348 xL37S	84.68	6.56	0.0196	96.0	25.75	0.0001	105.84	19.95	0.0003
L348 xL37R	10.32	0.80	0.3829	6.83	1.83	0.1927	8.64	1.63	0.2181
L351 xL37S	7.28	0.56	0.4623	26.46	7.10	0.0158	25.63	4.83	0.0413
L351 xL37R	2.04	0.16	0.6955	1.50	0.40	0.5339	5.61	1.06	0.3175

As previously described, symptoms intensity was higher in Venado Tuerto than in Balcarce. Thus, the inoculated plants in Venado Tuerto showed a significant reduction in all parameters in the S hybrids (without resistant gene) and in the R hybrids (with resistant gene) except plant height of R hybrids and leaf size (when L348 female was crossed with L37 R) (Table 3). On the contrary, in Balcarce, inoculation showed a significant reduction in all parameters in the S hybrids but it did not affect R hybrids (Table 3).

The presence of the incorporated gene did not modify the morphological characteristic of non-inoculated isohybrids (S vs R non-inoculated) (Table 4).

Table 4. Orthogonal contrast for morphological traits, between hybrids differing in *Rcm-1* gene inoculated and non-inoculated with SuCMoV in two locations.

Venado Tuerto

Pedigree	Plant height			**Leaf length**			**Leaf width**			**Head diameter**		
	MS	F Value	p-value	MS	F Value	p-value	MS	F Value	p-value	MS	F Value	p-value
L16 xL37S vs L16xL37R.inoc	684.7	23.0	0.001	24.8	28.9	0.0002	22.8	28.1	0.0002	0.2	0.1	0.7553
L16 xL37S vs L16xL37R.non inoc	170.7	5.7	0.040	1.4	1.6	0.225	0.8	1.0	0.339	9.8	5.9	0.0379
L348 xL37S vs L348xL37R. inoc	2140.6	71.8	<0.0001	40.0	46.7	<0.0001	49.3	60.7	<0.0001	61.3	37.0	0.0002
L348 xL37S vs L348xL37R. non inoc	64.4	2.2	0.176	0.03	0.03	0.863	1.7	2.1	0.173	0.5	0.3	0.6111

Balcarce

Pedigree	**Plant height**			**Leaf length**			**Leaf width**		
	MS	F Value	p-value	MS	F Value	p-value	MS	F Value	p-value
L16 xL37S vs L16xL37R.inoc	234	18.1	0.0005	36.5	9.8	0.006	39.5	7.5	0.0137
L16 xL37S vs L16xL37R.non inoc	33.8	2.6	0.1232	5.6	1.5	0.236	1.9	0.4	0.5542
L348 xL37S vs L348xL37R. inoc	200.3	15.5	0.0010	29.0	7.8	0.012	36.5	6.9	0.0172
L348 xL37S vs L348xL37R. non inoc	66.7	5.6	0.0355	3.2	0.9	0.365	1.7	0.3	0.5776
L351 xL37S vs L351xL37R. inoc	2.1	0.2	0.6889	55.2	14.9	0.001	43.7	8.3	0.0101
L351 xL37S vs L351xL37R. non inoc.	0.04	0.003	0.9580	12.3	3.3	0.086	15.4	2.9	0.1060

Complete resistance to SuCMoV has not been detected up to now. The *Rcm-1* gene produced a qualitative modification of symptom expression, which could be affected by the environment and the specific hybrid combination. Slight differences in the inoculation time could be excluded as the cause of the intensity differences between locations, because the phenotypic data used for gene mapping was obtained under field inoculation in Balcarce at younger stages and the symptoms of resistant plants were equal to those obtained for this location in the present study (Lenardon et al., 2005).

The use of this resistance gene could attenuate the effect of SuCMoV on morphological traits such as leaf area, and therefore, on some of the yield components.

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Study of resistance to *Sclerotinia* head disease in sunflower genotypes

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ABSTRACT

In Hungary, the most dangerous head diseases of sunflower are white and grey rots, caused by *Sclerotinia sclerotiorum* (Libert de Bary) and *Botrytis cinerea* (Persoon) respectively. In recent years, the role of fungal species well-disposed to higher temperatures (above 30°C) has also become more important. The intensity of attack by pathogens is not the same from year to year. Nevertheless, in certain years, they cause heavy yield losses and reduced quality. In 2005, the heaviest damage in seed and commercial grain production was caused by *Sclerotinia* head rot. Protection of the sunflower head has some difficulties and, in many cases, treatments do not produce any results. One of the most important aspects of our breeding work is the development of hybrids tolerant to head diseases. Heavy epidemic in 2005 made it possible to confirm the success of selection aimed at tolerance to *Sclerotinia* head rot. Genetic differences in susceptibility to *Sclerotinia* head rot were assessed in performance trials at 4 locations with 17 experimental hybrids and 3 check varieties of MGSZH (Central Agricultural Office, Directorate of Plant Production and Horticulture). Impact of sowing date on degree of infection was studied in two experiments with an earlier (15th April) and a later (2nd May) sowing time. Differences in the level of infection were scored in the plots of nearly isogenic lines and with their hybrids flowering at different dates. Efficiency of chemical treatment was evaluated on the basis of data obtained in treated and untreated plots. Temperature, humidity and rainfall were systematically recorded. Results of trials reflected differences in tolerance between hybrids. Planting date had an indirect influence because the level of damage was highly dependent on the average temperature and quantity of rainfall during bloom. Differences in percentage of infection in nearly isogenic lines and their hybrids flowering at different dates, as well as those in performance trials of hybrids sown on two dates, showed a close significant correlation r=0.84*** with the quantity of precipitation from the beginning to the end of the flowering period. Combining ability of 5 CMS female lines and 5 male restorer lines was studied by coupling model of Comstock and Robinson and analysis of variances was used for the evaluation of the experiment.

Key words: head rot – flowering – *Sclerotinia sclerotiorum* – sunflower – susceptibility – weather.

INTRODUCTION

Sclerotinia sclerotiorum (Lib.) de Bary is one of the most important pathogens in sunflower widely spread all over the world. Korf and Dumont (1972) assigned the species to *Whetzelinia*, a new genus, but objections to this disposition were raised by Dennis (1974). Not only *S. sclerotiorum*, but also *Sclerotinia minor* and *Sclerotinia trifoliorum* Fuckel, are pathogenic to sunflower (Cormack, 1946). After ontogenetic studies of *Sclerotinia* (Willets and Wong, 1971) and electrophoretic investigations (Wong and Willetts, 1973), it was concluded that *S. scelorotiorum, S. trifoliorum* and *S. minor* are three different species.

Originally identified on sunflower in 1861, the fungus has been reported from all sunflower-growing regions of the world (Gulya et al., 1997). Depending on the environmental conditions, it attacks the seedling, root, petiole, stem, and inflorescence. Since there is no 100% efficient chemical protection, hybrids should have good field resistance against this pathogen as well. As chemical control of *S. sclerotiorum* is difficult and uneconomical (Mestries et al., 1998), genetic control appears to be of great value. In the literature, there are no articles showing total resistance to *Sclerotinia sclerotiorum* in cultivated sunflower. However, reports on the identification of sunflower genotypes with low susceptibility or partial resistance are common worldwide. Some wild species include important *Sclerotinia* resistance genes (Seiler and Rieseberg, 1997; Köhler and Friedt, 1999; Degener et al., 1999). Resistance is a polygenic trait (Castaño et al., 1993.) There are two types of resistance in sunflower: (i) resistance to penetration, and (ii) resistance to mycelial extension in the tissues.

Breeders have a better opportunity to assess genetic resistance. A first approach is to make use of the natural infection, but the intensity of pathogen attacks is not the same from year to year. A second approach is to produce artifical infection (Tourvieille and Vear, 1984; Rodríguez et al., 2004). These

authors used the following procedure: Ascospores were suspended in sterile distilled water with Tween 80 (0.05%) to a concentration of 5×10^3 spores/ml (10 ml per inflorescence). Plants were checked regularly for flowering. Sunflower heads were sprayed with inoculum when the anthesis of the two outer rows was completed, and so inoculation was produced at anthesis. Control plants were sprayed with water and drops of detergent.

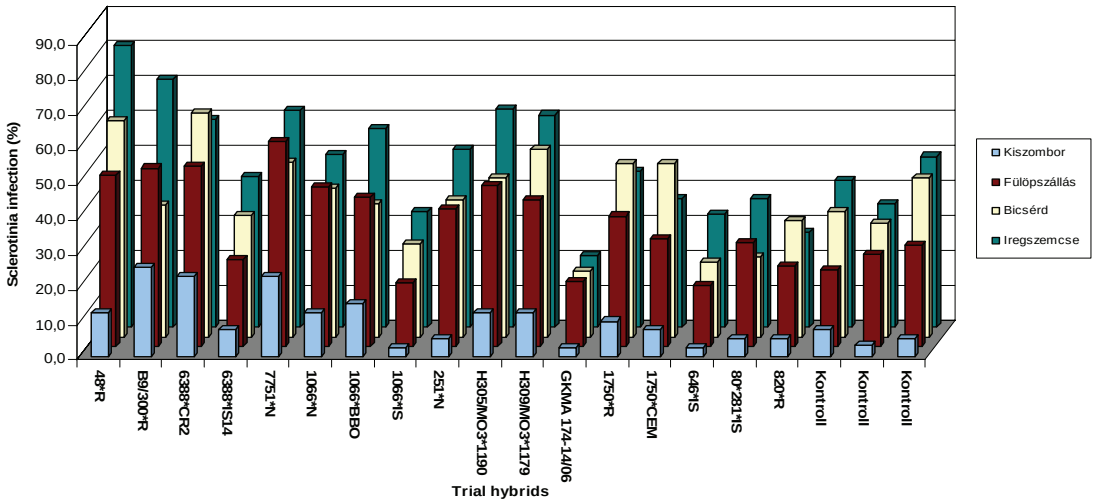
The aim of this study was to analyse genetic differences in susceptibility to *Sclerotinia* head rot at different locations.

MATERIALS AND METHODS

Genetic differences in susceptibility to *Sclerotinia* head rot were assessed in performance trials at 4 locations with 17 experimental hybrids and 3 check varieties of MGSZH (Central Agricultural Office, Directorate of Plant Production and Horticulture). Impact of sowing date on degree of infection was studied in two experiments with an earlier (15th April) and a later (2nd May) sowing time. Differences in the level of infection were scored in the plots of nearly isogenic lines and their hybrids flowering at different dates. Efficiency of chemical treatment was evaluated on the basis of data obtained in treated (6 pair of leaves, initial reproductive stage, and full flowering) and untreated plots. Temperature, humidity and rainfall were systematically. Combining ability of 5 CMS female lines and 5 male restorer lines was studied by coupling model of Comstock and Robinson (1948) and analysis of variances was used for the evaluation of the experiment.

RESULTS AND DISCUSSION

The four locations showed different levels of head infection. The highest and lowest average of attack were 47.7% and 10.2%, respectively. Degree of infection in the most susceptible genotype at the location with the heaviest infection pressure attained 80%, whereas that of the most tolerant hybrid was only 15.1% (Fig. 1.)



LSD 5%=9.3

Fig. 1. *Sclerotinia* infection of trial-hybrids at four locations, 2005

The *Sclerotinia* head rot resistance of hybrids was studied as a function of sowing time and chemical treatment. In the experiment, the mean infection was 14.3%, the highest was 30% and the lowest was 1.5% (Fig. 2). There was no significant difference between chemically treated and untreated plots.

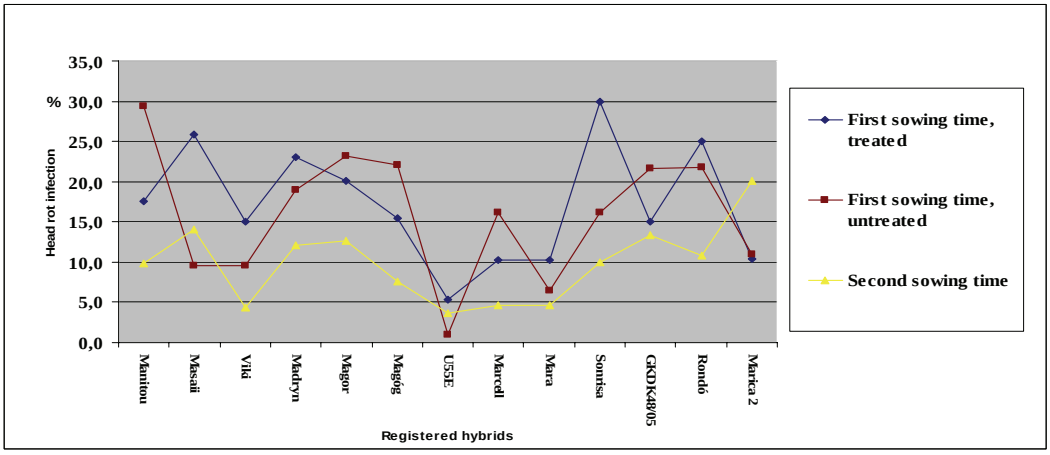


Fig. 2. The *Sclerotinia* head rot resistance of registered hybrids as a function of sowing time and chemical treatment.

Differences for disease incidence between the two sowing dates was analysed with the data of temperature and rainfall at the beginning of flowering, 50% flowering, and full flowering Significant difference was found between the percentages of infection at the two sowing times, the lower infection being on the second sowing date (Fig. 2).

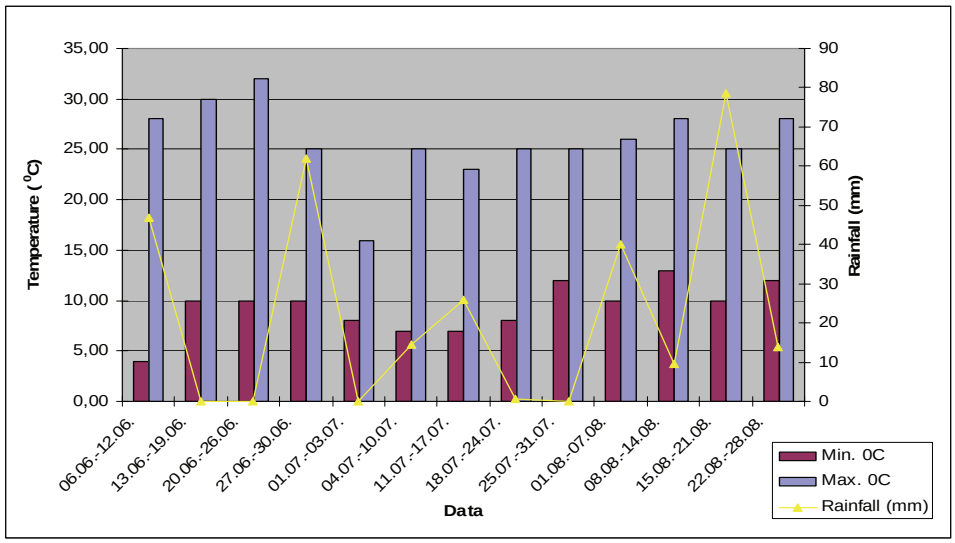


Fig. 3. The ecological conditions during flowering time.

The results of the experiments of the registered hybrids were assessed compared to the results of nearly isogenic lines and their hybrids. The hybrids sown on 28th April were flowering between 10-20 July and their average infection was 17.2%. The hybrids sown on 14th May were flowering between 18-27 July and the average infection was 9%. The ecological conditions during flowering time were examined on the basis of the data (Fig. 3).

The weather during the flowering time of the genotypes sown on 1st April was hot (30 °C), there was no rain, and the humidity was under 40%. After flowering, there was 62 mm rainfall but it did not affect the extent of infection.

The head rot infection was significantly higher in the case of the genotypes sown on 30th April, the temperature during the flowering time of these genotypes was lower, the relative humidity was more then 60% all day and the rainfall was 40 mm, compared to the plots flowering before and afterwards. In the experimental area the rainfall was 0.5 mm and there were no hot days. The degree of head rot infection of the genotypes flowering this time was less than that of the genotypes flowering till mid July.

The observations have shown that the weather during flowering time had a huge influence on the head rot infection. Besides genotype sensitivity, the degree of infection was determined by favourable weather conditions for the germination of the ascospores. The failure of the artifical infection can be attributed to the high temperature during the period after infection. Many researchers claim that the optimal temperature for spores germination and infection is 16-25°C. In the experiments at more locations, the considerable differences in the genotype susceptibility were determined by the ecological conditions duing the flowering of a given genotype. Differences in infection percentage in nearly isogenic lines and their hybrids flowering at different dates, as well as those in performance trials of hybrids sown at two dates, showed a close significant correlation (r=0.84***) with the quantity of precipitation recorded from the beginning to the end of the flowering period.

In relation to combining ability, there was a significant difference with respect to general combining ability (GCA) in the mother lines and specific combining ability (SCA) between parental lines. There was one restorer line which had a good GCA to the head rot infection, and this line transmitted the resistance to its hybrids.

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Puccinia helianthi Schw., infecciones en híbridos comerciales en Argentina y su evolución durante dos décadas

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RESUMEN

La roya negra del girasol, causada por *Puccinia helianthi* Schw., puede reducir el rendimiento y calidad de híbridos de girasol. En la República Argentina se la reconoce como endémica a la región girasolera Norte (Chaco y Santa Fe). Sin embargo, en la campaña 2006/7 ocurrió una epifítia sin precedentes en la región girasolera Centro (sur de Córdoba, La Pampa y Norte de Buenos Aires) y Sur (sur de Buenos Aires), de temprana aparición y elevada intensidad. El objetivo del presente trabajo es caracterizar la interacción entre genotipos de girasol (cultivares comerciales) y ambientes de la región centro y sur, donde se presentó roya negra. De la caracterización de esta interacción se aportará valiosa información sobre la posible existencia de razas de *P. helianthi* en la República Argentina y su directa implicancia en los planes de mejoramiento y manejo de esta enfermedad. Los genotipos resistentes identificados fueron estables a través de los ambientes; cinco genotipos presentaron comportamiento variable entre ensayos. La falta de interacción cruzada entre GxA para la manifestación de roya negra sugiere la presencia en la región Centro y Sur de girasol de una única raza de *Puccinia helianthi*.

Palabras clave: *Helianthus annuus* enfermedades - híbridos - *Puccinia helianthi* - roya negra girasol.

ABSTRACT

Sunflower rust caused by *Puccinia helianthi* is an important disease of sunflower in Argentina with a potential for causing significant yield losses in susceptible hybrids. In Argentina it is most frequently encountered affecting sunflower in the northern area (Chaco and Santa Fe states), but during the 2006/07 growing season early and severe rust outbreaks occurred in the central states of Cordoba, La Pampa and Buenos Aires. The objective of this study was to characterize the interaction between commercial cultivars of sunflower in diverse localities where the epidemic was severe. It is important to know the existence of pathotypes of *P. helianthi* in a given area to improve the effective lifespan of sunflower commercial cultivars. Results indicated that the resistant sunflower genotypes identified were stable at the different localities. Lack of cross interaction between Genotypes x Environment for the rust sunflower disease revealed the presence of only one rust pathotype in the Central and South sunflower growing areas.

Key words: diseases - *Helianthus annuus* – hybrids – *Puccinia helianthi* – rust – sunflower.

INTRODUCCIÓN

La roya negra del girasol (RN), causada por *Puccinia helianthi* Schw., puede reducir el rendimiento y calidad de híbridos de girasol (Gulya et al., 1997). En la República Argentina (RA) se la reconoce como endémica a la región girasolera Norte (Chaco y Santa Fe). Sin embargo, en la campaña 2006/7 ocurrió una epifitia sin precedentes en la región girasolera Centro (sur de Córdoba, La Pampa y Norte de Buenos Aires) y Sur (sur de Buenos Aires), de temprana aparición y elevada intensidad (Huguet et al., 2007). El manejo de la enfermedad se basa en la utilización de genotipos con resistencia genética. Las fuentes de resistencia utilizadas son las provenientes de cruzamientos y selecciones de la "Mezcla Precoz" (Bertero de Romano y Norberto Vázquez, 2003) que dio origen a la variedad "Charata INTA" también ha dado origen a líneas importantes para el mejoramiento del girasol tales como: Pergamino 71/538 (INTA Pergamino), HA-R1 y HA-R4 (USDA, Fargo, ND, USA) y MP555 y MP557 (INTA Castelar). HA-R1 se utiliza como diferencial internacional para las razas de *Puccinia helianthi*, y HA-R4 para las razas de

Puccinia helianthi y *Plasmopara halstedii* (Gulya and Masirevic, 1995; Miller and Gulya, 1995). En la RA las infecciones de roya negra han variado considerablemente año a año, la evolución en las últimas dos décadas se observa en la Fig. 1.

Fig. 1. Evolución de la severidad de Roya Negra en la República Argentina entre los años 1982 y 2008. Evaluaciones realizadas sobre híbridos comerciales que participaron en los Ensayos Comparativos de Rendimientos de la Red Nacional de Girasol en las campañas 1982-1984 y 2006-08 y Ensayos de NUZEA-INTA Campañas 1992-98.

Entre los principales objetivos del la Red Nacional de Cultivares Comerciales de Girasol de INTA se destacan la caracterización del comportamiento sanitario de los genotipos. A partir de varios ensayos ubicados en las zona centro y sur se identificó a un grupo acotado de cultivares comerciales resistentes a la Roya negra (18 de 85 cultivares evaluados), como así también no fueron identificados cultivares con niveles altos de susceptibilidad (Huguet et al., 2007). Aproximadamente el 10% del total de los cultivares evaluados no fueron caracterizados por su comportamiento debido a la variabilidad entre ensayos. El objetivo del presente trabajo es caracterizar la interacción entre genotipos de girasol (cultivares comerciales) y ambientes de la región Centro y Sur, donde se presentó Roya negra. De la caracterización de esta interacción se aportará valiosa información sobre la posible existencia de razas de *P. helianthi* en la RA y su directa implicancia en los planes de mejoramiento y manejo de esta enfermedad.

MATERIALES Y MÉTODOS

Cincuenta y dos híbridos comerciales de girasol fueron evaluados en cinco ensayos con presencia de la enfermedad, ubicados en la zona sur y centro de producción de la RA. El diseño utilizado fue de tres bloques completos aleatorizados. En el estadio de fin de floración se evaluó la severidad de Roya negra (ASAGIR, 2002). Se realizó análisis de la varianza donde se estimó el efecto del genotipo (G), el ambiente (A) y la interacción GxA, todos estos factores fueron considerados como de efectos aleatorios (Procedimiento GLM, SAS, Institute, Cary, NC, USA). Se estimó los componentes de la varianza mediante el procedimiento RELM (SAS, Institute, Cary, NC, USA). Para la identificación de genotipos de comportamiento variable se realizó el análisis de interacción GxA adaptado por Massiero y Castellano (1991).

RESULTADOS

Se identificó el efecto de la interacción genotipo-ambiente (*P*=0,0001), del ambiente (*P*=0,0001) y el genotipo (*P*=0,001). Del total de la variabilidad fenotípica, la interacción representó el 12%, el genotipo el 82% y el ambiente el 6%. Los cultivares Paraíso 33 (Nidera sa, Junin, Argentina), Paihuén (El Cencerro, Coronel Suárez, Argentina), CF31 (Advanta semillas sa, Venado Tuerto, Argentina), PAN7031 y PAN7047 (PANNAR Argentina sa, Venado Tuerto, Argentina) presentaron comportamiento variable entre ensayos. Ninguno de estos híbridos presentó comportamiento cruzado a través de los ambientes, de las interacciones detectadas solo existió cambio de magnitud en niveles de Roya Negra. Los genotipos resistentes identificados fueron estables a través de los ambientes.

CONCLUSIONES

Las fuentes de resistencia de Roya Negra incluida en los híbridos comerciales de girasol de la RA son estables en ambientes del la región Centro y Sur de girasol.

La falta de interacción cruzada GxA para la manifestación de Roya Negra sugiere la presencia en la región Centro y Sur de girasol de una única raza de *Puccinia helianthi*. Son necesarios posteriores trabajos para confirmar y actualizar las mismas.

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Results regarding the influence of *in vitro* stress induced by the *Phomopsis helianthi* filtrate on some physiological indices and on sunflower oil quantity and quality

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ABSTRACT

During 1997-2005 at ARDI Fundulea, many experiments for *in vitro* testing and selection of some Romanian sunflower genotypes with tolerance to *Phomopsis helianthi* have been performed. Fourteen out of the 30 tested genotypes were selected for their good response to the *in vitro* culture. Following the treatment applied on MS culture medium supplemented with 150ml/l filtrate, and, on the basis of the results obtained regarding the leaf index, chlorophyll content, 1000-kernel weight, seed oil percentage and its composition, genotypes with increased resistance to this pathogen have been selected. The determinations were performed by the Minolta Chlorophyll meter (SPAD units) for chlorophyll contents, nuclear magnetic resonance for oil content, and gas-chromatography for fatty acid composition of the seed oil.

Key words: chlorophyll content – *in vitro* culture – *in vitro* testing and selection – *Phomopsis helianthi*.

INTRODUCTION

Phomopsis helianthi (Diaporthe helianthi), causal agent of stem canker, is one of the most important pathogens of sunflower in Europe. It can cause significant losses in yield (10±50%) and in oil content (10±15%) when the environmental conditions are favorable for disease development. Stem canker was noticed for the first time in Yugoslavia in 1980 and in Romania in 1981 (Vrânceanu et al., 1992; Vrânceanu, 2000). In 1994, the inocula of *Phomopsis* were present in all the areas where sunflower is grown (Vear et al., 1997).

Using *in vitro* screening, the goals of this study were to contribute to the knowledge regarding the influence of stress induced by *Phomopsis helianthi* filtrate on some Romanian inbred lines and to the identification of inbred lines with a high level of tolerance to the pathogen (Raducanu et al., 1997a, 1997b; Raducanu, 1998; Hagima and Raducanu, 1998; Raducanu et al., 2002; Raducanu and Moraru, 2003; Raducanu et al., 2005).

MATERIALS AND METHODS

For *in vitro* testing to *Phomopsis helianthi* pathogen, a total of 14 Romanian inbred lines were used. As explants, immature embryos collected 10 days after pollination were inoculated on an MS medium, supplemented with 150ml/l *Phomopsis helianthi* filtrate and incubated for 21 days at 27ºC, 12/12 light/dark. After this period, phenotypically normal plants were transplanted into pots with a mixture of heavy soil and sand in 1:1 proportion and they were grown under controlled conditions until maturity.

On these plants, under different stages of vegetation, the following data were recorded: leaf index, chlorophyll content, TKW (thousand kernel weight), seed oil content and its composition.

The determinations were performed by the Minolta chlorophyll meter (SPAD units) for chlorophyll content, nuclear magnetic resonance (NMR) for oil content, and gas-chromatography (Shimadzu-GC-14B) for seed oil fatty acid composition.

The fatty acids were analyzed according to the conventional method (Schulte and Weber, 1989). The transesterification of triglycerides to fatty acid methyl esters was performed with trimethylsulfoniumhydroxid (TMSH). A capillary column (25 MX 0.32 MM ID) of 25m length on a Shimadzu gas chromatograph with flame ionization detector (FID) was used. Injector and detector were kept at 270 and 280 ºC, respectively. The carrier gas was nitrogen, with a flow rate of 20 ml/min. To calculate the total area of the peaks, an electronic integrator was used. The area of each fatty acid peak was expressed as a percentage of the total area.

The leaf index was calculated by the following formula:

L x l x 0.66 (L=length; l=width; 0.66=correction coefficient).

RESULTS

The ANOVA analyses and effects of *Phomopsis helianthi* filtrate on leaf index, chlorophyll content, 1000-kernel weight, seed oil percentage and its composition are presented in Tables 1 to 9.

Table 1. ANOVA of the leaf area of some Romanian sunflower genotypes after *Phomopsis helianthi* treatment

Source of variation	SS	DF	MS	F value
Genotypes (A)	34198.51	13	2630.65	60.049***
A error	1139.02	26	43.808	-
Treatment (B)	2584.12	1	2584.12	67.034***
A x B	4020.47	13	309.267	8.022***
B error	1079.37	28	38.549	

Table 2. The effects of *Phomopsis helianthi* filtrate on leaf area in some Romanian sunflower genotypes

NO.	**Genotypes**	Average leaf area (cm^2 / genotype)			
		Control		treatment	
		Average	Difference from average[1]	Average	Difference from control[2]
1	LC 4001	17.366	-16.125^^	22.800	-10.691
2	LC 4002	12.933	-20.558^^^	162.000	-17.291**
3	LC 4005	27.566	-5.925	25.466	-8.025
4	LC 4006	41.800	8.308	26.000	-6.891
5	LC 4007	23.866	9.625	14.100	19.391***
6	LC 4010	62.166	28.675^^^	44.000	10.508
7	LC 4011	36.166	2.675	34.833	1.341
8	LC 4016	14.600	-18.891^^	15.166	-18.325**
9	LC 4018	37.433	3.914	16.100	-17.391**
10	LC 4019	55.633	22.141^^^	31.833	-1.658
11	LC 4020	110.666	77.175^^^	62.566	29.075***
12	LC 4022	16.266	-17.225^^	11.533	-21.958***
13	LC 4024	68.300	34.802^^^	49.866	16.375***
14	LC 4025	21.766	-11.725^	20.166	-13.325*
	Average	39.037		35.216	

[1]^, ^^, ^^^ Significantly different from average for P<0.05; P<0.01; P<0.001, respectively.
[2]*, **, *** Significantly different from control for P<0.05; P<0.01; P<0.001, respectively.

Table 3. ANOVA of the chlorophyll content in some Romanian sunflower genotypes after *Phomopsis helianthi* filtrate treatment

Source of variation	SS	DF	MS	F value
Genotypes (A)	3418.58	13	262.968	19.575***
A error	349.271	26	13.433	-
Treatment (B)	460.615	1	460.615	43.124***
A x B	593.004	13	45.615	4.271***
B error	299.075	28	10.681	

Table 4. The effects of *Phomopsis helianthi* filtrate on chlorophyll content in some Romanian sunflower genotypes

NO.	**Genotypes**	Average chlorophyll content (SPAD/units)			
		Control		Treatment	
		Average	Difference from average[1]	Average	Difference from control[2]
1	LC 4001	24.500	-5.508	28.500	-1.508
2	LC 4002	22.433	-7.575	17.600	-12.408**
3	LC 4005	26.633	-3.375^^	20.766	-9.241**
4	LC 4006	30.233	0.225	23.866	-6.141**
5	LC 4007	30.400	0.391^^^	25.400	-4.608
6	LC 4010	35.800	5.791	36.000	-6.141**
7	LC 4011	35.800	5.791	39.866	9.858**
8	LC 4016	38.800	8.917^^	31.933	4.925
9	LC 4018	42.733	12.725	31.566	1.558
10	LC 4019	42.866	12.725	36.800	6.792**
11	LC 4020	42.733	12.725	31.266	-1.258
12	LC 4022	26.166	-3.841^^^	24.266	-5.741
13	LC 4024	38.500	8.492^^^	20.800	-9.408
14	LC 4025	22.866	-7.142^	18.900	-11.108***
	Average	32.890		27.680	

[1]^, ^^, ^^^ Significantly different from average for P<0.05; P<0.01; P<0.001, respectively.
[2]*, **, *** Significantly different from control for P<0.05; P<0.01; P<0.001, respectively.

Table 5. ANOVA of the TKW in some Romanian sunflower genotypes after *Phomopsis helianthi* filtrate treatment.

Source of variation	SS	DF	MS	F value
Genotypes (A)	819.046	13	63.003	21.632***
A error	75.726	26	2.912	-
Treatment (B)	150.934	1	150.934	117.678***
A x B	77.645	13	5.972	4.657***
B error	35.912	28	12.826	

Table 6. The effects of *Phomopsis helianthi* filtrates on TKW in some Romanian sunflower genotypes

NO.	**Genotypes**	Average TKW(g)			
		Control		Treatment	
		Average	Difference from average[1]	Average	Difference from control[2]
1	LC 4001	13.766	-0.408	13.533	-0.640
2	LC 4002	18.733	4.599^^^	18.366	4.192**
3	LC 4005	20.966	6.792^^^	18.900	4.726***
4	LC 4006	21.223	7.059^^^	15.600	1.426
5	LC 4007	12.300	-1.874	10.366	-3.807**
6	LC 4010	16.800	2.626^	10.600	-3.574**
7	LC 4011	17.566	3.392^^	15.466	1.292
8	LC 4016	14.666	0.492	13.033	-1.106
9	LC 4018	14.000	-0.174	10.933	-3.240**
10	LC 4019	14.033	-0.140^^^	10.466	-3.707
11	LC 4020	18.933	4.759^^^	12.933	-1.240
12	LC 4022	12.700	-1.474	11.100	-3.074**
13	LC 4024	11.700	-2.474^	9.400	-4.744***
14	LC 4025	9.700	-4.474^^^	8.966	-5.207***
	Average	14.459		12.833	

[1]^, ^^, ^^^ Significant different from average for P<0.05; P<0.01; P<0.001, respectively.
[2]*, **, *** Significantly different from control for P<0.05; P<0.01; P<0.001, respectively.

Table 7. ANOVA of the oil content in some Romanian sunflower genotypes after *Phomopsis helianthi* filtrate treatment

Source of variation	SS	DF	MS	F value
Genotypes (A)	1302. 19	13	100.169	48.209***
A error	54.022	26	20. 778	-
Treatment (B)	516.031	1	512.031	160.344***
A x B	195.958	13	15.073	4.684***
B error	90.111	28	3.218	

Table 8. The effects of *Phomopsis helianthi* filtrate on the oil content in some Romanian sunflower genotypes

NO.	**Genotypes**	Average oil content (%)			
		Control		Treatment	
		Average	Difference from average[1]	Average	Difference from control[2]
1	LC 4001	29.333	- 4.972^^^	27.200	- 7.104***
2	LC 4002	37.700	3.395^	27.600	6.671***
3	LC 4005	39.266	4.961^^^	33.633	0.671
4	LC 4006	39.200	4.895^^^	36.333	2.028
5	LC 4007	27.200	- 7.104^^^	29.033	- 5.271***
6	LC 4010	41.900	7.595^^^	36.000	1.695
7	LC 4011	29.933	- 4.371^^^	27.366	-6.938***
8	LC 4016	36.233	1.928	28.433	-5.871***
9	LC 4018	38.033	3.728^^^	33.333	1.005
10	LC 4019	41.100	7.795^^^	38.500	4.195*
11	LC 4020	43.866	9.561^^^	35.400	1.095
12	LC 4022	38.866	4.561^^^	30.000	- 4.305*
13	LC 4024	33.700	- 0.538	30.133	-4.171*
14	LC 4025	37.566	3.261^	34.033	-2.272
	Average	36.706		31.928	

[1]^, ^^, ^^^ Significantly different from average for P<0.05; P<0.01; P<0.001, respectively.
[2]*, **, *** Significantly different from control for P<0.05; P<0.01; P<0.001, respectively.

Table 9. Retention time for fatty acids from the standard solution

No. peak	Retention time (min.)	Fatty acid (formula)	The fatty acid
1	12.55	C 16:0	Palmitic acid
	17.48	C 18:0	Stearic acid
2	17.48	C 18:1	Oleic acid
3	20.36	C 18:2	Linoleic acid
4	22.65	C 18:3	Linolenic acid

DISCUSSION

The results obtained by gas chromatography underlined the fact that of the five fatty acids from sunflower oil, oleic acid decreases after treatment in all genotypes, excepting the LC 4010 line. At the same time, the linoleic acid percentage increases after treatment in nine out of the tested lines. We positively noticed the fact that the linolenic acid, which reduces oil stability, was detected only in three genotypes but in very small quantities. ANOVA for the leaf index emphasized a very different behavior of the tested lines, with significant positive or negative differences between genotypes, depending on both tolerance degree to disease and response to the *in vitro* culture. Eight genotypes in which the leaf area was not diminished by the treatment as compared with the control have been identified.

As regards the chlorophyll content, it was ascertained that for all tested genotypes, at the treatment variant, the average/variant was diminished with 5.2 SPAD units vs. the control.

In variants treated with the filtrate, TKW was drastically diminished in seven out of the 14 genotypes. The oleic acid content showed a higher decrease in comparison with the control in all lines excepting the LC 4010 line.

ACKNOWLEDGEMENTS

The authors thank Prof. Dr. h.c. A.V. Vrânceanu for providing sunflower lines and scientific support.

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The impact of the new races of broomrape (*Orobanche cumana* Wallr.) parasite in sunflower crop in Romania

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ABSTRACT

The pathogenic composition of broomrape populations has changed over the years, slowly at first, then rapidly in Eastern Europe, Turkey and Spain. In Romania there are three important areas infested with broomrape (*Orobanche cumana* Wallr.), which are different in their infestation degree and presence of different virulence groups. A new highly virulent population of broomrape has attacked sunflower in Romania in 2005. Many commercial hybrids from different companies lost their resistance to this parasite. In the sunflower germplasm collection of Fundulea Institute has been identified a restorer line, AO-548, fully resistant to this new broomrape population. Since this line could be used directly as a parent to produce commercial hybrids, as well as a source of resistance to broomrape in sunflower breeding programs, the inheritance of resistance to the new population of *Orobanche cumana* was studied. Using the cytoplasmic male sterile inbred line AD-66, very susceptible to this population of the parasite, as a female parent, progenies of the cross with AO-548: F_1, F_2 and BC_1 to both parents, as well as the parental lines, were analysed for their reaction to this broomrape population. The results of the observed resistant/sensitive plants vs expected ratio (15:1 and 3:1) indicated that the inheritance of resistance to broomrape in the line AO-548 is conferred by two independent dominant genes.

Key words: broomrape – inheritance – resistant – sensitive – sunflower – virulence.

INTRODUCTION

Broomrape (*Orobanche cumana* Wallr./*Orobanche cernua* Loefl.) attacks sunflower crop in parts of Europe, the Near East and China (Vrânceanu, 2000). In the last few years the parasite migrated to Western Europe (in 2007 this parasite attacked sunflower crops in France).

Sunflower breeding for resistance to this parasite was started by Pustovoit in 1912 at VNIIMK Institute in Krasnodar, Russia (Sackston, 1992). Using the method of growing all available sunflower material in heavily infested plots, Pustovoit had, by 1927, selected strains with up to 99% of resistant plants (Pustovoit, 1967). By that year, however, previously resistant sunflowers were succumbing to what turned out to be a complex of new races of broomrape. Repeated selection produced lines resistant to the new race complex. Pustovoit did not try to determine the nature of genes controlling this resistance.

Some early Soviet sunflower breeders did study genetic ratios. Meister (1936) reported that resistance to broomrape was inherited as a dominant character, and referred to simple segregation ratios. Later, scientists found that resistance to broomrape races A and B derived from the perennial *H. tuberosus* L. was controlled by a single simply inherited dominant gene (Burlov and Kostyuk, 1976; Pogorletsky and Geshele, 1976).

The virulence of the parasite populations has changed over the years. Vrânceanu et al. (1980) reported on five virulence groups (races or groups of races) of broomrape encountered in Romania, and five types of effective resistance against the respective groups. These investigators set up a series of differentials permitting identification of the five virulence groups, although not the individual races of the pathogen, as each resistance type was effective against a specific race group. The results of complex crossing studies demonstrated a gene-for-gene relationship between virulence groups in the broomrape and resistance in sunflower. They successfully introduced gene *Or5*, which gives resistance to all five race groups, into inbred lines with high combining ability that were the parents of existing or prospective hybrids, and released resistant hybrids.

The different reactions of resistance in varieties of different pathogen sensitivities in sunflower have been reported in recent years. Ciriaev (1987) reported the oligogenic resistance controlled by two genes. Domínguez (1996) has identified the line R-41 having resistance to broomrape controlled by two independent dominant genes. Melero-Vara and Fernández-Martínez (2004) have reported two independent recessive genes for resistance to broomrape.

In Romania, the race F was identified in 1997, as well as the gene (one dominant gene) conferring resistance to this race (Pacureanu-Joita et al., 1998)

Melero-Vara et al. (1989), and other authors quoted works indicating the chemical control of broomrape, but agreed that genetic resistance is the most important method for controlling the parasite. IMI resistance sunflower hybrids may be another way to control it.

This paper presents the results obtained in identifying a new race of *O. cumana* in sunflower crop in Romania, as well as a source of resistance and its inheritance.

MATERIALS AND METHODS

Different sunflower hybrids were tested in fields naturally infested with broomrape, in two important areas in Romania. The investigators set up a series of differentials permitting identification of different virulence groups of the parasite. The different sunflower genotypes (lines and populations) were tested for resistance to the broomrape attack, with a view to identifying new sources of resistance to the most virulent populations of the parasite. Crosses between sensitive lines and new resistant ones were performed in order to establish the inheritance of the genetic resistance. The testing was performed under artificial inoculation using broomrape seeds from two infested areas in Romania. The Panchenko (1975) method was used for testing the artificial infestation conditions.

RESULTS

In Romania, more than 55% of the sunflower cultivated area is infested with broomrape. There are three important areas with a high infestation degree and the presence of different virulence groups (Fig. 1). The high infestation degree in the first area, situated near the Black Sea, is given by race F, and race G has also been identified in this area. In the second area, situated in Ialomita-Braila, race F is well represented.

Fig. 1. The main areas of broomrape infestation in sunflower crop in Romania**.**

In recent years, the parasite *Orobanche cumana* has developed new races in a short time in sunflower crops in Romania, compared to the first period (Fig. 2). So, if 15 years have passed since the identification of races A and B until race E appeared, as well as from race E to race F, the races G and, may be, H, have appeared in a shorter time and spread quickly over a large area.

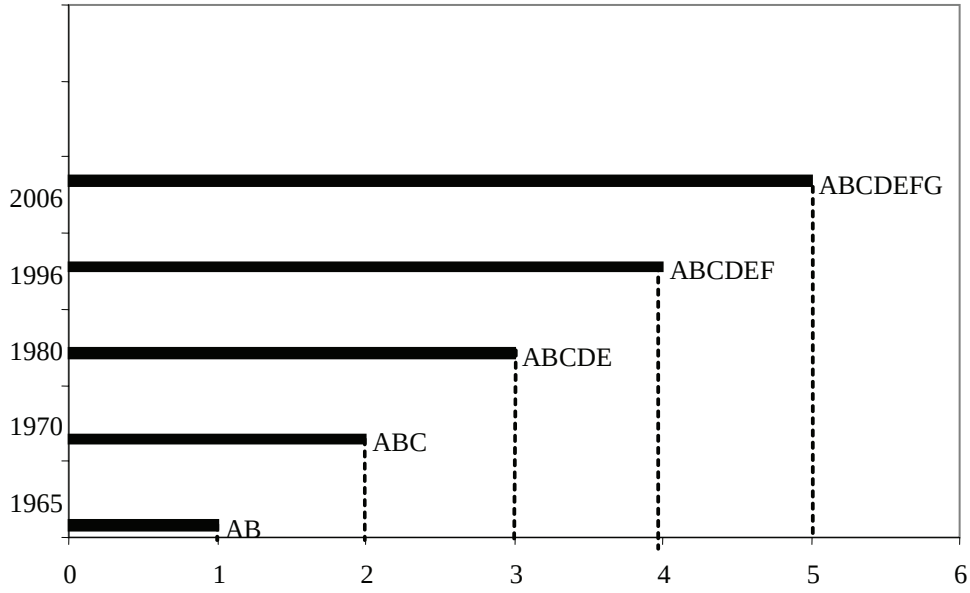

Fig. 2. The evolution of the broomrape races in sunflower crop in Romania.

In 2006, in a sunflower crop cultivated in Tulcea area, near the Black Sea, some of the hybrids resistant to the race F lost their resistance, being infested at a high percentage (Fig. 3). The hybrids having resistance to the races G or H , were fully resistant.

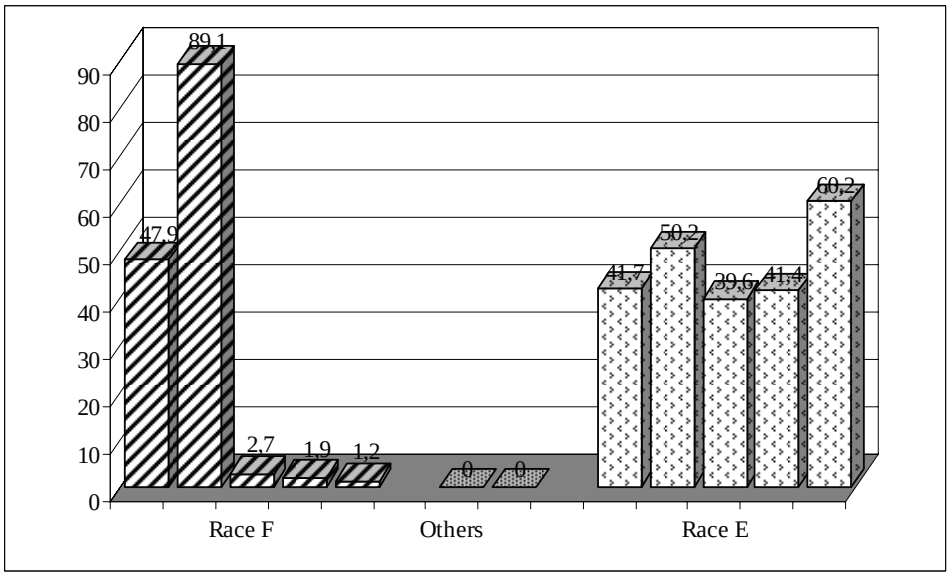

Fig. 3. The behaviour of some sunflower cultivated hybrids in an infested area with broomrape, Romania, 2006

Using broomrape seeds collected from this area, different inbred lines used as differentials for the races E, F and G have been tested, as well as some resistant hybrids, under artificial infestation conditions in the greenhouse. At the same time, the same genotypes were tested, using the broomrape seeds collected from Ialomita-Braila area. The results (Table 1) showed that the differential for the race F, the inbred line LC 1093, lost its resistance in Tulcea area, having full resistance in the Braila-Ialomita area. The inbred line AO-548 is resistant in both cases. The same behaviour was shown by the hybrids having as parents these two lines (Favorit and Daniel).

Table 1. The reaction to the broomrape attack in different sunflower genotypes (Fundulea, 2006-2007)

Sunflower genotype	Reaction to the broomrape races	Source of broomrape			
		Ialomita -Braila		Tulcea - Constanta	
		Number of infested sunfl. plants	Infestation degree (%)	Number of infested sunfl. plants	Infestation degree (%)
P-1380-2	E - A	10/10	41.7	10/10	77.4
LC-1093	F - A	0/10	0.0	3/10	1.9
Kd-3-2	F - A	0/10	0.0	2/10	1.2
AO-548	G - A	0/10	0.0	0/10	0.0
Od-832-2b	F - A	0/10	0.0	3/10	1.8
Favorit	F - A	0/10	0.0	5/10	2.3
F-225	F - A	0/10	0.0	7/10	2.9
PR64A83	(E)F - A	8/10	19.7	10/10	73.1
PR64A71	(G)H	0/10	0.0	2/10	0.9
Daniel	G - A	0/10	0.0	0/10	0.0

The test conducted under natural infestation conditions in Braila area, using some differentials for the races E, F and G, confirmed that race G was still not present in this area (Table 2).

Table 2. The reaction of the broomrape attack to sunflower under natural infestation conditions – Braila, Romania, 2007

Sunflower genotypes	Reaction to the broomrape races	Infestation degree (%)
P-1380-2	E - A	49.7
LC-1093	F - A	0.0
O-7455	E - A	8.7
Sel-10481	E - A	19.7
Kd-3-2	F - A	0.0
AO-548	G - A	0.0
AD-66	Sensitive	69.7

All tests for resistance to broomrape, which were performed in 2006 and 2007, have shown that, in all the infested areas, the restorer inbred line AO-548 was fully resistant. This line was crossed with AD-66, a CMS line, in order to establish the inheritance of resistance in this restorer line. The F_1 generation was obtained in the field, after which, the crosses and selfings to obtain BC_1 and F_2 generations were carried out under artificial infestation conditions, in pots, in the greenhouse. The plants were kept in pots until maturity, after that they were uprooted and their roots carefully washed to observe any established broomrape nodules. The plants free of nodules or stalks in the roots were considered resistant. The observed ratio of resistant and susceptible plants, in each generation, as well as the goodness of fit of observed – expected ratios are shown in Table 3. The F_2 progeny segregated at a ratio of 15:1 (resistant:susceptible), whereas the BC1 on the susceptible parent, AD-66, segregated according to 3:1

(resistant:susceptible), indicating that resistance to *Orobanche cumana* in AO-548 line is conferred by two single genes with independent action.

Table 3. Broomrape resistant and susceptible sunflower plants in the parental, F1, F2 and BC1 generations of crosses between AD-66 (cms) and AO-548, and the goodness of fit of observed (vs) expected ratios

Material (generations)	Plants		Expected ratio	P%
	Resistant	Susceptible		
AD-66 (P1)	-	15	-	
AO-548 (P2)	15	-	-	
F1	15	-	-	
F2	189	14	15:1	80-90
BC1 (AD-66)	50	15	3:1	50-70
BC1 (AO-548)	65	-	-	

DISCUSSION

The parasite *Orobanche cumana* has became more and more dangerous for the sunflower crop in Romania. In 2006, most resistant sunflower hybrids cultivated in an infested area with this parasite were attacked, some of them at a high attack degree (80%).

The behaviour of some sunflower genotypes regarding resistance to broomrape, under natural and artificial infestation conditions, has shown that the parasite virulence is increasing. The inbred line AO-548 was fully resistant.

The inheritance of resistance to broomrape in AO-548 line is conferred by two independent dominant genes. This line has a good combining ability, being used directly in the obtention of commercial hybrids.

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Distribution and dissemination of sunflower broomrape (*Orobanche cumana* Wallr.) race F in Southern Spain

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ABSTRACT

Sunflower broomrape (*Orobanche cumana* Wallr) is the most important problem in the sunflower crop in Southern Spain. The dissemination and dispersion of the virulent race F of this parasite has been evaluated during the last 10 years. The sunflower crop acreage in Southern Spain was divided into 8 large areas, where the presence and intensity of broomrape race F in sunflower fields was evaluated in the years 2001, 2003, 2005 and 2007. In two of these areas, irrigation lands and Antequera, broomrape race F was not found. In one area, Jerez, the progress of infection and dispersion showed a very slow advance. In the Seville-Huelva and Villamartín areas, the presence of race F accounted for 79% and 69% respectively, although without causing serious damage to the sunflower yield. In the other three areas studied, Córdoba, Écija and Carmona, the broomrape race F infestation was very high, causing important damage to sunflower production.

Key words: broomrape race F – broomrape distribution – *Orobanche cumana.*

INTRODUCTION

Sunflower broomrape (*Orobanche cumana* Wallr.) is a major disease in southern Europe, the Black Sea Region, Ukraine and China (Sackston, 1992; Parker, 1994). This obligate holoparasitic angiosperm attaches itself to the roots of infected plants depleting them of nutrients and water and causing important yield losses. In cases of severe infections, these losses can reach up to 50% and near 100% of sunflower production. Throughout sunflower broomrape history, different races of this holoparasite have developed in infested areas where sunflower has been traditionally cultivated (Vrânceanu et al., 1980).

In Spain, the broomrape was detected for the first time in Toledo province in 1958, attacking confectionery sunflower (González Torres et al., 1982). At the beginning of the 1980's the first broomrape infections in oil sunflower were noticed in Cuenca and El Coronil (Sevilla). In the early 1990's, in the surroundings of Écija (Sevilla) and La Almarcha (Cuenca), all the available commercial hybrids at that time were affected. Race studies showed that these infections were caused by broomrape race E (Melero-Vara, 1999; Domínguez, 2004). After 1993, several sunflower hybrids resistant to this race, most of them carrying the Or5 gene as the resistance source, were developed and commercialized. These hybrids are still being grown currently in most of the areas where sunflower is sown in Spain.

In 1995, a serious broomrape attack on resistant hybrids carrying the Or5 gene was detected for the first time in Spain near Écija (Sevilla) (Alonso et al., 1996). This new broomrape pathotype was determined as race F (Alonso et al., 1996). From that moment until the end of the 1990's, several spots of race F broomrape have been detected in Sevilla, Córdoba and Cuenca provinces (Domínguez, 1999). We report here a study carried out to evaluate the dissemination and dispersion of broomrape race F in the South of Spain during the present decade.

MATERIALS AND METHODS

The sunflower crop acreage in Southern Spain was divided into 8 large areas, where the presence and intensity of the broomrape virulent race F in sunflower fields was evaluated for four years. These areas are shown in Fig. 1 and Table 1. In each selected area, several sunflower fields were chosen at random. The sampling method consisted of selecting roads and ways including each area. Then the sunflower fields close to them located at a distance of 2-3 km. between each field were visited and evaluated. The total numbers of evaluated fields were 453 in 2001, 629 in 2003, 602 in 2005 and 565 in 2007.

For each tested field the following ranks of attack were considered:

- Large plots infected with race F: these are the sunflower fields with presence of plots larger than 1 Ha, highly infected by broomrape and associated with high yield losses.

- Small plots infected with race F: these are sunflower fields with more than 30-50% of infected sunflower plants with a low number of broomrapes per plant or with presence of small plots with a high broomrape infection. The incidence in sunflower yield is low.
- Presence of race F: these are the sunflower fields with a 10-25 % of infected sunflower plants with a low number of broomrapes per plant. The incidence in sunflower yield is practically void.
- Broomrape absence: sunflower fields with an under 10% broomrape infection in sunflower plants. In 2001 and 2003, these fields were clearly associated with non broomrape-infected fields. However, with the presence from 2005 onwards of sunflower race F-resistant hybrids in some studied areas, the absence of broomrape could be associated either with a non infected field or with a race F-resistant sunflower hybrid. In 2005 and 2007 assessments, the classification of a field with broomrape absence was assigned to one category or another by two methods. The first was a direct one, by obtaining information from the farmer about the hybrid sown. The second was a non direct one, on the basis of the information supplied by commercial staff of seed companies.

RESULTS AND DISCUSSION

The evaluation of the evolution of sunflower broomrape race was carried out in Andalusia (Southern Spain). The sunflower crop in this region accounts for approximately 45% of the total sunflower production in Spain, also being the area in which the mean performances are higher. Moreover, Andalusia is the region where broomrape race F has spread most, causing large losses in sunflower production. In order to study the evolution and dispersion of race F of broomrape, the sunflower growing area was divided into 8 large areas (Fig. 1), basically considering their record of the presence of former broomrape races. Table 1 shows for each area the surface taken up by sunflower in the four years of the study.

Fig. 1. Distribution of sunflower crop in Andalusia, subdivided into 8 large areas.

The results of the evaluation showed that throughout these last years, there were only two areas (E and G) where broomrape race F was not found in any field. This may be due to different reasons. In area E, irrigated land, the sunflower is a minor crop that is sown in rotation with corn and cotton. This area is relatively close to areas with a large broomrape presence, and, therefore, the broomrape seed dissemination by short distance movement, wind, water, tillage operations or infested farm equipment (Eplee et al., 1998), could be possible. The absence of *Orobanche* race F in this area could be due to the fact that both cotton and corn are trap crops, that promote the germination of broomrape seeds but without starting any infection in these crops (Rodríguez-Ojeda et al., 2001), resulting in the reduction of broomrape inocula in the soil. The second area with the absence of broomrape was area G, Antequera. This was probably due to the fact that this area is isolated from the rest of sunflower crop areas by large olive groves.

Table 1. Sunflower areas evaluated in Andalusia, location name and number of hectares cultivated in each area during 4 years.

Area	Location name	2001	2003	2005	2007
A	Sevilla-Huelva	28,381	26,822	18,619	20,463
B	Carmona	47,818	47,494	25,125	36,834
C	Écija	47,818	47,494	25,125	36,834
D	Córdoba	50,792	50,822	23,780	36,854
E	Irrigated land	28,189	28,045	15,934	21,722
F	Villamartín	44,339	44,002	24,394	34,276
G	Antequera	19,473	17,050	10,612	10,743
H	Jerez-Arcos	34,582	34,061	26,615	27,574
Total		301,392	295,788	170,204	225,300

The six remaining studied areas can be divided into 2 groups (Fig. 2 and Fig. 3). The areas of Écija, Córdoba and Carmona, C, D and B, respectively, where at present the broomrape race F is considered to be a serious problem for the sunflower crop, and the areas including Villamartín, Jerez and Sevilla-Huelva, F, H and A, respectively, where broomrape race F has been detected later and could be a potential problem for sunflower crop within the next years.

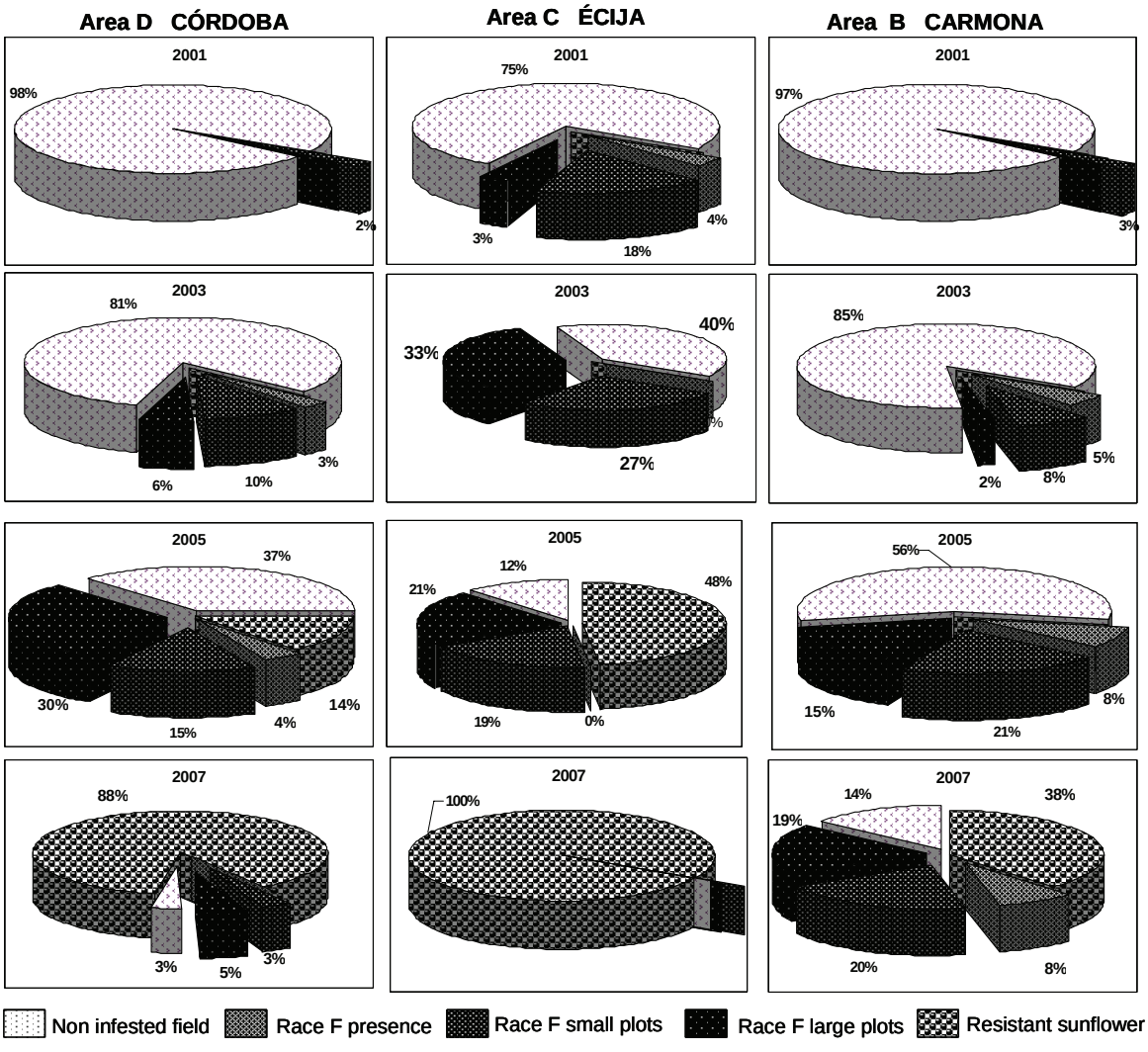

Fig. 2. Distribution of broomrape race F in Córdoba, Écija and Carmona areas during the years 2001, 2003, 2005 and 2007.

The first spots of race F were detected near Écija in the province of Sevilla in 1995 (Alonso et al., 1996). Within the following years, other small spots of this race appeared in new sunflower fields in a radius of about 20 km round Écija (Domínguez, 2004). In 2001 the fields affected by race F reached 25%, with large plots infested in 18% of the cases (Fig. 2). The spreading of race F in this area has been relatively slow, especially if compared with the broomrape race E evolution, which took place in the early 1990's, when the period from the appearance of the first fields with broomrape race E until its presence in over 80% of the fields, was only 4-5 years. From 2001, there has been a constant spread of race F in this area, from a complete absence of broomrape race F in 75% of the fields in 2001 to 40% in 2003, 12% in 2005 and, finally, with the whole sunflower cropped area sown with race F resistant hybrids in 2007 (Fig. 2).

The epidemic rate of growth in the Carmona area has followed a similar pattern to the one in Écija, although with a later appearance of the first race F infections. On the contrary, even though the presence of the first disease spots in Córdoba was simultaneous to that in Carmona, the spreading of race F towards Córdoba has been much faster than in the two previous areas (Fig. 2). One of the most important vectors in broomrape dissemination is the machinery movement among the different growing areas (Eplee et al., 1998). The higher rate of growth of race F in Córdoba may be due to the movement of combine-harvesters from Écija to the Córdoba area, where the harvest is up to 10-15 days later with regard to Écija.

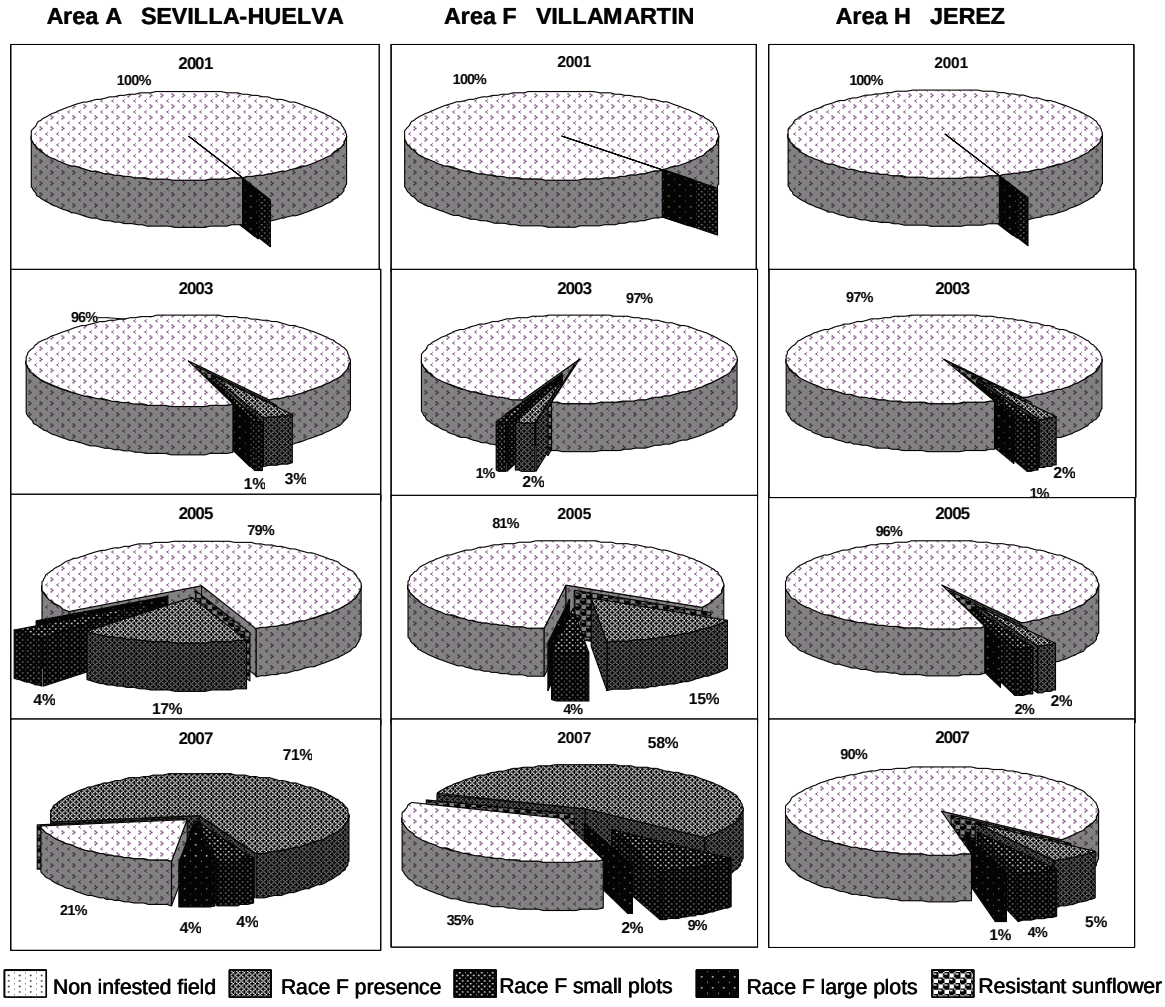

Fig. 3. Distribution of broomrape race F in Seville-Huelva, Villamartín and Jerez areas from 2001 to 2007.

The areas A and F, corresponding to Sevilla-Huelva and Villamartín, showed a similar behaviour regarding the appearance and dispersion of race F both in space and time. In both cases the first infections were detected in 2003, and four years later, the percentage of infected fields reached 79% and 69%, respectively (Fig. 3). It is worth mentioning that in these areas the infection with race F is present in many sunflower fields with 10-25 % of infected plants and a low broomrape number per plant, in contrast to B, C and D areas, where, in the first epidemic stages, the broomrape appears in small plots with a high broomrape infection. This fact may be due to multi-infestations with broomrape seed from the most infected areas (Écija and Carmona) through the combine-harvester movement. In areas A and F the sunflower harvest is carried out around 10 and 15 days later than in B and C areas, with the resulting machinery movement in this direction during harvest.

In the H area, around Jerez, the race F appearance took place in 2003 and with a similar intensity to that of A and F areas. Nevertheless, the rate of growth has been much lower and, in 2007, this race was present in only 10% of the sunflower fields. This situation is similar to that observed in the early 1990's regarding broomrape race E dispersion, when Jerez was the area in which broomrape appeared the latest in comparison to the rest of Andalucía and the spread was quite slow (unpublished results). The reason why broomrape presence and its expansion in the Jerez area is slower than in other sites, both for race E and race F, is unknown, at least by the authors.

The distribution knowledge and the broomrape F race rate of growth in a large area such as Southern Spain, with a sunflower surface of over 250000 has in recent years, may be considered as a model for designing strategies both for farmers and plant breeders. For the former, so that they can prevent broomrape seed dispersion among farms. For the latter, to be able to design alternative systems in order to fight the damage caused by broomrape in sunflower crop. This could be especially interesting in Andalusia, where the presence of a more virulent race (race G) which attacks race F resistant hybrids (Molinero-Ruiz and Melero-Vara, 2005) has already been reported.

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Development of resistance to insect pests attacking the stem and head of cultivated sunflower in the central and northern production areas of North America

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ABSTRACT

There is a need to provide successful and economical management tools for the sunflower producer to reduce losses from the spectrum of insect pests that attack the crop in the major production regions. The use of plant resistance can be a useful strategy in a long-term integrated pest management approach for crop protection. The goal of this project was to investigate host plant resistance as a potentially valuable management resource and screen sunflower accessions, interspecific crosses, and lines for those having reduced seed damage from larval feeding by the sunflower moth, red sunflower seed weevil, and banded sunflower moth and reduced densities of sunflower stem weevil larvae in the stalks. Trials were conducted in the central and northern Plains of the U.S. to screen germplasm in the areas where the different insects have caused economic losses. The discovery of germplasm that has lower insect damage can provide the seed companies with breeding material to be incorporated into hybrids targeted to locations where specific insect problems occur. A long-term goal is to identify germplasm with resistance or tolerance to more than one insect pest. The 2005 and 2006 trials revealed that the most resistant lines had a 70-90% reduction in weevil or moth seed damage or numbers of weevil larvae in stalks compared to the most susceptible lines evaluated. After each year of testing, lines with low damage have been retested to confirm their resistance to attack. Trials were conducted again for all four insect pest species in 2007.

Key words: banded sunflower moth – germplasm – pest management – red sunflower seed weevil – sunflower moth –sunflower stem weevil.

INTRODUCTION

The major insect pests attacking cultivated sunflower include the sunflower stem weevil, *Cylindrocopturus adspersus* (LeConte), the sunflower moth, *Homoeosoma electellum* (Hulst) (Lepidoptera: Pyralidae), the red sunflower seed weevil, *Smicronyx fulvus* LeConte (Coleoptera: Curculionidae), and the banded sunflower moth, *Cochylis hospes* Walsingham (Lepidoptera: Tortricidae). (Charlet et al., 1997; Knodel and Charlet, 2007). Strategies to reduce crop losses for these pests have concentrated on insecticidal control, but host-plant resistance would provide producers with a sustainable integrated pest management approach for crop protection with lower input costs.

The sunflower stem weevil has caused yield losses in North Dakota, Colorado, Kansas, and Texas (Charlet et al., 1997; Armstrong, 1996; Charlet et al., 2002). Mature larvae overwinter in sunflower stalks and adults emerge in early summer. After mating, females deposit eggs in the stem at the base of the plant. Weevil larvae feed inside the stalk, descending to the lower portion of the stalk or root crown by late August. Larvae construct overwintering chambers by chewing cavities into the stem cortex. High larval populations in a plant can weaken the stem through pith destruction, tunneling, or overwintering chambers, causing it to break at soil level resulting in a loss of the sunflower plant prior to harvest. Stalk breakage is most severe during drought stress or when high winds occur as plants are drying prior to harvest (Charlet, 1987; Knodel and Charlet, 2002).

The sunflower moth causes yield losses to cultivated sunflower in the southern and central Plains. Larvae overwinter in the soil in Texas and adults are carried on northerly winds to the central and northern Plains. Female moths deposit eggs in blooming sunflower heads. Larvae feed and develop in the sunflower head, destroying seeds and reducing oil content. Feeding damage in the head may provide an entrance site for the Rhizopus head rot fungal pathogen. Larvae exit the seed when mature and drop into the soil to overwinter (Rogers, 1978, 1992; Charlet et al., 1997).

The banded sunflower moth has been a persistent pest of sunflower in the northern Plains and populations also are present in the central Plains. Adults emerge from the soil in mid-July and are present in the field until mid-August. Adults congregate in field margins on weeds or adjacent crops during the day and then fly into the sunflower field in the evening. Females lay eggs on the outside of the bracts of the sunflower head and larvae feed on the florets, developing seed, and mature seeds. After completing development, larvae drop from the heads and spin cocoons in the soil and overwinter as mature larvae (Charlet and Gross, 1990; Charlet et al., 1997).

The red sunflower seed weevil is a pest of cultivated sunflower in both North and South Dakota, but is also present in the central Plains (Charlet and Glogoza, 2004). Larvae overwinter in the soil, emerge in July, and after mating, females deposit eggs inside the developing sunflower seeds. Larvae feed and develop in the seeds, destroying a portion of the kernel and reducing oil content. When mature, the larvae exit the seeds and drop into the soil in late August or September to overwinter (Brewer, 1991; Rogers, 1992; Charlet et al., 1997).

Plant resistance is an important strategy in a sustainable pest management program for sunflower. Our goal was to evaluate selected sunflower accessions, interspecific crosses, and lines for reduced seed damage from larval feeding by the sunflower moth, red sunflower seed weevil, and banded sunflower moth, and lower populations of stem weevil larvae in stalks. Lines that have less insect damage can provide germplasm for incorporation into hybrids targeted to locations where specific insect problems occur. Our long-term objective is to identify germplasm with resistance or tolerance to more than one insect pest. This will increase grower confidence in the crop and facilitate maintenance and expansion of sunflower acreage in both the central and northern Plains production regions of the United States.

MATERIALS AND METHODS

Sunflower stem weevil

Plots were established at the Northwest Research Extension Center, Kansas State University, Colby, KS. Field trials in 2005 screened 14 selected sunflower hybrids, 8 accessions or Plant Introductions (PIs) obtained from the USDA, ARS, Plant Introduction Station at Ames, Iowa, 12 interspecific crosses, and hybrid '894'. In 2006 we screened 9 selected commercial sunflower hybrids, 5 retested accessions, 4 accessions that had previously shown low levels of seed damage from the banded sunflower moth, 4 accessions and an interspecific cross that previously had shown low sunflower moth damage, 5 retested interspecific crosses, 2 susceptible checks, and hybrid '894'. The lines were planted each year in single rows 7.6 m long and each was replicated three times in a randomized block design and planted on 9 and 8 May in 2005 and 2006, respectively. As a result of a phenotypic recurrent selection program that genetically combined lines with quantitatively-controlled insect tolerance factors from earlier trials, 60 S_1 line progeny rows also were subjected to insect infestation in 2006 in a separate trial planted on 8 May. The lines were planted in single 7.6 m rows in a block design with checks randomly placed within the trial. Five other hybrids, crosses, or lines (Hir 1734-1, HA 89, Str 1622-2, PI 497939, hybrid '894') were included as checks within the trial. Five stalks (~ 46 cm length plus the root crown) per row were removed in October each year and sent to the USDA, ARS, Northern Crop Science Laboratory, Fargo, ND, for evaluation. Stalks were held in the cold until evaluated. The stalks were then split and the numbers of weevil larvae in each stem determined. Because of time constraints, only one half of each stalk was evaluated and then converted to number per stalk. The degree of resistance or tolerance was measured by comparing the number of weevil larvae per stalk with the germplasm having the lowest number of insects in the trial.

Sunflower moth

Plots were planted at Colby, KS. Sunflower moth feeding damage in the 2004 trials was very low with an average of 0 to 2% in the material evaluated. Because of the reduced amount of damage, the trial was repeated in 2005. Germplasm selected for testing included retested accessions and interspecific crosses with less than 4% feeding damage in 2003, selected susceptible checks and hybrid '894'. Other

accessions were added because of low damage in sunflower stem weevil, red sunflower seed weevil, and banded sunflower moth screening trials. Seven new accessions also were added. Germplasm selected for evaluation in 2006 included retested accessions and interspecific crosses with less than 4% feeding damage in 2005. The susceptible checks Hir 1734-1 and 01-4094-1 (04-628) were included as was hybrid '894'. All accessions were obtained from the USDA Plant Introduction Station. The entries were replicated three times in a randomized block design and were planted 9 and 8 May in 2005 and 2006, respectively. In a separate trial in 2006, 58 S_1 line progeny rows also were subjected to insect infestation. Five other hybrids or lines (04-628, Cropland 378, HA 89, Str 1622-2, Hybrid '894') were included as checks within the trial. The lines were planted in single 7.6 m long rows on 8 May in a block design with checks randomly placed within the trial. Physiologically mature heads were harvested between 23 August and 12 September each year. Five heads were removed from each row and shipped to Fargo, for evaluation. The heads were dried, threshed, the seed cleaned, and subsamples of 100 seeds per head evaluated for number of seeds damaged by moth larval feeding. The degree of resistance or tolerance to the sunflower moth was measured by comparing the percentage of seeds damaged among those tested.

Red sunflower seed weevil

Plots in 2005 were established at two locations: Highmore, SD, and Prosper, ND. Field trials at each site screened the same germplasm: 2 interspecific crosses, 17 accessions obtained from the USDA Plant Introduction Station and hybrid '894'. Plots in 2006 were planted at the same locations. Field trials at each site screened the same germplasm: 2 interspecific crosses, 4 retested accessions, 5 accessions with low banded sunflower moth damage, 2 interspecific crosses and 5 accessions with low sunflower moth damage from previous trials, and hybrid '894'. The entries were planted in a randomized block design with three replications on 16 June at Highmore and on 20 May at Prosper in 2005 and 7 and 9 June at Highmore and on 18 May at Prosper in 2006. In a separate trial in 2006, 60 S_1 line progeny rows also were subjected to insect infestation at the same two locations. Four other hybrids, crosses or lines (PI 431542, Hir 828-3, HA 89, and hybrid '894') were included as checks within the trial. The lines were planted in single 7.6 m long rows on 7 and 9 June (Highmore) and on 18 May (Prosper) in a block design with checks randomly placed within the trial. At Highmore because of very dry conditions in 2005, up to ten heads from each row were harvested in October and shipped to Fargo for evaluation. At Prosper, five heads were randomly removed from each row from mid-September to early October each year and taken to Fargo for evaluation. Harvest occurred in early November at Highmore and heads were sent to Fargo for evaluation. The heads from both locations were dried, threshed, and the seed cleaned. Subsamples of 100 seeds per head from each nursery were evaluated for number of seeds damaged by seed weevil larval feeding and the percentage of damaged seeds determined. Resistance or tolerance to the banded sunflower moth was measured by comparing the percentage of damaged seeds among the germplasm evaluated in the trials.

Banded sunflower moth

In 2005, plots were established at Prosper, ND. Five interspecific crosses, one new line, 17 accessions obtained from the USDA Plant Introduction Station and hybrid '894' were screened. The entries were planted in a randomized block design with three replications on 20 May. Plots in 2006 also were planted at Prosper, ND. Field trials screened 2 interspecific crosses, a new line, 11 retested accessions, 6 new accessions, an accession with low sunflower moth damage and two accessions with low seed weevil damage from previous trials, and hybrid '894'. The treatments were planted in a randomized block design with three replications on 18 May. In a separate trial, 60 S_1 line progeny rows also were subjected to insect infestation. Five other hybrids or lines (PI 251902, Par 1673-2, HA 89, P21VRI, and hybrid '894') were included as checks within the trial. The lines were planted in single 7.6 m long rows on 18 May in a block design with checks randomly placed within the trial. Five heads were randomly removed from each row when plants were physiologically mature in mid-September both years and taken to Fargo for evaluation. The heads were dried, threshed, the seed cleaned, and subsamples of 100 seeds per head evaluated for number of seeds damaged by moth larval feeding. The degree of resistance or tolerance to the banded sunflower moth was measured by comparing the percentage of seeds damaged among those tested.

RESULTS AND DISCUSSION

Sunflower stem weevil

In the 2005 trial, the mean number of sunflower stem weevil larvae occurring in the germplasm tested ranged from 7 to 70 larvae per stalk. Among all the individual stalks evaluated, numbers ranged from 0 to a high of 166 per stalk. Among the 35 lines or hybrids tested, 13 were below 25 and five below ten weevil larvae per stalk. The line with the best performance in the trial was accession PI 431516 with a mean of only 6.6 larvae per stalk. This was the first year in which this line was tested. Three interspecific crosses Str 1622-2, Hir 828-2 and Hir 828-3 had less than 20 weevil larvae per stalk. Accession PI 497939 only had 9 larvae in 2005, 12 in 2004, and only six in 2003. The accession PI 386230 had only 9 larvae per stalk and was among the ten lowest in 2004. Hybrid '894', which had only an average of 16 larvae per stalk in 2004, had over 30 larvae per stalk in 2005. The commercial hybrid with the lowest density among those tested was Fontanelle 902NS with 21 larvae per stalk.

In 2006, the mean number of larvae occurring in the material tested ranged from 5 to 51 larvae per stalk. Among the 31 lines or hybrids tested, 21 were below 25 and three below ten weevil larvae per stalk. One of the two accessions with the best performance in the trial was accession PI 431516 with a mean of only 6.5 larvae per stalk. This was the second year in which this line was tested, and in 2005 it had the lowest number of larvae in the trial. The line with the lowest number of larvae in the trial was PI 386230 with a mean of 5 larvae per stalk; in 2005 it was among those with the lowest larval density per stalk and was among the ten lowest in 2004. The accession Ames 3454 had 9 larvae per stalk, the same as in 2005. The results from the trial evaluating the S1 lines showed high numbers of larvae occurring in some of the stalks with means from 0 to140 larvae per stalk among those tested. However, a total of 22 showed average larval densities of less than 25 per stalk. Thirty-two were selected for reevaluation in 2007.

Sunflower moth

Other than two lines which showed over 30% damage, the remaining 34 tested showed an average of less than 10% seed damage per head in the 2005 trial. Although some inconsistencies in the results were evident compared to those in previous years, a number of lines that have repeatedly had low damage also were among those tested with reduced percent seed damage again in 2005. The susceptible line 01-4094-1 was again the most damaged of those evaluated. Eleven lines with low damage in 2003 sustained an average of 2% or less damage per head in 2005. This group included hybrid '894'. Others with less than 2% damage included four that had previously shown reduced seed damage in trials for banded sunflower moth (PI 505651, PI 291403, PI 494861 and PI 494859), one in trials for sunflower stem weevil (Ames 3391), and one in trials for red sunflower seed weevil (Ames 3269). Two of the accessions that were new in the 2005 trial also had less than 2% seed damage from sunflower moth feeding (PI 170405 and PI 193775).

Insect pressure from the sunflower moth was very heavy in 2006 as shown by the amount of seed damage in the trial; the damage ranged from 1 to 81% seed damage among the selected accessions and lines evaluated. The amount of damage sustained by the accessions tested was surprising because, other than the susceptible checks, those included in the 2006 trial only had shown 4% or less damage in 2005. However, hybrid '894', which had the lowest amount of damage in the trial, also was among the lowest in 2005 with only 0.3% damage. Others in the 2006 trial with lower damage levels included PI 170385 (9.6%) and Ames 3269 (11.6%) which averaged 2.5% and 1.1% damage, respectively, in 2005. PI 170414 averaged only 10.6% damage in 2006 and had averaged 0% damage in 2005, although only 3 heads were evaluated. The results from the trial evaluating the S1 lines showed feeding damage levels from 0.2 to 70% among those tested. A total of 36 showed average percentage damage of less than 10%. The check, hybrid '894' again showed lower damage from moth feeding in this trial. The best of these lines were retested in 2007 to confirm their resistance to damage by the sunflower moth.

Red sunflower seed weevil

The damage at Highmore in 2005 indicated high levels of weevil infestation, with a range of 2 to 59% seed damage among the germplasm tested at this location. Those showing damage levels of 18% or less included the three accessions PI 431545, Ames 3269, and PI 431542; however, the results were from only a limited number of heads evaluated. Ames 3269 had been tested in both 2003 and 2004 and showed only 13% damage each year. PI 431542 had the least damage of all germplasm in 2005 as well as in 2004. Hybrid '894' averaged 43% seed damage which was higher than the 2004 trial in which it averaged 24% damage. The density of red sunflower seed weevil at the Prosper trial was much lower than the Highmore location, based on the amount of seed damage. Percentage damage ranged from a high of 4% in accession PI 431569 to 0.7% in Ames 3269. Hybrid '894', which scored near the middle of the selected germplasm

evaluated at Highmore, was near the bottom in level of seed damage at Prosper at 1.5%. The accessions 431542 and Ames 3269 scored near the bottom in percentage of damage at both locations. Some others showed inconsistent results, but the differences were likely because of the lower levels of damage that occurred at Prosper.

High levels of red sunflower seed weevil occurred in the 2006 trial with a range of 7 to 52% seed damage among the germplasm tested at Highmore. Eight lines showed damage levels of 15% or less. Ames 3269 had the lowest amount of damage in both 2003 and 2004, was one of the lowest in 2005, and showed only 13% damage in 2006. Three of the least damaged accessions had shown low damage from sunflower moth in earlier trials (PI 175728, PI 162453, and PI 193775). The results from the trial evaluating the S1 lines showed feeding damage levels from 0.3 to 40% among those tested. Of the 59 evaluated, 25 showed average percentage damage of less than 13%. The best of the lines were retested in 2007 to confirm their resistance to red sunflower seed weevil damage. The density of red sunflower seed weevil at the Prosper trial was lower compared to the Highmore location based on the amount of seed damage from the germplasm evaluated. Percentage damage ranged from a high of 0.9% to 24%. There were a number of inconsistencies at the two sites. Hybrid '894', which scored near the middle of the selected germplasm evaluated at Highmore (34%), was at the bottom in the level of seed damage at Prosper with 0.9%. The accession Ames 3269 scored at the bottom in percentage of damage at Highmore, but was the most damaged of those tested at Prosper. Some others, however, were similar in amount of damage between the two locations likely because of the lower levels of damage at Prosper. Only 47 of the S1 lines were evaluated for damage due to lodging from a wind storm. The results from the trial showed feeding damage levels from 20 to 0.6% among those tested. Of those evaluated, 31 showed average percentage damage of less than 4%. The best of the lines were retested in 2007.

Banded sunflower moth

The percentage of banded sunflower moth feeding damage ranged from 8% in hybrid '894' to 58% in accession PI 431542 in 2005. The accessions PI 251902 and PI 170391, which had less than 11% damage in 2005, had only 8% damage in 2004 and less than 5% in the previous year. PI 265503 which sustained only 11% in the 2005 trial incurred 12% damage in 2004 and less than 7% in the 2003 trial. Hybrid '894', which sustained the least amount of damage in the trial, had less than 9% damage in the 2004 trial and only 4% in the 2003 trial. All of the interspecific crosses had greater than 18% feeding damage.

In the 2006 trial, the percentage of banded sunflower moth feeding damage ranged from a low of 0.5% in accession PI 432516 to 29% in interspecific cross Par 1673-2. The majority of the tested germplasm sustained less than 10% damage from moth larval feeding. PI 162453 which had shown reduced sunflower moth damage in previous trials sustained less than 6% feeding damage from banded sunflower moth. The accessions PI 170401 and PI 505651 had less than 2% damage and were also low in 2005. Three of the new PIs tested in 2006 (PI 195573, PI 219649, PI 432516) were the lowest in the trial, showing less than 2% feeding damage. The results from the trial evaluating the S1 lines showed feeding damage levels from 0.4 to 14% among those tested. A total of 19 were lost to lodging from wind. Of the remaining 41 S1 lines, 17 showed average percentage damage of less than 3%. The best of these lines were retested in 2007 to confirm their resistance to banded sunflower moth damage.

CONCLUSIONS

Evaluation of sunflower germplasm for resistance to important sunflower seed-feeding and stem-infesting pests has been conducted in regions where these insects have caused economic losses. Nurseries for the sunflower moth and sunflower stem weevil were located in KS, for the banded sunflower moth in ND, and nurseries for the red sunflower seed weevil were placed in ND and SD. Results from both 2005 and 2006 identified promising resistance in germplasm against the four insects studied. There was a reduction in seed damage of 90% and 80% between the most susceptible and the most resistant line in the sunflower moth and banded sunflower moth trials, respectively. The red sunflower seed weevil trials in both locations had genotypes with a 70% to 80% reduction in seed damage and 90% fewer larvae per stalk in the stem weevil trials. After each year of testing, lines, accessions, or interspecific crosses with low damage are retested to confirm their resistance to attack. Trials were again conducted for all four insect pest species in 2007 and the results are currently being evaluated. The lines that are determined to be the most resistant in the 2007 trials will be random-mated to begin development of the next cycle of S_1 progeny lines.

ACKNOWLEDGEMENTS

We thank Theresa Gross (USDA, ARS, Fargo, ND) for assistance in the field and laboratory, Larry Dible (Kansas State University, Colby, KS) for planting and plot maintenance, and Ralph Wolf (Kansas State University, Colby, KS) for field sampling and data collection. Sunflower accessions for the studies were provided by the USDA-ARS, North Central Plant Introduction Station, Ames, IA. The High Plains Sunflower Committee, the National Sunflower Association, and the State Board of Agricultural Research & Education provided financial support for this project.

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Integrated pest management of the banded sunflower moth in cultivated sunflower in North Dakota

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ABSTRACT

Banded sunflower moth, *Cochylis hospes* Walsingham (Lepidoptera: Tortricidae), is a key insect pest of cultivated sunflowers in North Dakota. We investigated pest management strategies to reduce feeding injury caused by the banded sunflower moth in commercial oilseed and confection sunflower fields located in north central North Dakota during 2005-2006. Seed damage from banded sunflower moth was more concentrated on field edges than at 20 m, 40 m and 150 m in the field. As a result, edge spraying was as effective as whole field spraying in controlling banded sunflower moth when populations were low to moderate. Early planted sunflower had a higher percentage of seed damage than later planted sunflower regardless of sunflower type. There was a positive linear relationship between the percent of damaged seed and the subsequent number of banded sunflower moth larvae emerging from heads. The presence of sunflower in adjacent fields had a diluting effect on field densities of banded sunflower moth. In contrast, when sunflower was not present in adjacent fields, fields had a concentrating effect with higher densities of banded sunflower moth. Sixty-one percent of banded sunflower moth reared were parasitized by two species of parasitoids: *Glypta prognatha* Dasch (Hymenoptera: Ichneumonidae) and *Chelonus phaloniae* (Mason) (Hymenoptera: Braconidae). Parasitism rates were negatively impacted by insecticide spraying in field edges. Parasitoids were effective in searching from field edges to 40 m into the field and were not dependent on the presence of sunflower in the landscape.

Key words: banded sunflower moth – biological control – *Cochylis hospes* Walsingham – insecticide control – integrated pest management – sunflower.

INTRODUCTION

Banded sunflower moth, *Cochylis hospes* Walsingham (Lepidoptera: Tortricidae), is a major pest of sunflower in the northern Plains and populations have been increasing in recent years in North Dakota. Adults begin to emerge from the soil about mid-July and are present in the field until mid-August (Mundal et al., 2006). Adults tend to congregate in field margins on weeds or adjacent crops during the day and then move to the crop in the evening. Eggs are deposited on the outside of the bracts of the sunflower head. Larvae feed in the florets and developing seeds, and also destroy mature seeds. At maturity, larvae drop to the ground and spin cocoons in the soil to overwinter (Charlet and Gross, 1990; Charlet et al., 1997). The primary management strategy for control of banded sunflower moth has been the use of insecticides, although research has also shown that delayed planting can reduce feeding damage (Knodel and Charlet, 2007). In addition, crop management programs relying primarily on insecticide usage can be detrimental to parasitoid diversity and activity. Several parasitoid species attack banded sunflower moth (Charlet, 1999, 2001). However, in most years the control exerted by parasitoids is inadequate to maintain banded sunflower moth populations below economic injury levels. Understanding the population dynamics of the pest and its natural enemies will provide valuable information that could improve control of banded sunflower moth in cultivated sunflower. The integration of different pest management strategies has the potential to provide more effective control with reduced input costs for sunflower producers.

The goal of this project was to investigate the integration of pest management strategies to reduce input costs and overall feeding injury caused by banded sunflower moth in commercial confection and oilseed sunflower fields. The effectiveness of treating only the margins of sunflower fields was evaluated for reducing economic losses from banded sunflower moth in early and late planted fields. In addition, the impacts of landscape and parasitoid complex were determined on populations of banded sunflower moth. The discovery of the most effective combination of control tactics to manage banded sunflower moth will enable producers to reduce yield loss and save money by lowering insecticide treatment costs.

Cooperation from a certified crop consultant ensured that commercial sunflower producers could readily adapt results. This helps to validate the field research in a real-life setting.

MATERIALS AND METHODS

Sunflower fields in north central North Dakota were selected in cooperation with a certified crop consultant. During 2005, eight commercial oilseed sunflower fields in Renville and Bottineau counties of ND were either treated or untreated only around the perimeter of the field with a registered sunflower insecticide, Lorsban (chlorpyrifos, Dow AgroSciences LLC, Indianapolis, IN, USA). Lorsban was applied at 1 pt per acre and 3 GPA by air when the crop was at the 10% ray petal stage. In the past several years, many fields in this region have been treated with insecticides only on the outer 61 m of the field. The influence of landscape, including sunflowers in adjacent fields, was also studied to determine the impact on abundance and field distribution of both the pest and its parasitoids. A total of eight fields were sampled on 31 August 2005. Five randomly selected sunflower heads containing mature banded sunflower moth larvae were collected from the edge, 20 m and 40 m into the field on each side of the field (a total of 60 heads per field). The heads were bagged individually, labeled, and returned to the USDA, ARS laboratory at Fargo. Banded sunflower moth larvae were extracted from the heads and reared in the laboratory to determine pest and parasitoid density, parasitoid species richness, and parasitism rates. Each head was dried, threshed, and subsamples of 100 seeds were evaluated for seed damage.

In 2006, commercial fields were selected from both confection and oilseed sunflower that were either treated or untreated, and planted early (prior to mid-May) or late (late May to mid-June). There were three replicates of the following treatments: (1) early planted, sprayed, oilseed fields; (2) late planted, sprayed, oilseed fields; (3) early planted unsprayed, oilseed fields (only two fields sampled); (4) late planted, unsprayed, oilseed fields; 5) early planted, sprayed, confection fields; (6) late planted, sprayed, confection fields; (7) early planted unsprayed, confection fields; and (8) late planted, unsprayed, confection fields. Field were aerial sprayed using 3 GPA, and the insecticide Asana (esfenvalerate, E. I. du Pont de Nemours and Co., Wilmington, DE, USA) applied at 9 fl oz per acre or the insecticide Baythroid XL (beta-cyfluthrin, Bayer Crop Sciences, RTP, North Carolina, USA) applied at 2.8 fl oz per acre. Applications were made at the 10% ray petal stage (or when early instar larvae of banded sunflower moth were present). A total of 23 fields were sampled on 25-26 September 2006. Ten randomly selected sunflower heads were collected at distances of edge (5 m), 40 m, and 150 m from two sides of each field for a total of 60 heads per field. The heads were bagged individually, labeled, and returned to the USDA, ARS laboratory at Fargo. Each head was dried, threshed and subsamples of 100 seeds were evaluated for damage by banded sunflower moth.

The effect of treated and untreated sunflower fields were compared by determining the percent of damaged seed within each sunflower field for both years. In 2005, landscape, parasitoid species richness, percent parasitism, and density of banded sunflower moth larvae also were compared. In 2006, planting date and sunflower type also were also analyzed. Data were evaluated at different sampling distances from the field edge for both years. Data were analyzed using ANOVA and Fisher's Protected LSD to separate means at the 5% significance level. Linear regression was used to determine the relationship between damaged seed in sunflower heads and the number of emerged larvae. Before analysis, banded sunflower moth data for larvae and damaged seeds were square root transformed due to non-normal distributions of residuals and non-homogeneity of variance.

RESULTS

A total of 5,242 tortricid larvae emerged from the 480 sunflower heads collected from the eight field sites in 2005. Thirty-six percent of emerged larvae were identified as *Cochylis* spp., 61 percent were parasitized and the remaining three percent died from unknown factors. Of the *Cochylis* species, 69 percent were *C. hospes* and 31 percent were *C. arthuri*. Of the parasitoids reared from *Cochylis* spp.: 53 percent were *Glypta prognatha* Dasch (Hymenoptera: Braconidae), 45 percent were *Chelonus phaloniae* (Mason) (Hymenoptera: Ichneumondiae), and 2 percent were a hyperparasitoid *Perilampus robertsoni* Crawford (Hymenoptera: Perilampidae).

In this study, untreated fields were monitored for populations of banded sunflower moths and were not treated because population levels were below the economic threshold level. There were significantly lower percent of damaged seed ($F = 1.10$; $df = 1, 82$; $P = 0.2971$), mean number of larvae ($F = 0.37$; $df = 1, 82$; $P = 0.5033$) and percent parasitism ($F = 1.42$; $df = 1, 82$; $P = 0.2369$) in the treated fields compared

to the untreated fields in 2005 (Table 1). There were no significant differences in head diameter between untreated and treated sunflower fields. Results indicate that spraying on edges can successfully reduce moth damaged seed and mean number of banded sunflower moth larvae emerging from the seed. However, these results could vary depending on locality and year-to-year population densities of banded sunflower moth. As anticipated, insecticide spraying had a negative impact on the parasitoid complex, reducing parasitism by 6.9 percent.

Table 1. Effects of spraying edges of sunflower fields on head diameter, percent seed damaged by banded sunflower moth, mean number of larvae and percent parasitism in 2005

Location	Head diameter (cm)	% Damaged Seeds[1]	Mean number of larvae[1]	% Parasitism
Treated[2]	18.6 a	1.6 a	42.4 a	19.1 a
Untreated	17.9 a	2.5 b	74.1 b	26.0 b

Means within a column followed by the same letter are not significantly different (P< 0.05), Fisher's Protected LSD.
[1]Data transformed using square root, untransformed means presented.
[2]Lorsban applied at 1 pt per acre during 10% ray petals by air using 3 GPA, edge spray application.

For 2006, there also were significantly lower percent of banded sunflower moth damaged seed in treated fields compared to the untreated fields for both confection ($F = 88.78$; df = 1, 60; $P =< 0.0001$) and oilseed ($F = 10.54$; df = 1, 60; $P = 0.0019$) sunflower (Table 2). There were no significant differences for head diameter between untreated and treated field regardless of sunflower type. Results indicated that whole field spraying was successful in reducing damaged seed when populations of banded sunflower moth were moderate to high.

Table 2. Effects of spraying whole sunflower fields on head diameter and percent seed damaged by banded sunflower moth in confection and oilseed sunflowers in 2006

Location	Confection		Oilseed	
	Head diameter (cm)	% Damaged Seeds[1]	Head diameter (cm)	% Damaged Seeds[1]
Treated[2]	18.2 a	2.3 a	17.8 a	2.3 a
Untreated	17.6 a	10.6 b	17.7 a	3.1 b

Means within a column followed by the same letter are not significantly different (P< 0.05), Fisher's Protected LSD.
[1] Data transformed using square root, untransformed means presented.
[2] Asana (9 fl oz per acre) or Baythroid (2.8 fl oz per acre) was applied during 10% ray petals by air using 3 GPA, whole field spray application.

Comparison of early versus late-planted sunflower fields indicate that early planting dates had a significantly higher percent of damaged seed ($F = 20.15$, df = 1, 60, $P =< 0.0001$) than late planting dates for oilseed sunflower in 2006 (Table 3). There were no significant differences in head diameter between the two planting dates regardless of the sunflower type.

Table 3. Effects of early versus late planted sunflowers on head diameter and percent seed damaged by banded sunflower moth in confection and oilseed sunflowers in 2006

Location	Confection		Oilseed	
	Head diameter (cm)	% Damaged Seeds[1]	Head diameter (cm)	% Damaged Seeds[1]
Early planted	18.2 a	6.6 a	17.9 a	3.6 a
Late-planted	17.6 a	6.5 a	17.5 a	2.0 b

Means within a column followed by the same letter are not significantly different (P< 0.05), Fisher's Protected LSD.
[1] Data transformed using square root, untransformed means presented.

In 2005, the comparison of sampling locations revealed that the edge sample had significantly higher percent damaged seed ($F = 8.35$; df = 2, 82; $P = 0.0005$) and mean number of larvae emerging from heads ($F = 12.24$; df = 2, 82; $P =< 0.0001$) than the 20 m and 40 m samples (Table 4). There were no significant differences among the sampling locations for head diameter or percent parasitism. When data for treated and untreated sunflower fields were analyzed separately (results not presented), results were identical to the combined analyses. Results indicate that field edges harbor higher numbers of banded sunflower moth larvae than the samples collected at 20 m and 40 m in fields. These data validate why field edge spraying can be effective in controlling banded sunflower moth when populations are low to moderate. Parasitoids

were as proficient in searching for banded sunflower moth larvae in the edge as they were in 20 m and 40 m within fields.

Table 4. Effects of sampling locations from combined (treated and untreated) sunflower fields on head diameter, percent damaged seed by banded sunflower moth, mean number of larvae and percent parasitism in 2005

Location	Head diameter (cm)	% Damaged Seeds[1]	Mean number of larvae[1]	% Parasitism
Edge	18.2 a	3.4 a	102.8 a	23.8 a
20 M	18.2 a	1.6 b	43.6 b	21.0 a
40 M	18.3 a	1.0 b	28.3 b	22.8 a

Means within a column followed by the same letter are not significantly different (P< 0.05), Fisher's Protected LSD.
[1] Data transformed using square root, untransformed mean presented.

Edge samples in 2006 had significantly higher percent damaged seed than the 40 m and 150 m samples for both confection ($F = 8.12$; df = 2, 60; $P = 0.0008$) and oilseed ($F = 5.25$; df = 2, 60; $P = 0.0080$) sunflower (Table 5). There were no significant differences among sampling locations for head diameter. When data for untreated or treated confection or oilseed sunflower fields were analyzed separately (results not presented), results were identical to the combined analyses. The 2006 results were identical to results in 2005 and further support that field edges have higher numbers of banded sunflower moth than the samples collected in the field.

Table 5. Effects of sampling locations from combined (treated and untreated) sunflower fields on head diameter and percent seed damaged by banded sunflower moth in confection and oilseed sunflowers in 2006

Location	Confection		Oilseed	
	Head diameter (cm)	% Damaged Seeds[1]	Head diameter (cm)	% Damaged Seeds[1]
Edge	18.0 a	9.5 a	17.5 a	3.5 a
40 m	17.5 a	5.7 b	17.7 b	2.0 b
150 m	18.2 a	5.3 b	18.0 a	2.6 b

Means within a column followed by the same letter are not significantly different (P< 0.05), Fisher's Protected LSD.
[1] Data transformed using square root, untransformed means presented.

Landscape effects were evaluated only for 2005 (Table 6). Fields without sunflower fields nearby had a significantly higher mean number of banded sunflower moth larvae ($F = 3.61$; df = 1, 82; $P = 0.0611$) than fields that were adjacent to sunflower fields. However, there were no significant differences for percent damaged seeds, percent parasitism or head diameter. When data were analyzed separately by treated and untreated sunflower fields (results not presented), results were identical to the combined analyses. Since fields with non-sunflower fields nearby had higher mean number of larvae, this suggests that the presence of the host plant is a density-dependent factor for banded sunflower moth populations. The opposite was observed for parasitoids with no landscape effects on parasitism, which suggests that parasitoids are not density-dependent on sunflower in the landscape.

Table 6. Landscape effects on head diameter, percent damaged seeds by banded sunflower moth, mean number of larvae and percent parasitism in 2005

Adjacent Fields	Head diameter (cm)	% Damaged Seeds[1]	Mean number of larvae[1]	% Parasitism
Sunflower	17.7a	1.3a	23.8a	17.6a
Non-sunflower	18.3a	2.1a	63.5b	23.3a

Means within a column followed by the same letter are not significantly different (P< 0.05), Fisher's Protected LSD.
[1] Data transformed using square root, untransformed means presented.

Mean number of larvae that emerged from the seed resulted in a significant parameter estimates and a significant relationship to percent damaged seeds [square root of mean number of larvae emerged from seed = 1.4870 (square root of percent damaged seeds) + 1.1415; N = 89, $R^2 = 0.498$, P< 0.0001] (Fig. 1). This indicates a positive relationship between the number of larvae emerging from seed and the percent of damaged seeds.

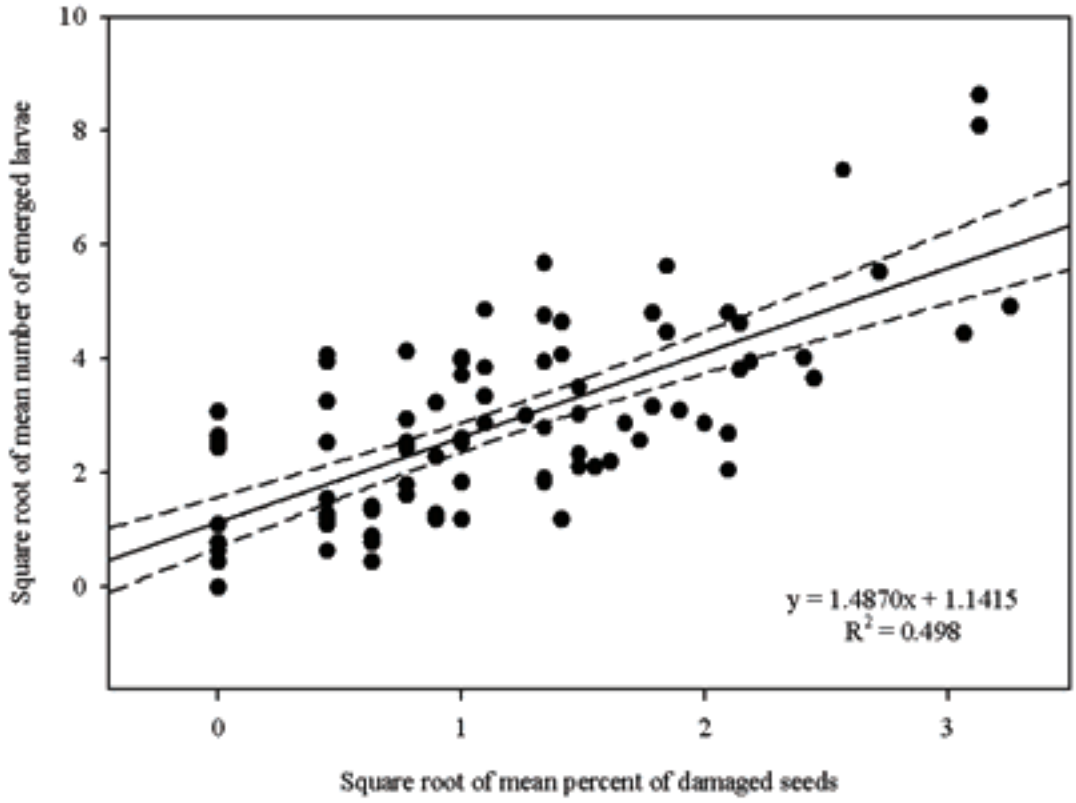

Fig. 1. Relationship between mean percent of damaged seeds and mean number of emerged larvae (n=89) across all locations and treatments. The solid line represents the best fit linear equation. Dashed lines represent 95% confidence intervals.

DISCUSSION

Since the early 1980s, cultivated sunflower fields in North Dakota, Minnesota and South Dakota have had frequent economic damage caused by banded sunflower moth (Charlet and Busacca, 1986; Charlet and Glogoza, 2004). Insecticide spraying decisions are based on sampling for eggs or adults of banded sunflower moth in fields during mid to late July (Knodel and Charlet, 2007; Knodel et al., 2008). In our study, edge spraying was effective in controlling banded sunflower moth because populations of banded sunflower moth were found to be concentrated in field edges. However, edge spraying was only effective in controlling banded sunflower moth when population levels were low to moderate. When populations of banded sunflower moth were higher in 2006, whole field spraying was required to control banded sunflower moths. A positive linear relationship was established between percent damaged seed and the subsequent number of banded sunflower moth larvae emerging from heads. Manipulating planting dates to avoid oviposition minimized damage caused by banded sunflower moth. Late planting sunflower fields into June could provide producers with a cultural control tactic to mitigate banded sunflower moth damage. Oseto et al. (1989) also reported that sunflower planted late (early June) in southeastern North Dakota had fewer damaged seeds than sunflower planted early (first week in May). The presence of sunflower in the landscape had a diluting effect on field densities of banded sunflower moth. In contrast, when sunflower was not present in the landscape, a concentrating effect was observed with higher densities of banded sunflower moths in that sunflower field.

Charlet (1999, 2001) identified several species of parasitoids attacking banded sunflower moth. Sixty-one percent of banded sunflower moth larvae reared were parasitized by two species of parasitoids: *Glypta prognatha* and *Chelonus phaloniae*. Parasitism rates were negatively impacted by insecticide spraying in field edges. Parasitoids were effective in searching from field edges to 40 m into the field and were not dependent on the presence of sunflower in the landscape.

In summary, this research supports the concept of integrating cultural control, biological control and insecticide control, which together can be used effectively to reduce banded sunflower moth damage in cultivated sunflowers in North Dakota.

ACKNOWLEDGEMENTS

We thank Mike Hutter, crop consultant, for his assistance with field selection and producer cooperation, and Theresa Gross and Bruce Goren (USDA, ARS, Fargo, ND) for their assistance in the laboratory and field. We also thank Patrick B. Beauzay for the parasitoid identifications. The National Sunflower Association provided support for this project.

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Epicuticular wax content in the pericarp of sunflower fruits (*Helianthus annuus* L.) grown under moderate water deficit

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ABSTRACT

The effect of a moderate water deficit (MWD), imposed on field grown plants in two sunflower hybrids from early anthesis (reproductive stage 6 or R6) to harvest maturity (HM), on the development of epicuticular waxes (epw; mg/g) of the fruit's pericarp, was studied in the present work. The experiment was repeated during two consecutive years. In both hybrids and experiments, plants grown under MWD showed an epw content higher than the 'controls. A decrease in the epw from stage R6 to HM was observed. This could be attributed to the erosive action on the surface of the pericarp by particulate solids carried by wind or rain. These results constitute valuable information for sunflower breeders to further investigate about the mechanisms that regulate wax content in the fruit's pericarp.

Keys words: epicuticular wax - *Helianthus annuus* - pericarp - sunflower - water deficit.

INTRODUCTION

After the sunflower oil has been industrially obtained and cooled, a crystalline sediment can be observed which affects its commercial quality (Rivarola et al., 1988). This sediment is mainly composed of waxes of epicuticular origin (epicuticular waxes or epw). They come from the fruit's pericarp (hull; 83%) (Martin and Juniper, 1970; Morrison, 1983), from the seed teguments (16%) and the embryo (1%) (Morrison et al., 1994).

The amount of waxes passing to the oil during the extraction process depends on the relative hull content of the fruit and the amount of wax it carries. In modern hybrids with high oil content, a thin pericarp is strongly adhered to the seed increasing epw transfer to the oil (Morrison et al., 1984). In these hybrids, fruit's hull content is inversely correlated with oil wax content (Morrison, 1983).

Although waxes constitute a problem for the oil industry, no studies on the development of epw in the sunflower hull are available to date. So, there is no information about the variability in the epw content among hybrids or the effect that different environmental factors and agronomical practices could produce on the epw genesis.

It is known that thermal and water stress can trigger and enhance epicuticular wax synthesis in several plant organs (Premachandra et al., 1992) and that the level of response is phenotipically sensitive and genetically controlled (Koornneef et al., 1989; Jenks et al., 2002). So in this work we have analyzed the evolution of epw content in the pericarp through different developmental stages of two sunflower hybrids grown under two water regimes.

MATERIALS AND METHODS

Plant material

Two sunflower hybrids, Dekasol (DK) 3900 and DK4030, were sown at the Department of Agronomy, UNS, experimental field (Bahía Blanca. Argentina, Lat. S., 38° 45'; Long. W, 62°11') during two consecutive growing seasons (Experiment I: 2003/2004; Experiment II: 2004/2005). The crop was grown under drip irrigation and managed according to recommended conventional agronomical practices (Pereyra and Farizo, 1981). Plant density was adjusted at 5.6 plants/m^2. Fruit samples taken from the capitulum's periphery during reproductive stages R6, R9 and harvest maturity (HM) (Schneiter and Miller, 1981) were analyzed (Table 1).

Treatments
During the reproductive stages R4 to R6 a moderate water deficit (MWD) was generated by interrupting irrigation. It was monitored by measuring the relative water content of plant leaves (RWC_{leaf}) in each treatment at different crop developmental stages.

Determination of epw content
Epw content was measured in the pericarp of the fruits at each sampling stage, for each hybrid and experiment, following the technique described by Franchini and Hernández (2006) using carbon tetrachloride as extracting agent. The epw content was expressed in mass of epw by mass of pericarp dry weight (mg/g).

Experimental design and statistical analysis
Both experiments consisted of complete randomized split plots, with water status assigned to main plots and hybrids to subplots. To determine differences between treatments and hybrids, experimental results were processed by ANOVA and differences between means were evaluated with LSD test.

Table 1. Days from first anthesis to attain reproductive stages R6, R9 and HM (Schneiter and Miller, 1981) in each of the hybrids and experiments HM: harvest maturity

	Experiment I		Experiment II	
	Hybrid		Hybrid	
Stage	DK3900	DK4030	DK3900	DK4030
R6	8	12	13	12
R9	58	48	48	44
HM	71	68	60	56

RESULTS

Plant water status
In both experiments and at different sampling times, an overall decrease of RWC_{leaf} was observed in plants under MWD comparing to control plants (Figs. 1A and 1B). Nevertheless a significant reduction (Fig. 1B; $p<0.05$) in the RWC_{leaf} was only observed 79 days after crop emergence in Experiment II accompanied by a temporary leaf wilting. After irrigation was reestablished, leaves recovered their normal turgor.

Epw content in the pericarp.
In both hybrids and treatments a reduction in epw content was observed from R6 to HM (Figs. 2A and 2B). In fruits of DK3900, during Experiment I, the observed reduction was 28 % ($p<0,05$) from stage R6 to HM (Fig. 2A), while during the Experiment II, the observed reduction was not significant ($p = 0,09$; Fig. 2B).

Although a continuous reduction in the epw content of DK4030 fruits was observed from stage R6 to HM, this was not significant in Experiment I ($p>0,05$; Fig. 2A). In Experiment II, epw content was significantly reduced by 14% ($p<0,05$) from R6 to R9, with no significant differences detected between the latter stage and HM (Fig. 2B).

MWD and epw content
Since there was no hybrid x water regime interaction ($p>0,05$) for the variable epw content, only the average results for both hybrids (Table 2) in each experiment are presented. In both experiments and in each reproductive stage studied, epw of fruits from plants under MWD showed a 33% epw increase compared to control plants (Table 2). Nevertheless, it must be mentioned that during Experiment I water deficit was not as high as expected so the differences between treatments might not be so evident.

Table 2. Average content of epw (mg/g) of the pericarp of the sunflower hybrids DK3900 and DK4030 both in control and under moderate water deficit (MWD). R6, R9: Reproductive stages as described by Schneiter and Miller (1981). HM: harvest maturity.

Stage	Experiment I			Experiment II		
	Control	MWD	S.E.	Control	MWD	S.E.
R6	5,08 a*	6,24 a	0,3	5,25 a	7,08 b	0,3
R9	4,72 a	5,59 b	0,4	4,46 a	6,53 b	0,3
HM	3,64 a	4,44 a	0,4	4,24 a	6,22 b	0,3

* In a row, within each assay, means followed by the same letter are not significantly different at p>0,05. MDW: Moderate Water Deficit. S.E.:Standard error.

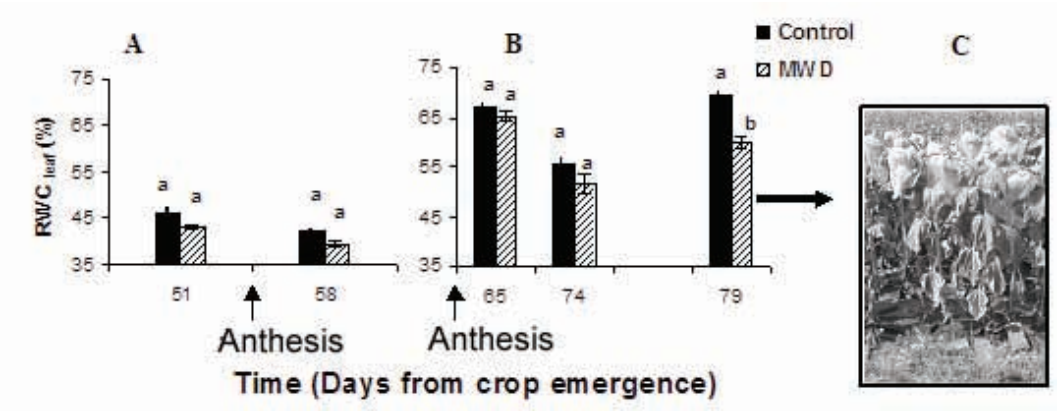

Fig. 1. Leaf relative water content (RWC_{leaf} %) in the sunflower hybrids DK3900 and DK4030 during experiment I (A) and II (B). C. Temporary wilting of leaves of plants under MWD during experiment II, 79 days after crop emergence (24 days after anthesis). Leaves became turgent once irrigation was reestablished. MDW: Moderate Water Deficit. Within each set, bars topped by the same letter are not significantly different at p>0,05.

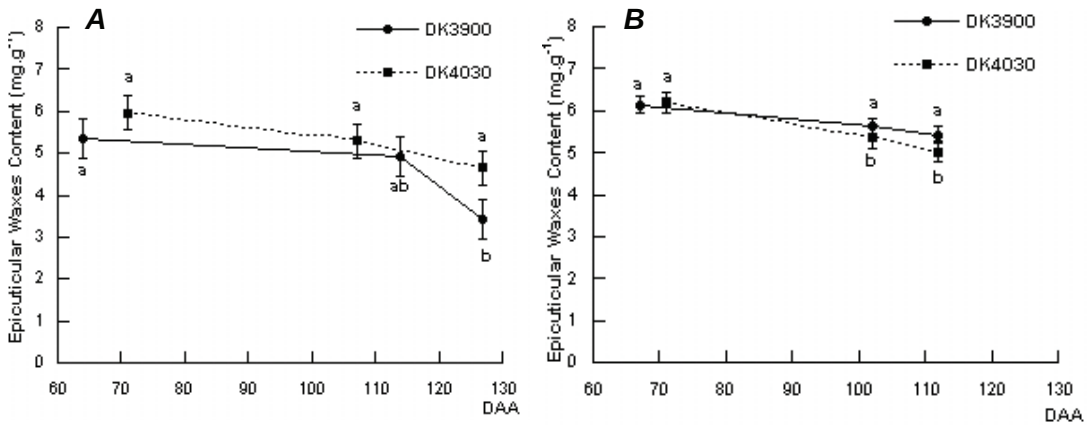

Fig. 2. Changes with time of epw (mg/g) in the pericarp of fruits of the sunflower hybrids DK3900 and DK4030, averaged across water treatments, from R6 to HM. A) Experiment I. B) Experiment II. DAA: Days after anthesis. For each hybrid, values followed by different letters indicate significant differences between sampling dates (p<0,05).

DISCUSSION

Plant water status

The observed RWC_{leaf} magnitudes (Fig. 1) show that, in both experiments, the procedure of irrigation shortage was sufficient to generate a suboptimal water status in the critical developmental stages of the formation of pericarp (stages R5 and R6; Lindström et al., 2000).

Epw content in the pericarp

The observed reduction in epw content from R6 to HM in both hybrids and experiments, could be attributed to the erosive action produced by several environmental factors, among which rainfall and wind are particularly common. They can transport abrasive particulate material removing wax crystals from the pericarp surface. The same effect has been observed in leaves of *Eucalyptus* sp. (Baker and Hunt, 1986), *Brassica* sp. and *Fraggaria* sp. (Neinhuis and Barthlott, 1997). Also, in both hybrids and experiments, the highest content of epw measured in R6, when the pericarp is still young and contains high water concentration (Rondanini et al., 2007), agrees with the phenomenon observed by Neinhuis et al. (2001). These authors demonstrated that cuticular transpiration allows the waxes attached to water molecules to move from the inner regions of the leaf to its outer surface. So, in young epidermis with a thin cuticle, such as that present in undeveloped fruits, with a lesser resistance for the passage of waxes through it compared with mature ones, a higher epw content can be expected.

MWD and epw content

In both experiments and in the three fruit developmental stages (Table 2), the imposed leaf water deficit induced a comparatively higher epw than in the controls. Similar results can be found in leaves of weeping lovegrass (*Eragrostis curvula* Schrad) (Echenique et al., 1986) and sorghum (*Sorghum bicolor* L.) (Premachandra et al., 1992), where a constant water stress led to an increase in the content of epw and a reduction in the cuticular transpiration rate.

CONCLUSIONS

A moderate plant water deficit during fruit development led to an increase of 33 % in the epw content in the pericarp, compared with that of the control plants.

From R6 to HM, epw content decreased, possibly due to the erosive action produced by wind and rain on the fruit surface.

The results shown here can be used as a physiological tool to define the dynamics of wax accumulation in the sunflower fruit pericarp, a variable that can be genetically modified (Jenks et al., 2002). Thus, breeders would be able to manipulate two characters, which are currently antagonists in the sunflower fruit: seed oil and pericarp wax content.

ACKNOWLEDGEMENTS

This work was funded by grants to L.F.H. of the Agencia Nacional de Promoción Científica y Tecnológica and the Argentine Sunflower Association (ANPCyT-ASAGIR, PICTOS-13151), the Secretaría Gral. de Ciencia Tecnología (SeGCyT-UNS) and the Comisión de Investigaciones Científicas (CIC, La Plata) Argentina. Sunflower seed was kindly provided by Monsanto of Argentina.

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The pattern of foraging paths of the Honey bee (*Apis mellifera* L.) can also explain the appearance of located regions with incompletely developed fruits in the sunflower capitulum

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ABSTRACT

The occurrence of fruits with absent or poorly developed embryos, also defined as seedless fruits or incompletely developed fruits (IDF), respectively, significantly impacts sunflower yield. Failures in pollination, fertilization and physiological or morphological defects in the ovary and embryo, either genotypic or environment-induced, post-pollination or post-fertilization, are among the most common reasons for the generation of this kind of fruits. A detailed study of the foraging pattern of diurnal pollinators in the sunflower crop, mainly honey bees, showed that there was a significant inverse correlation between the percentage of areas covered by foraging paths (ACP) and the total IDFs counted per capitulum's concentric sector. Almost a complete limitation of visitations in these sectors (0-30% ACP) resulted in poor seed set and IDFs ranging from 9 to 17%. At that level of ACP, a significant inverse correlation (r^2=0.61; p= 0.05) was found between the density of honeybee visitation and the percentage of IDFs. Partial limitation of the insect visitation (30 to 59% ACP) generated 5 to 9 % of IDFs. It is concluded that as much as 60% or more of the capitulum's area must be covered by pollinators to minimize the occurrence of IDFs.

Keywords: *Apis mellifera* - *Helianthus annuus* - pollen - seed set - sunflower.

INTRODUCTION

At maturity, the capitulum of sunflower [*Helianthus annuus* L., var. Macrocarpus (D.C.) Cockerell] usually has fruits with a different degree of pericarp and embryo development. In most of them, the embryo reaches its full size filling the internal cavity of the ovary. These fruits are defined as fully developed (FDF) (Lindström et al., 2006; 2007). On the other hand, many fruits often contain ovules that did not fully develop into seeds. In those fruits, growth processes stop at different moments, leaving the fruits with an incompletely developed pericarp and/or seed so being defined as seedless or incompletely developed fruits (IDF; Alkio et al., 2002; Alkio and Grimm, 2003; Lindström et al., 2004). Generally, IDFs can be seen to be randomly distributed over the capitulum surface (Hernández et al., 2002; Lindström et al., 2004).

The causes of the origin of IDFs are unknown but several proximate mechanisms put forward to explain the low seed to ovule ratio in many species of the *Angiospermae* can be applied to the sunflower. Poor seed set occurs mainly due to inadequate pollination, the competition for resources between developing ovaries, or vascular deficiencies at the ovary-receptacle interface (Birch and van der Sandt, 1985; Durrieu et al., 1985; Hernández and Orioli, 1991; Hernández and Palmer, 1992; Connor and Hall, 1997; Alkio and Grimm, 2003; Cantagallo et al., 2004, Lindström et al., 2006). Several studies have shown that the foraging activity of the honey bee (*Apis mellifera* L.) can increase seed set and yield (Parker, 1981a; Birch and van der Sandt, 1985; Fell, 1986; Skinner, 1987; Medan et al., 2003; DeGrandi-Hoffman and Chambers, 2006). Nevertheless, the bee foraging pattern on the sunflower capitulum has not been deeply studied (Parker, 1981b) and its relationship with seed set has not been totally established.

The aim of the present work was to determine the relationship between the path of daily visits of pollinators on capitula of the cultivated sunflower and the pattern of IDFs.

MATERIALS AND METHODS

The experiment was carried out at the Agronomy Department-UNSur, Bahía Blanca, Argentina (Lat. S. 38°45'; Long. W. 62°11') over one growing season. A low self-fertile experimental sunflower genotype, provided by Dow Agrosciences of Argentina, was sown starting the first week of October on three

successive dates, separated by 5 days, in order to obtain plants at the beginning of flowering (first anthesis [FA]; Schneiter and Miller, 1981) during several consecutive days and study them individually.

At seedling emergence, plant density was adjusted to 5.6 plants/m^2. Weeds, pests and irrigation were adequately controlled. The experimental plot was near (300 m) 20 bee colonies. This ensured that visitation at flowering was highly intense. Daily records of temperature and solar radiation were obtained from a meteorological station located 800 m from the experimental field.

Plant selection and pollinator visits observations

Two plants displaced 4 to 5 days in time for each seeding date were randomly selected in the stand (n=6). Before FA, the selected plants were staked with the florets oriented eastwards. At FA, the capitulum diameter was measured and four landmarks were placed at the periphery using colored pearl head pins. The capitulum of one plant at a time was then continuously recorded using a digital camera. The recording process took 2 to 3 days, from FA until the first 6 to 7 rows of peripheral flowers finished opening. Bee foraging on a head was continuously watched from 8.00 a.m. to 5.00 p.m. Recording was interrupted when visitors were absent. Only honey bees and sporadically carpenter bees (*Xylocopa* sp.) were observed. At dusk, each capitulum was covered with a mesh bag to avoid the action of night pollinators. After the study was completed, the procedure was repeated with another plant that by that time was at FA. The observed capitula were covered during the night until harvest.

Data processing

Digital files for each observed plant (n=6) were processed using the software VideoPoint v.2.5 (Lenox Softworks, Lenox, MA) to define, in Cartesian coordinates, the pattern of foraging routes of the pollinators (Fig. 1). The bee's thorax was the reference point of movement to digitize the route followed by the insect during its visit (arrival-departure) to the capitulum. The landmarks on the capitulum allowed the correct location and correspondence of the recorded paths at anthesis and at maturity (Fig. 1). Each image of the capitulum was then fractionated in 60 sectors and the pixel density corresponding to the foraging routes in each sector was quantified with the software Object-Image v.2.21 (Vischer et al., 1994) in a Macintosh platform. After calculating the area of each sector of the capitulum, the average pixel density was estimated for each sector (pixels% per sector) for each capitulum for each one of the six observed plants (Fig. 2).

At harvest, IDFs were identified on each mature capitulum and its location per capitulum sector defined using the reference landmarks. The IDF proportion was calculated per each capitulum sector and then compared with the intensity of visitations (Fig. 1), defined in this work as area covered by paths or ACP (Fig. 3).

RESULTS

The complete pattern in the capitulum generated by the routes followed during two consecutive visiting days for one of the six studied plants is shown in Fig. 1. Its corresponding density of visits per capitulum sector calculated according to the above methodology is presented in Fig. 2. The relationship between the % of areas covered by paths (ACP%) and the percentage of IDFs per sector in capitula of the six plants observed in this work is presented in Fig. 3.

The main floral visitors in all observations belonged to the order Hymenoptera (100% of total visits): *Apis mellifera* L., (98%), and *Xylocopa sp*. (2%). The path density was not homogeneous, showing zones with quite different densities (Figs. 1-2). There was an inverse relationship between the absence of visits or a low density of visits in a sector and the percentage of IDFs (Fig. 2). From Fig. 3 three intervals for the relationship between the ACP% and the IDF% can be defined. Thus, between 0 and 30% ACP an inverse relationship (r^2= 0.61; n=91; p= 0.05) was observed (Fig. 3). Between 30 and 100% ACPs, IDF magnitudes were distributed in two levels of a broad fluctuation, ranging from 5.0 to 9.0 % IDFs between 30 to 100% ACPs and 0% IDF or 6.0 to 9.5 % IDFs between 56 and 100% ACPs (Fig. 3).

DISCUSSION

Lack of sufficient pollen loads on the stigma to fertilize all the flowers (Zimmerman and Pyke, 1988) and physiological and/or anatomical alterations and source limitations to provide for seed development (Stephenson, 1981; Zimmerman and Pyke, 1988; Connor and Hall, 1997) have been most commonly attributed as causes for a low seed set.

According to the "non-uniform pollination hypothesis" (Thomson, 1989; Berry and Calvo, 1991) the

observed patterns of IDFs in the mature capitula may be attributable to variations in pollen receipt over the flowering period. Specifically, the relatively low seed set in central areas of the sunflower capitulum has usually been attributable to insufficient pollen quantity or pollinator visits.

The reason why some florets are left unvisited in the observed regions of the capitulum is not known. Recently, Giurfa (2004) demonstrated that the honey bee can discriminate color, and, regarding this, it has been noticed that some floret corollas in different capitulum locations of recently open florets, have a different color intensity compared with their neighbors (L.F. Hernández, unpublished). It is also known that honeybees avoid probing flowers that have been recently depleted by conspecifics, presumably repelled by foraging scent marks deposited by the previous visitor (Giurfa and Nuñez, 1992; Gawleta et al., 2005). Probably, if this is the case, the reason to leave some disc florets unvisited (Fig. 1) could be related with its proximity to already visited neighbor florets.

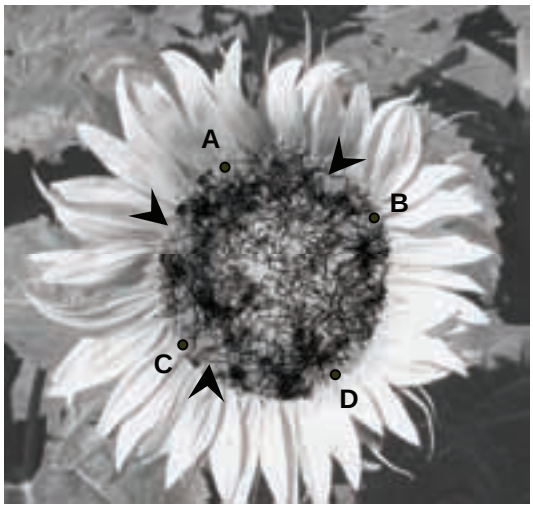

Figure 1. Pattern of routes of daily visits of honeybees (*Apis mellifera* L.) and, in a lesser proportion, carpenter bees (*Xylocopa sp*.) during two consecutive days after FA, on the capitulum of one plant studied in this work. The tracing of the image of 2 pixel width was accomplished after processing the digital images with the software VideoPoint. Arrows show some of the unvisited regions. Circles noted with letters A, B, C and D correspond to the lansmarks defoned with colored pearl head pins. The routes followed towards the central region of the capitulum were not considered in this study because these flowers were not open at the time of the analysis.

In this work, climate conditions during capitulum maturation were optimal. No rain occurred during the observation period, which could induce pollination failures by pollen lixiviation, and air temperature was always near or below 30^{o} C, a thermal level known to affect sunflower pollination (DeGrandi-Hoffman and Chambers, 2006).

Sunflower genotypes vary in their attractiveness to the honey bee. Short corolla length, non pigmented stigmas, many nectaries, and high sucrose content of the nectar are preferred by bees. If the flower was never visited it could be indicating that perhaps the floret *per se* was responsible for the lack of attractiveness due to some intrinsic difference that made it special and "unvisitable", compared with the surrounding ones. Sammataro et al. (1984; 1985) found intragenotypical differences in the quality, quantity and anatomy of nectaries. Perhaps some interplant differences could also exist.

The availability of resources can vary in both space and time for an individual flower, due to local competition for the resources (Stephenson, 1981). Hence, within a single plant, resources may be limited for some flowers but not for others. Nevertheless, perhaps this was not the case for the external flowers in the capitulum. It has been observed that at early anthesis, recently open flowers in the capitulum are not deprived of an assimilate supply (Hernández and Orioli, 1991; Alkio et al., 2002; Alkio and Grimm, 2003). Finally, according to the "architectural effects hypothesis", the pattern of seed production can also be caused in some plants by intrinsic factors, biological or physical, limiting the ripening of ovules located in some inflorescence positions (Diggle, 1995). The proximate causes of these architectural effects are still unknown (Diggle, 1997), although accumulating evidence is showing that it can have an

important effect on the observed pattern of seed production (Medrano et al., 2000 and references therein).

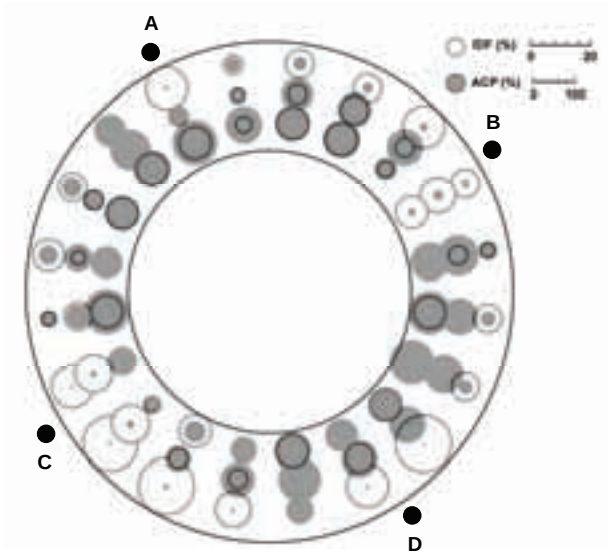

Figure 2. Emerging relationship between the area covered by visit paths (◯ ; ACP%) and the IDF% (⬤) produced in each sector for the capitulum of Fig. 1. The scales indicate the length of the diameter of each circle with the percentage magnitude for each variable. A,B,C and D, as in Fig. 1.

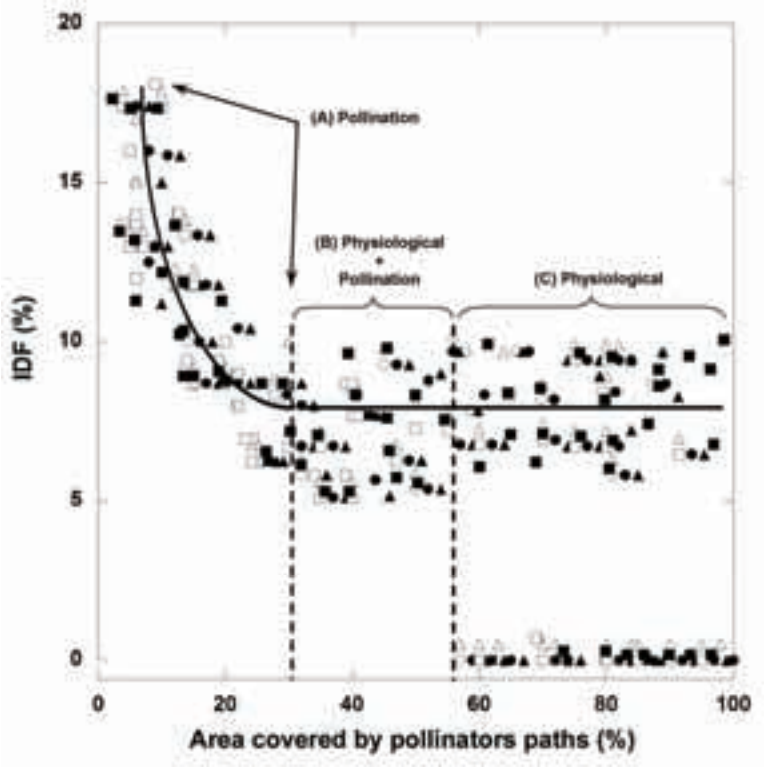

Figure 3. Relationship between the area covered by paths (ACP%) and the percentage of incompletely developed fruits (IDF%) observed in each sector (n=60) in which each capitulum of the 6 sunflower plants was divided for analysis. The sum of observations of IDF% are divided into three intervals ranging from 0% to 30%, 31% to 59 and 60% to 100% of ACP to discriminate the causes acting in the generation of IDF: (A), pollination failures, associated with the lack of pollinator visits: $IDF\% = 31.48 \times ACP^{-0,43}$; $r^2 = 0.61$; n=91; p= 0.05), (B), physiological plus pollination failures and (C), mainly physiological causes. Different markers indicate individual plants.

Considering the conditions under which the experiment and the observations were conducted and assuming that no contribution from other pollinators occurred from anthesis to fertilization, the analysis of Fig. 3 revealed at first glance three intervals which can separate different causes for the generation of IDFs. From 0% to 30% of ACP, the negative correlation found between ACP% and IDF% (r^2= 0.61) suggests that within this range, the lack of visits had a high incidence on fruit set (Fig. 3).

Approximately from 30% of ACP and above this value, the stable level of IDFs, fluctuating from 5 to 10% suggests that we would have to consider other variables. Probably there was a combination of a low occurrence of bee visits and factors related to the floral biology (physiological factors) of the tested genotype (Fig. 3).

Above 60% of ACP, the absence of IDFs (0%) in several sectors (Figs. 1 and 2) and the occurrence of sectors with a fluctuating level of IDF% ranging from 6 to 10% of the total value, would suggest that the IDFs generated in that region were produced by physiological causes, which were neither detected nor studied in this present work. Probably, they were associated with the low self-compatibility of the genotype used. Given the present information, it would be expected that in sunflower genotypes with high self-compatibility, the IDF fraction, although fluctuating, could descend to levels under 5% per sector.

The correlation between the percentage of IDFs per sector and the ACP (%) over 30% was then weak (Fig. 3), probably because the data was masked with other variables, which would act to generate IDFs.

Another weakness is the fact that the ACPs were sampled only after the bees settled during the day, without quantifying the behavior of other pollinators during the night. Nevertheless, the positive relationship between the density of foraging routes and the development of IDFs in several areas of the capitulum, confirms the important role of day-sheltering bees as sunflower pollinators. Unvisited areas are positively correlated with the presence of seedless or incompletely developed fruits at maturity. This suggests that some flowers from those areas are inclined to show an absence or delay in pollination with respect to the adjacent flowers. Due to night covering, we could assume that nocturnal pollinators would substitute the lack of visited sites by areas that bees did not visit during the day. Nevertheless if a deficiency in the number of nectaries or floret functionality occurred, these sites would not be visited at night either.

ACKNOWLEDGEMENTS

This work was funded by grants to L.F.H. of the Agencia Nacional de Promoción Científica y Tecnológica and the Argentine Sunflower Association (ANPCyT-ASAGIR, PICTOS-13151), the Secretaría Gral. de Ciencia Tecnología (SeGCyT-UNS) and the Comisión de Investigaciones Científicas (CIC, La Plata) Argentina. The valuable comments on the manuscript made by Dr. D. Sammataro (Dept. Entomology, Univ. Arizona, USA) are greatly appreciated. The invaluable cooperation of N. Vischer (Swammerdam Institute for Life Sciences, University of Amsterdam) in developing a special plug-in for his software Object Image and Mrs. G.M. Abrego for collaborating in the field experiments are also specially appreciated. Sunfower seed was kindly provided by Dow Agrosciences of Argentina.

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The influence of weather conditions on economic characteristics of sunflower hybrids in macro experiments from 1997 to 2007

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ABSTRACT

Over the period of 11 years (1997-2007), macro experiments with sunflower hybrids were set up at the location of Vinkovci. The experiments included hybrids of recognized world companies, from 13 to 30 per each test. During 11 years, the average head diameter, stem height, disease incidence of *Sclerotinia sclerotiorum*, grain yield, oil yield and oil content were recorded for each hybrid. The highest plant height was recorded in 1997 and 1998 (204 cm), while the lowest plant height was observed in 2006 (169 cm). Average plant height throughout the experiments was 188 cm. Head diameter ranged from 18.1 to 22.9 cm, averaging 19.6 cm. The greatest incidence of white rot disease (*Sclerotinia sclerotiorum*) occurred in 2005 (39.6% of infected plants). The average grain yield was 3 t ha^{-1} and it varied from 1.13 t ha^{-1} in 2005 to 4.56 t ha^{-1} in 2000. The oil content was between 42.05 and 48.17 %, with an average over experiments of 44.42%. The lowest oil yield was obtained in 2005 (0.49 t ha^{-1}) and the highest in 2000 (2.04 t ha^{-1}), with an average of 1.31 t ha^{-1}).

Key words: economic characteristics – sunflower – weather conditions.

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the four most significant oleaginous plants, planted on more than 20 million hectares worldwide with grain yields varying from 0.5 to 3.6 t ha^{-1} and an average grain yield of 1.16 t ha^{-1} (FAOSTAT, 2002). Production of sunflower in Croatia has significantly varied over the past 25 years. Average grain yield was between 1.9 to 2.5 t ha^{-1}, depending on the climate conditions of the production year (Liovic et al., 2006). Numerous pathogens can attack sunflower and one of the most important ones is white rot, *Sclerotinia sclerotiorum* (Lib.) de Bary (Duvnjak et al., 2005; Hudec, 2006). The highest disease incidence can be expected in years with cold and wet months during the vegetation period. Sunflower breeding programs must be based on creation of new genetic variability and hybrids with high oil and grain yield potential (Krizmanic et al., 2004; Hunyadi et al., 2007). Through several years of field experiments in Croatia, many hybrids have been tested and only those with the best economic characteristics were recommended to producers. Experiments also gave a direction to breeding program and seed production work. An overview of such experiments is given in this manuscript.

MATERIALS AND METHODS

During 11 years, experiments were set up in the experiment field of PIK Vinkovci. Experiments included hybrids of well-known world companies (Pioneer, RWA, KWS, Monsanto, Agricultural Institute Osijek, Institute for Crops and Vegetables of Novi Sad). The size of the main parcel was 250 m^2. Sowing was done with pneumatic sowing machines with the same crop density for all hybrids (65,000 grains per ha). Common sunflower agrotechnique was used. Protection was given with fungicide Konker (vinclosolin 20% + carbendasim 16.5%) with dosage of 1.5 l ha^{-1}. Spraying was done with field sprayer in R1-R2 sunflower stage (head size 2 cm) (Schneiter and Miller, 1981). The intensity of white rot (*Sclerotinia sclerotiorum*) incidence was determined in stage R8, by counting infected plants (40 of each hybrid). Grain yield was determined after the harvest with electronic measurer (Schrran Engeneering, model 715) and presented as tons of dry grain per hectare (grain moisture 9%, 2% ingredients). Grain moisture was determined with Dickey John measurer, model GAC 2000 (grain analysis computer).

Weather conditions: In Table 1, amount of rainfall and average temperature over the vegetation (April - August) and over the year (1997-2007) are presented. The driest year was 2007, with 94.1 mm of rainfall over the vegetation period and 238.7 mm over the year, average temperature during the vegetation period was 19.8 ºC. The largest amount of rainfall was in 2001, with 939.7 mm over the year and 514.2 mm over

the vegetation period. The lowest average temperature in 2001 (17.8 ºC). The amount of rainfall over the vegetation period 1997-2007 was 149.6 mm lower and temperature 0.8 ºC higher compared to historical records (1970-2005).

Table 1. Amount of rainfall and average temperature over the vegetation period (April - August) and over the year for the Vinkovci location

Year	Amount of rain fall (mm)		Average temperature ºC	
	Vegetation	Year	Vegetation	Year
1997	384.2	672.8	17.9	11.7
1998	375.4	683.9	18.6	11.3
1999	504.7	867.6	18.7	11.5
2000	153.4	315.2	19.7	12.9
2001	514.2	939.7	17.8	11.4
2002	483.6	682.4	18.7	12.3
2003	105.8	513.6	18.5	11.7
2004	312.8	911.9	20.1	10.9
2005	496.7	859.2	18.0	10.6
2006	461.3	639.7	18.2	11.6
2007	94.1	238.7	19.8	12.9
Average 1997-2007	353.3	665.9	18.7	11.7
Average 1970-2005	502.9	628.5	17.9	11.4

RESULTS AND DISCUSSION

Grain and oil yield, oil content, plant height, head diameter, and incidence of white rot disease varied depending on the year of production (temperature, amount and distribution of rainfall) and hybrid (Table 2). Plant height and head diameter were mostly influenced by the hybrid. In the course of the breeding program, head diameter was reduced from 22.9 cm (1997) to close to 18 cm. The objective was to reach a lower stem with smaller head diameter to facilitate a high crop density and reduced risk of lodging due to head weight, especially in wet years. White rot incidence mostly depended on rainfall and temperatures over the vegetation period. In accordance with this, the lowest disease incidence was determined in very dry years (2003 - 2.1% of infected plants; 2007 - 3.4% of infected plants) and the highest disease incidence in wet and cold years (2005 - 39.6% of infected plants; 2001 - 37.2% of infected plants). The average grain yield was 3 t ha^{-1}. The lowest average grain yield was in 2005 (1.13 t ha^{-1}) and the highest in 2000 (4.56tha^{-1}). The average oil content (1997-2007) was 44.42%. Average oil yield was 1.31 t ha^{-1}, the lowest record in 2005 (0.49 t ha^{-1}) and the highest in 2000 (2.04 t ha^{-1}).

Table 2. Results of research on economic characteristics of sunflower hybrids in macro experiments from 1997 to 2007

Year	No. of hybrids	Plant height (cm)	Head range (cm)	*Sclerotinia sclerotiorum* (%)	Grain moisture (%)	Grain yield (tha^{-1})	Oil content (%DMC^{-1})	Oil yield (tha^{-1})
1997	20	204	22.9	12.5	11.3	2.81	42.05	1.18
1998	17	204	21.1	11.1	10. 6	2.76	44.31	1.26
1999	13	184	19.8	12.2	9.7	2.39	44.17	1.06
2000	20	196	19.4	4.1	7.8	4.56	44.77	2.04
2001	24	184	18.1	37.2	11.8	2.31	44.55	0.96
2002	30	196	19.1	5.6	7.4	2.84	43.64	1.24
2003	20	170	18.7	2.1	8.0	4.35	45.32	2.03
2004	26	185	20.1	9.8	13.2	2.94	43.76	1.20
2005	30	181	19.9	39.6	15.7	1.13	42.46	0.49
2006	24	169	18.2	12.4	12.8	2.97	45.47	1.21
2007	25	198	18.3	3.4	8.7	3.89	48.17	1.75
Average	**23**	**188**	**19.6**	**13.6**	**10.6**	**3.00**	**44.42**	**1.31**
Min	13	169	18.1	2.1	7.4	1.13	42.05	0.49
Max	30	204	22.9	39.6	15.7	4.56	48.17	2.04

CONCLUSIONS

Based on the result of a macro field experiment from 1997 to 2007 in Vinkovci, the following conclusions can be reached:

1. Grain and oil yield of sunflower hybrids was significantly influenced by temperatures and the amount and distribution of rainfall.
2. Weather conditions have less influence on plant height, head diameter and oil content.
3. White rot (*Sclerotinia sclerotiorum*) incidence is significantly influenced by weather conditions (rainfall and temperatures)
4. The breeding program reached important results on creation of new hybrids with increased yield and oil potential.

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The appropriate technique for collecting and measuring the amount of floral nectar in sunflower (*Helianthus annuus* L.)

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ABSTRACT

The available techniques for collecting and measuring the amount of floral nectar are applicable but often found to be unrepresentative. Centrifugation yields larger samples but they also include nectar that is not actually accessible to insects, the capillary method has been described as unsuitable because of possible damage to the nectary tissue, the method including filter paper is considered to be unreliable because of evaporation and nectar extraction methods including washing are considered limited because the solution may include sugars from plant tissue cells. We have found that capillary tubes with inner diameter of 0.25-0.5mm and outer diameter of 0.5-0.75mm are suitable for nectar collection in sunflower. To determine the amount of nectar, we isolate five inflorescences per sunflower line at the start of flowering and collect the nectar two days after the isolation. The capillary tube is inserted between the style and filaments down to the nectary. After the level of nectar stops rising the next flower is processed. The tubes can be measured on an analytical scale and the amount of nectar is obtained as the weight increase in comparison to the empty tube. Faster determination of floral nectar amount can be provided by using calibrated capillary tubes of a known and uniform inner diameter. The appropriate outer diameter of the capillary tubes reduces the risk of tissue damage and allows more precise collecting so that the capillary method is preferable to others for nectar collecting in sunflower.

Key words: capillary technique – nectar quantity – sunflower

INTRODUCTION

Sunflower is one of the plant species that produces pollen which is too heavy for wind dispersal (Putt, 1940). Even though the cultivated sunflower has a reasonable percentage of self-compatibility it still benefits from insect pollination. One of the major components influencing pollinator choice is certainly the production of nectar, whose amount and quality are often studied.

The nectaries in *Asteraceae* family form on top of the ovary and surround the style base (Mani and Saravanan, 1999), (Fig. 1). The nectar can be accessed for quantification purposes by capillary tubes (Hocking, 1953), volumetric centrifugation (Bosi, 1973), filter paper strips (McKenna and Thomson, 1988), flushing of water into the corolla (Cresswell and Galen, 1991) or floating the flowers inverted in water (Manetas, 2000) depending on flower structure. These techniques were used with a variable success, but the overall conclusion is that no single method can be considered satisfactory for all plant species (Mesquida et all, 1988). The capillary method can be used in sunflower but it is necessary to use capillary tubes of appropriate dimensions. If the outer diameter is too large it is not possible to access the nectar without destroying the surrounding corolla tissue (Fig. 1.) and if the tube is too thin then the intake of nectar is slower and the strong capillary force may make the extraction of nectar from the tubes difficult.

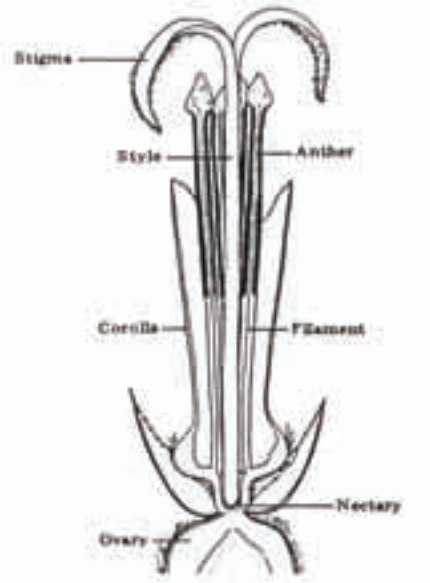

Fig. 1. Longitudinal section of a sunflower disk flower showing the location of the nectary

MATERIALS AND METHODS

We found that capillary tubes with inner diameter of 0.25-0.5mm and outer diameter of 0.5-0.75mm are suitable for nectar collection. The length of the tube then determines the capacity and can be picked for its suitability to the collecting design but should not be smaller than 15 μl.

To determine the amount of nectar, we isolate five inflorescences per sunflower line at the start of flowering with linen bags to prevent insects from collecting nectar. The best moment is when the first two rows of disk flowers have opened. Two days after the isolation, at approximately 8 AM, the inflorescences are cut and taken to the laboratory in a portable refrigerator to minimize evaporation and the change in nectar volume. It is advisable for the transport duration to be as short as possible. The following should be prepared to analyze one inflorescence:

1. Four capillary tubes (previously weighed on an analytical scale) each placed in a separate tube labeled with sample and replication to ease the work of collecting and measuring
2. A clean vegeglass (previously weighed on an analytical scale)
3. A clean HPLC vial with sample label on it filled with 1 ml mixture of AcCN:H_2O in a ratio of 75:25
4. A plastic dish with sample label on it for deep freezing

Four groups of five analyzed flowers are equally far from each other on an inflorescence. We collect nectar from 5 fully opened disk flowers with one non calibrated capillary tube. The tube is inserted between the style and filaments down to the nectary (Fig. 1.). After the level of nectar stops rising the next flower is processed. When a total of 20 flowers are finished, the tubes can be measured on an analytical scale and the amount of nectar is obtained as the weight increase in comparison to the empty tube. Faster determination of nectar amount in flowers can be provided by using calibrated tubes (capillary tubes with a uniform known inner diameter) for nectar collecting, in which case the height of nectar in tubes can be correlated with the nectar volume. This method is suitable when it is necessary to determine the amount of nectar in field conditions, without cutting the sunflower head and taking it to the lab.

The next step in method developing is a qualitative and quantitative HPLC analysis of nectar extracted from a single inflorescence as a collection from 20 disk flowers. For this purpose, ten disk flowers are also pulled off with tweezers, put in a glass and weighed on an analytical scale to obtain the information about the flower mass and possible correlation with nectar production.

The nectar collected in capillary tubes, after weight measurement, can also be kept for subsequent analysis. The contents of all capillary tubes from a single inflorescence are transferred into a HPLC vial (2 ml) filled with 1 ml mixture of AcCN:H_2O in a ratio of 75:25 and placed in a refrigerator for

subsequent HPLC nectar quality analysis. Twenty flowers are pulled out of the disk with tweezers, frozen in liquid nitrogen and then kept at -72ºC.

DISCUSSION

The rest of the techniques cited are applicable but often unrepresentative. Centrifugation yields larger quantities but they also include nectar that is not actually accessible to insects and modified chemical composition due to tissue lesion (Mesquida et al., 1988). The method including filter paper is considered to be unreliable because of evaporation (Livtzieva, 1954). Nectar extraction methods including washing are considered limited because the solution may include sugars from plant tissue cells (Kenoyer, 1917).

A combination of capillary method and filter paper can be used so that the nectar is extracted with capillary tubes and than ejected on to a filter paper, which is measured for total nectar. After the evaporation has finished the amount of sugars is obtained as a difference between wet and dry filter paper.

The capillary method has been used on sunflower (Pham-Delegue et al., 1985) but it has also been described as unsuitable for collecting nectar amounts less than 1 µl and to cause damage to the nectary tissue (McKenna, 1988). The appropriate outer diameter of the capillary tubes reduces the risk of tissue damage and allows more precise collecting so that the capillary method is preferable to others for sunflower nectar collecting.

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Sunflower and peanut emergence: initial development under sugarcane mulch

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ABSTRACT

The research aimed to evaluate the effect of residual sugarcane mulch on sunflower and peanut plant emergence and initial development. Containers of 4.0 L were disposed in a randomized experimental block design, with four replications, in a factorial arrangement of five mulch amounts and three cultivars of each crop. The mulch treatments consisted of four increasing amounts (5, 10, 15 and 20 t ha^{-1}) and a control with no mulch. The sunflower cultivars were the varieties IAC-iarama and Catissol and the hybrid H-358; the peanut cultivars were the runner-type varieties IAC-Caiapó and Runner 88, and the erect type Tatu. The speed emergence index and final emergence percentage, the plant height and shoot dry mass were evaluated. The presence of different levels of sugarcane mulch negatively influenced the emergence and initial plant development mainly in peanut but also in sunflower. The negative effects were particularly stronger for the runner-type cultivars of peanut, while cultivar Tatu was less influenced by the mulch thickness.

Key-words: *Arachis hypogaea* L. - *Helianthus annuus* L – mulch - oilcrops - seedling development.

INTRODUCTION

The sugarcane crop expansion is a reality in Brazil due mainly to the bio-energy or renewable energy concern. This expansion is now associated with the biodiesel agriculture chain, favored by the possibility of sugarcane rotation with oil crops. In Sao Paulo state, sugarcane rotation with peanut crop is a reality (Borsari Filho, 2006), but there is also a great potential for sunflower crop. There is increased interest in the oil crops that can be used for biodiesel production (MAPA, 2006). Thus, together with the sunflower rotation benefits on sugarcane of about 50% in the sugar yield (Ambrosano et al., 2005), the energetic benefits of fuel association are expected, due to the use of the biodiesel produced by the sugarcane chain in the agriculture and transportation vehicles from the sugar mills and farms.

With the implementation of laws that prohibited sugarcane burning, the harvest leaves a large amount of mulch on the soil surface. This presence of mulch can modify physical soil characteristics like water content and thermic extent (Vasconcelos, 2002), which contribute to soil conservation but, on the other hand, can cause problems to crop management (Furlani Neto et al., 1997).

The mulch layer in sugarcane areas can be as high as 10cm in thickness on the soil surface, which corresponds to 20 t ha^{-1} of residues; these form a physical barrier that reduces the light incidence and modifies the local climate conditions (Velini and Negrisoli, 2000). Those alterations are able to affect the emergence and plant development due to the influence on dormancy and seed emergence processes (Trezzi and Vidal, 2004). With the increased use of oilcrops in the sugarcane rotation, the study of the influence of mulch on different crops is a necessity. In this sense, the aim of the present research was to evaluate the influence of sugarcane residual mulch on the emergence and initial development of different sunflower and peanut cultivars.

MATERIALS AND METHODS

A greenhouse experiment was done at Ecophysiology and Biophysics Center of Instituto Agronômico (IAC), Campinas, SP, Brazil. Plastic containers of 4.0 L capacity, filled with 2.7 L of an argilous sieved soil, with the following chemical composition: pH ($CaCl_2$) = 5.2, organic matter = 25 g dm^{-3}, P (resin) = 1 mg dm^{-3}, K = 0.9, Ca = 23, Mg = 6, H+Al = 28, SB=29.9, CTC=57.7, expressed in $mmol_c$ dm^{-3} and V = 52% were used. The soil was amended according to Van Raij et al. (1997).

Before sowing, seeds of sunflower and peanut were physiologically characterized by determining the germination, emergence (MARA, 1992), and speed emergence index according to Maguire (1962).

The treatments were arranged in a factorial scheme (5 x 3), in a randomized block design, with four replications, combining five amounts of sugarcane mulch (0, 5, 10, 15 e 20 t ha^{-1}) and three cultivars of each crop, separately. For sunflower we used the open pollinated cultivars IAC-iarama and Catissol and the hybrid Helio-358, while for peanut the runner type varieties IAC-Caiapó and Runner 88, and the erect type Tatu were used.

In each pot, ten seeds previously treated with Thiram 0.2% were sown at 3cm deep, followed by the addition of the mulch of sugarcane cultivar SP 803280, which was cut into small pieces before scattering it on the soil surface. The layer thicknesses in the containers were 4, 6, 9, and 10 cm, which corresponded to 5, 10, 15, and 20 t ha^{-1}, respectively.

The final plant emergence (EM) was evaluated 15 days after sowing (MARA, 1992). For the speed emergence index (SEI), the number of normal seedling was counted daily up to a constant number, according to Maguire (1962). The initial plant development was evaluated 30 days after sowing by harvesting the plants and measuring the plant height (PH) and, after drying the aerial part in an oven at 65°C through constant mass, the shoot dry mass (SDM) was obtained.

The data were analyzed using the variance analysis with F test. The data in percentage were transformed to arc $\sin\sqrt{x/100}$ before the statistical analysis, although the original means are reported in the tables and figures. The Duncan test was used for the comparison of means among cultivars. For the mulch analysis, a regression analysis was utilized.

RESULTS AND DISCUSSION

The initial characterization of the physiological potential for sunflower and peanut cultivars (Table 1) showed that all cultivars fitted the commercialization patterns. The three sunflower cultivars presented the same physiological level while the peanut cultivar IAC-Caiapó showed a slightly lower level. Those results indicated the adequate physiological quality of all cultivars to be studied.

Table 1. Characterization of physiological potential for the seeds of sunflower and peanut cultivars in relation to initial germination level (G), final emergence (EM), and speed emergence index (SEI).

	G (%)	EM (%)	SEI
Sunflower			
IAC-iarama	99a[1]	100a	1.66a
Catissol	95a	100a	1.74a
Helio 358	98a	100a	1.51a
Peanut			
IAC-Caiapó	88b	86a	0.95a
Runner 886	92a	83a	0.87a
Tatu	97a	84a	1.04a

[1]Means followed by the same letter in column, for each specie, did not differ from Duncan ´s test at 5% probability

For sunflower there were significant interactions between mulch amount and cultivars only for shoot dry mass (SDM), while for peanut the interactions were significant only for seedling emergence (EM). There were almost no variations in the shoot dry mass of any of the sunflower cultivars with the increasing amount of mulch on soil surface (Table 2). The higher data presented by Helio-358 could be associated with the genetic vigour of the hybrid.

Table 2. Means of sunflower shoot dry mass affected by mulch (M) and cultivars (C). Campinas-SP, Brazil

Cultivars	Sugarcane mulch on the soil surface (t ha^{-1})					Adjustment equation and coefficient of determination (%)	
	0	5	10	15	20		
Sunflower –	Shoot dry mass – SDM (g)						
IAC-iarama	1.6b[1]	1.5b	1.6b	1.4b	1.4b	$Y = 1.59 – 0.01x$	r^2=69
Catissol	1.5b	1.4b	1.4b	1.4b	1.7b	$Y = 1.51 – 0.03x + 0.002x^2$	r^2=86
Helio-358	2.0a	2.1a	1.9a	2.0a	1.9a	not significant	
M x C	0.05*						

[1]Means followed by the same letter in column, for each species, did not differ from Duncan ´s test at 5% probability; *Significant at P=0.05.

The seedling emergence of all peanut cultivars was negatively affected by the mulch presence (Table 3). The cultivar Tatu was the least affected by the mulch on the soil surface, which indicates that this cultivar would be the one most indicated for the ploughed out sugarcane areas.

Table 3. Means of peanut seedling emergence affected by mulch (M) and cultivars (C). Campinas-SP, Brazil.

Cultivars	Sugarcane mulch on the soil surface (t ha^{-1})					Adjustment equation and coefficient of determination (%)	
	0	5	10	15	20		
Peanut -	Seedling emergence - EM (%)						
IAC-Caiapó	64.6a[1]	37.8b	23.2a	8.4b	2.6b	Y = 51.04 – 2.20x	r^2=99
Runner 886	60.8a	27.0b	16.5a	7.2b	7.3b	Y = 50.40 – 3.91x	r^2=99
Tatu	60.5a	57.1a	25.9a	48.4a	33,9a	Y = 51.22 . 0.97^x	r^2=68
M x C	442.11**						

[1]Means followed by the same letter in column, for each species, did not differ from Duncan´s test at 5% probability
**Significant at P=0.01.

The isolated effect of mulch amount and cultivars interfered with the evaluated parameters both for sunflower and peanut. The sunflower seedling emergency percentage (EM) and the speed emergency index (SEI) were directly affected by the mulch increasing on the soil surface (Fig. 1). The greater seedling emergence reduction occurred with the introduction of 5 t ha^{-1} (4 cm) of mulch on soil surface in relation to the tester with no mulch at all; between 15 t ha^{-1} and 20 t ha^{-1} the values did not change. The SEI followed the same pattern of reduction presented by seedling emergence (Fig. 1B). According to Teasdale (1996), the mulch deposition on soil surface can cause chemical, physical and biological alterations in the environment and, depending on the plant species, it can affect the seedling emergence and plant development. Mulch deposition is responsible for delay in the soil heat absorption, which interferes in the thermic difference between day and night, leading to a delay in the speed emergence index, in same cases, like what happened in the present research.

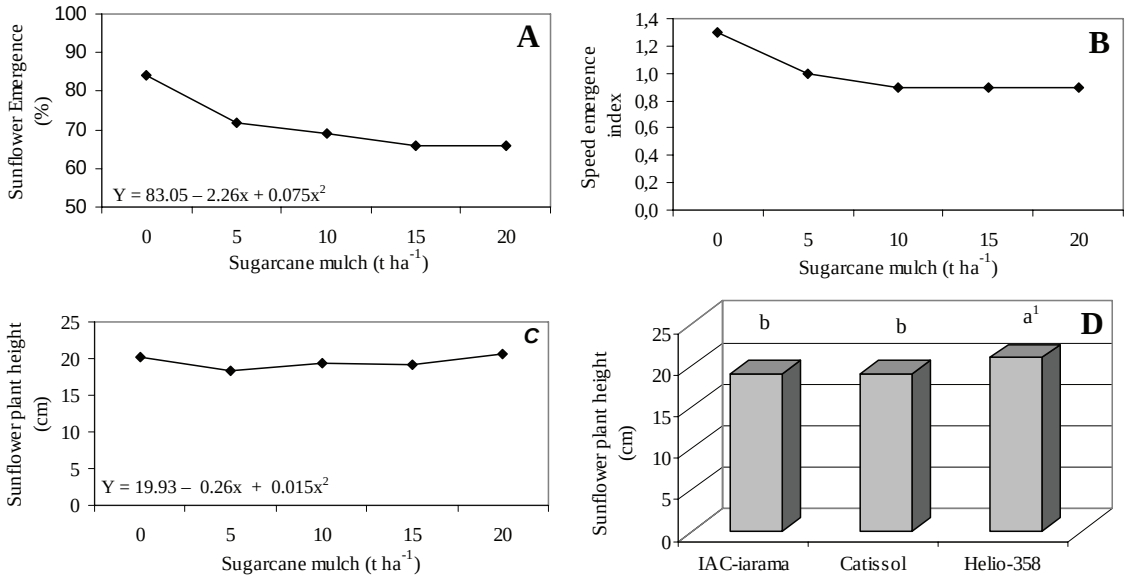

Fig. 1. Sunflower percentage of emergence (A), speed emergence index (B) and plant height (C) affected by the presence of sugarcane mulch on the soil. Sunflower plant height in the studied cultivars (D). Campinas-SP, Brazil.

Differently from other parameters, the plant height of sunflower (Fig. 1C) showed a small increase with the mulch thickness increase. This was an expected behavior since with the increasing of mulch thickness the seedling elongates in the sunlight direction (Carvalho and Nakagawa, 2000). In field conditions, plant shading leads to less biomass accumulation and the plants become more sensitive to lodging (Correia and Durigan, 2004). There were also differences between cultivars (Fig. 1D), with Helio-358 presenting the highest values for plant height in comparison to IAC-iarama and Catissol, which did not differ between each other. The better performance of Helio-358 could be related to its genetic vigour. Both seedling emergence and SEI did not vary between cultivars.

In relation to peanut crop (Fig. 2), there was a significant negative effect of the sugarcane mulch on SEI (Fig. 3A), plant height (Fig. 2C), and dry shoot mass (Fig. 2E). SEI was negatively influenced by the thickness of the mulch layer; with mulch 10-cm thick, the SEI was 78% lower. In peanut, the mulch negative effect was much more pronounced than in sunflower, probably due to the higher temperature necessary for seedling emergence because this is a tropical species whose center of origin is Brazil. Also, the DSM was reduced with the mulch layer increasing up to 10t ha^{-1}, being constant after this thickness. Like sunflower, the peanut plant height was positively influenced by increasing the mulch layer, with the elongation of the plants, already described by Carvalho and Nakagawa (2000) for seedlings under light deficits.

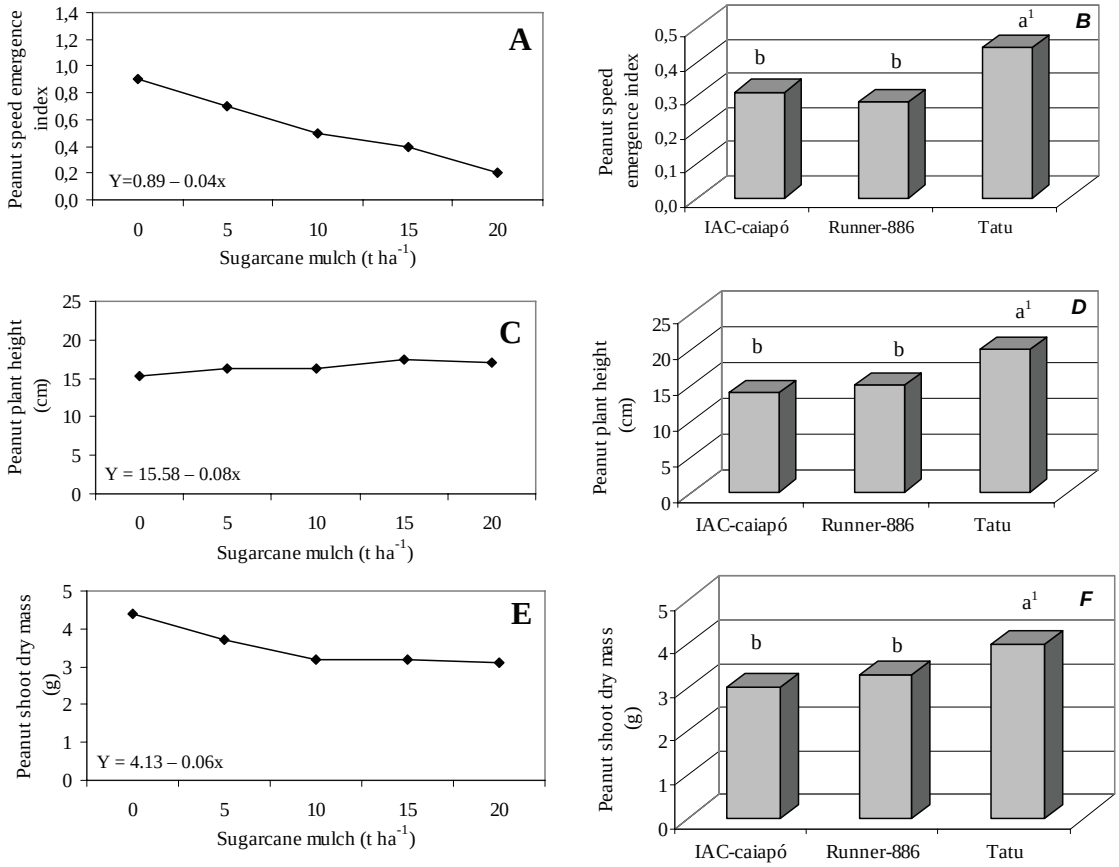

Fig. 2. Means of peanut speed emergence index (A and B), plant height (C and D) and shoot dry mass (E and F) affected respectively by sugarcane mulch (A, C and E) and cultivars (B, D, and F). Campinas-SP, Brazil.

The isolated effect of cultivars is also presented in Fig. 2. The cv. Tatu presented the best performance in comparison to IAC-caiapó and Runner-886 for SEI (Fig. 2B), HP (Fig. 2D), and DSM (Fig. 2F). This superiority could be related to its growing habit classified as erect, while IAC-Caiapó and Runner 886 are classified as runner type. The erect plants have a tendency to grow up faster than the

runner type. In the present research work, cultivars with different growing habits were evaluated in order to verify if the erect cultivars would perform better initially in comparison to the runner type which has a tendency to be more productive than the erect cultivar. In the past, the erect type was the one most cultivated in Brazil and it will likely become an option for the ploughed out sugarcane areas with the mechanical harvest obligation.

The research showed that both emergence and initial sunflower development was less negatively influenced by the presence of sugarcane mulch than peanuts. So, in the first approach, sunflower seems to be under better conditions for giving a good performance in areas with high levels of sugarcane mulch. Otherwise, it would be of interest to carry out field evaluations in the future.

CONCLUSION

Under greenhouse conditions it is possible to conclude that the presence of different levels of sugarcane mulch on the soil surface can negatively influence both sunflower and peanut emergence and initial plant development. The negative effects are stronger for peanut cultivars, especially for the runner type; the cultivar Tatu was less influenced by the mulch thickness.

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La agricultura de conservación como sistema viable para combatir el jopo en el girasol

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RESUMEN

Con objeto de estudiar la influencia que tienen dos sistemas de manejo de suelo (laboreo y no laboreo) sobre el desarrollo y propagación de las infestaciones de jopo en el cultivo del girasol y su producción, se ha llevado a cabo durante tres años un experimento utilizando tres variedades de girasol (Olimpia, Vanko y Peredovik). Los resultados obtenidos muestran que tanto el número de plantas de girasol atacadas por jopo, como el número de jopos por planta son significativamente menores en aquellas parcelas con el tratamiento de no laboreo. Estas diferencias se suavizan cuando se repite el cultivo de girasol más de dos años en la misma parcela. La producción de las tres variedades resultó siempre mayor en los tratamientos de no laboreo. Sin embargo la producción de la variedad tolerante al jopo (Olimpia) no mostró diferencias significativas con los tratamientos de laboreo.

Palabras clave: agricultura de conservación - girasol - jopo- manejo del suelo - susceptibilidad – tolerancia.

ABSTRACT

A study has been carried out to assess the influence of soil management systems on the occurrence and propagation of broomrape in the sunflower crop under dry-farming. Three sunflower varieties, Olimpia, Vanko and Peredovik have been cultivated during three years in a heavy clay soil of Andalusia under direct drilling and conventional tillage. The results of the last two years indicate that both the number of infested plants and the density of parasites per plant are significantly reduced in the direct drilling system with respect to the conventional tillage system. The differences between treatments are reduced when the sunflower crop is maintained during the three years in the same plot. The highest sunflower yield was obtained under the direct drilling system. Nevertheless, yield differences were not significant with the broomrape-resistant sunflower variety Olimpia.

Keywords: conservation agriculture - soil management - sunflower cropping - susceptibility - tolerance

INTRODUCCIÓN

El girasol (*Helianthus annuus* L.) es una planta oleaginosa cultivada en España desde la década de los 60 y que se caracteriza por su adaptabilidad a una gran diversidad de medios ambientes. Es un cultivo de primavera-verano, y con rendimientos bastante aceptables aunque dependen en gran medida de las temperaturas y pluviometria en el periodo que va de floración a maduración.

El jopo (*Orobanche cumana* Wallr.) es una planta parásita que ataca el sistema radicular del girasol y depende completamente de éste para su nutrición y desarrollo, siendo tal su agresividad que ha puesto en peligro la supervivencia del cultivo en amplias zonas de Andalucía, causando siempre perdidas económicas de importancia.

Las plantas de jopo florecen y maduran a la misma vez que el girasol. Los jopos alcanzan una altura variable en su único tallo que tiene escamas y brácteas y en cuyas axilas se forman flores coloreadas, que dan lugar a cápsulas que al madurar liberan miles de pequeñas semillas.

Las semillas de jopo germinan en el suelo en respuesta a los exudados radicales del girasol y los tubos germinativos penetran en las raíces del girasol estableciendo conexiones vasculares. Las células de esta zona responden intensificando su división con lo que la parte atacada de la raíz aumenta de tamaño. Estas conexiones vasculares entre las raíces del girasol y el jopo permite al parásito quedar integrado en la

fisiología de su huésped, tomando de éste nutrientes y agua por lo que reduce su vitalidad y su capacidad productiva (Melero y Alonso, 1988).

Las plantas atacadas forman capítulos pequeños, y con muchas de las semillas vacías. Si el ataque es muy intenso, las plantas parasitadas se marchitan ya que se incrementa la transpiración y disminuyen las reacciones de oxi-reducción.

Las pérdidas que ocasiona el jopo en el cultivo del girasol varían según la severidad de la infección y ésta a su vez depende de la cantidad de semilla de jopo que se encuentra en el suelo y del nivel de susceptibilidad o resistencia genética de la variedad. En variedades muy susceptibles, la pérdida de cosecha puede ser total ya que la planta no llega incluso a florecer.

El cultivar un híbrido con resistencia al jopo es el medio más eficaz y económico para prevenir o controlar la infestación.

No obstante, la continua aparición de nuevas razas de jopo del girasol, cada vez más virulentas, está poniendo en evidencia la vía de la resistencia genética ya que cuando se obtienen nuevos genes de resistencia para razas inéditas, no tardan en aparecer otras razas que vencen la nueva resistencia introducida por aquellos.

La posibilidad de utilizar material vegetal resistente a un herbicida, cuya resistencia no es de origen transgénico, aporta una nueva vía de lucha contra el jopo del girasol.

Adicionalmente se puede recurrir a los sistemas de manejo del suelo. Considerando que con la Agricultura de conservación (siembra directa) el lecho de siembra permanece casi intacto, las semillas de jopo tendrán más dificultades en alcanzar niveles más profundos del suelo para localizar las raíces del girasol. Por ello se ha planteado este experimento para estudiar la influencia que la agricultura de conservación y más concretamente la siembra directa, ejerce en el desarrollo y expansión de las nuevas razas de jopo, así como en el rendimiento del cultivo de girasol.

MATERIALES Y MÉTODOS

El estudio se ha realizado en la Estación Experimental de Tomejil, perteneciente al IFAPA Centro Las Torres- Tomejil en la provincia de Sevilla. Las coordenadas del punto central de la finca son 37º 24´ 07´´ N y 05º 35´ 10´´ W, localizada en la Vega de Carmona. El suelo es muy arcilloso, bujeo en la denominación local, clasificado como Chromic Haploxerent en el sistema de taxonomía del USDA (Ordóñez y col. 2007). Por su elevado contenido en arcilla, superior al 60%, la mayor parte expansible, el suelo retiene el agua durante la estación seca, lo que le hace adecuados para los cultivos de primavera (Giráldez y González, 1995).

El ensayo ha consistido en la prueba de tres variedades de girasol bajo dos regímenes diferentes de manejo de suelo: laboreo tradicional y siembra directa. Se ha realizado durante las campañas agrícolas 2005/06 y 2006/07. El experimento se comenzó en la campaña 2004/05, pero no se obtuvieron resultados ya que apenas aparecieron plantas de jopo en ninguna variedad y en ningún tratamiento.

Este ensayo se incluye dentro de los ensayos que realiza la Red Andaluza de Experimentación Agraria (RAEA) de girasol y cuyos resultados se publican anualmente en las revista serie RAEA y están disponibles en la dirección www.ifapa.cice.junta-andalucia.es.

Para la realización del ensayo se han usado dos híbridos de girasol: Olimpia (tolerante a la raza F de jopo y con rendimientos muy regulares en años anteriores, según los resultados de los ensayos de la RAEA de girasol) y Vanko (muy susceptible a la raza F de jopo, pero con unas producciones muy altas en zonas sin infestaciones del parásito, según las fuentes citadas anteriormente), y una variedad población Peredovik, adaptable a diversos ambientes y susceptible a la raza F de jopo (García Ruiz, 2003, 2004 y 2005).

La preparación del terreno en el ensayo con labor, en ambos años, consistió en un pase de chisel en el mes de septiembre del año anterior, un pase de cultivador en enero, un pase de vibrocultivador en marzo para incorporar el herbicida (Trifluralina 1,5 l/ha) y a continuación un pase de rulo.

En el ensayo de siembra directa se aplicó un tratamiento de 0,5 L/ha de glifosato + 0,5 L/ha de MCPA en presiembra.

La siembra de ambos ensayos se realizó con una sembradora de experiencias a alta densidad. La semilla se depositó a chorrillo y posteriormente se realizó un aclare manual (cuando las plantas tenían dos pares de hojas verdaderas) dejándose 4 plantas por metro lineal. En el momento de la siembra se incorporo junto con la semilla un insecticida de suelo (Clorpirifos 5%).

La parcela elemental estaba formada por cuatro líneas de siembra de 10 m de longitud y 0.70 m de separación entre ellas. El diseño experimental fue de bloques al azar con 8 repeticiones en dos sistemas de cultivos (laboreo y no laboreo).

El análisis conjunto de los resultados de los dos años de ensayos se ha realizado como el de un experimento factorial de bloques al azar combinado con años (Mcintosh, 1983).

La campaña agrícola 2006/07, se ha caracterizado por una precipitación abundante, 560mm concentrada durante los meses de invierno y primavera (Fig. 1), especialmente en el mes de mayo, que con 123 mm, representa el 23% del total anual, lo que favorece el desarrollo y producción del cultivo del girasol.

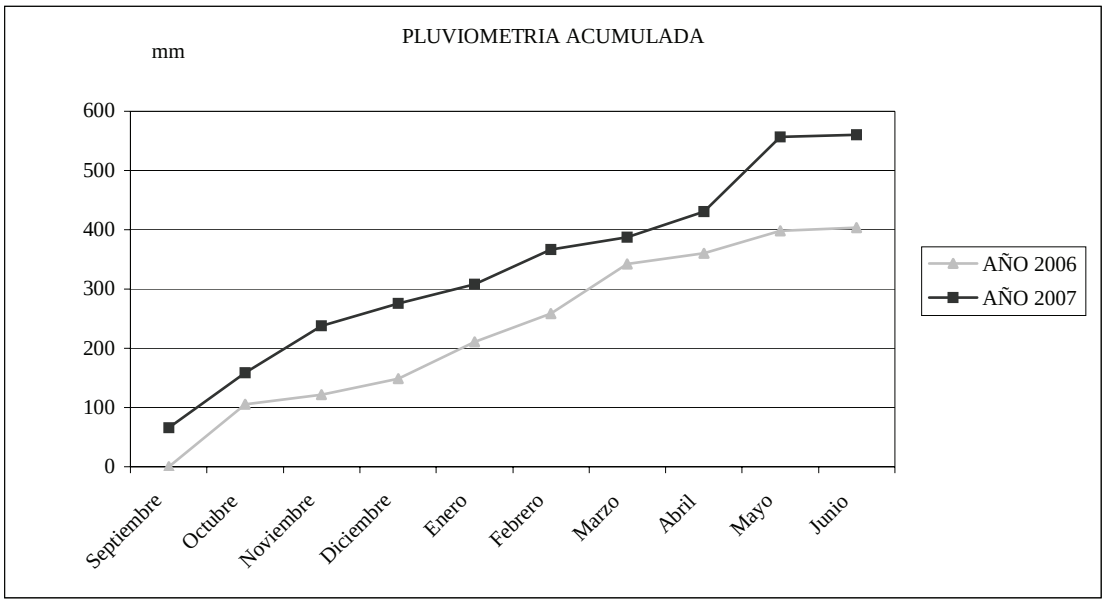

Fig. 1. Distribución de la pluviometría en los años agrícolas 2005/06 y 2006/07.

RESULTADOS Y DISCUSIÓN

La Fig. 2 representa la evolución en el porcentaje de plantas atacadas por jopo para las tres variedades, los dos sistemas de manejo de suelo y las dos campañas agrícolas consideradas en el experimento.

En dicha figura se puede apreciar, independientemente de la variedad y del sistema de manejo del suelo utilizado, un aumento significativo en el porcentaje de plantas infestadas por jopo en el año 2007 y, de forma especial en el tratamiento de no laboreo, con respecto a este mismo tratamiento en el año 2006.

En el sistema de laboreo tradicional, los aumentos han sido muy importantes, aunque la variedad Vanko ya presentaba en el año 2006 un 90% de plantas atacadas, pero Peredovik ha pasado del 56 al 97,5%(un aumento del 74%) y Olimpia ha pasado del 2.5 al 9% (lo que representa un incremento del 261%).

En el sistema de siembra directa, el incremento de plantas infestadas en el año 2007 ha sido muy alto alcanzándose cifras muy parecidas a las obtenidas con el sistema de laboreo tradicional. La variedad Peredovik ha pasado a tener del 23 al 89% de plantas infestadas, Vanko de 25 al 100% y Olimpia del 0 al 8%.

Se observa como en el primer año de ensayo, el factor no laboreo evita el aumento del número de plantas atacadas por jopo, manifestándose en la variedad Olimpia con 0 plantas y en Vanko con una disminución del 65%. Sin embargo considerando los dos años en conjunto se observa, que en el segundo año, además de existir un aumento significativo del porcentaje de plantas con jopo, de manera especial en las variedades susceptibles, desaparecen las diferencias entre los dos sistemas de manejo de suelo observada en el primer año. Esto se podría deber a un aumento considerable del inóculo de jopo en el suelo por haber mantenido el cultivo continuado de girasol durante tres años.

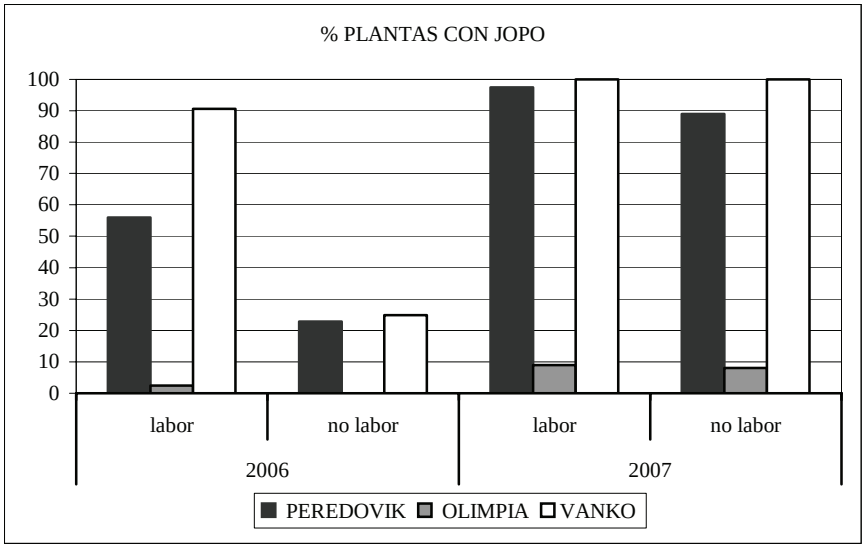

Fig. 2. Evolución en los dos años del porcentaje de plantas de girasol atacadas por jopo para las tres variedades y los dos sistemas de manejo del suelo.

La Fig. 3, representa el número de jopos por planta en cada una de las tres variedades, los dos años de ensayos y para los dos sistemas de manejo de suelo. En ella se puede apreciar cómo el mayor número de jopos por planta en ambos años se produce en el sistema de laboreo tradicional.

Tanto en el primer como en el segundo año al cambiar del sistema de laboreo tradicional a siembra directa se produce un drástico descenso en el número de jopos por planta, un 84, 91 y 100 % para las variedades Peredovik, Vanko y Olimpia, respectivamente, en el año 2006, y un 63, 49 y 48% en el año 2007.

Durante el año 2007 se aprecia un aumento considerable en el número de jopos por planta en ambos sistemas de manejo del suelo (Fig. 3).

La siembra durante tres años consecutivos de girasol en la misma parcela de ensayo ha producido una mayor cantidad de inóculo en el suelo, lo que ha podido tener incidencia no sólo en el número de plantas con jopo sino también en el número de jopos por planta.

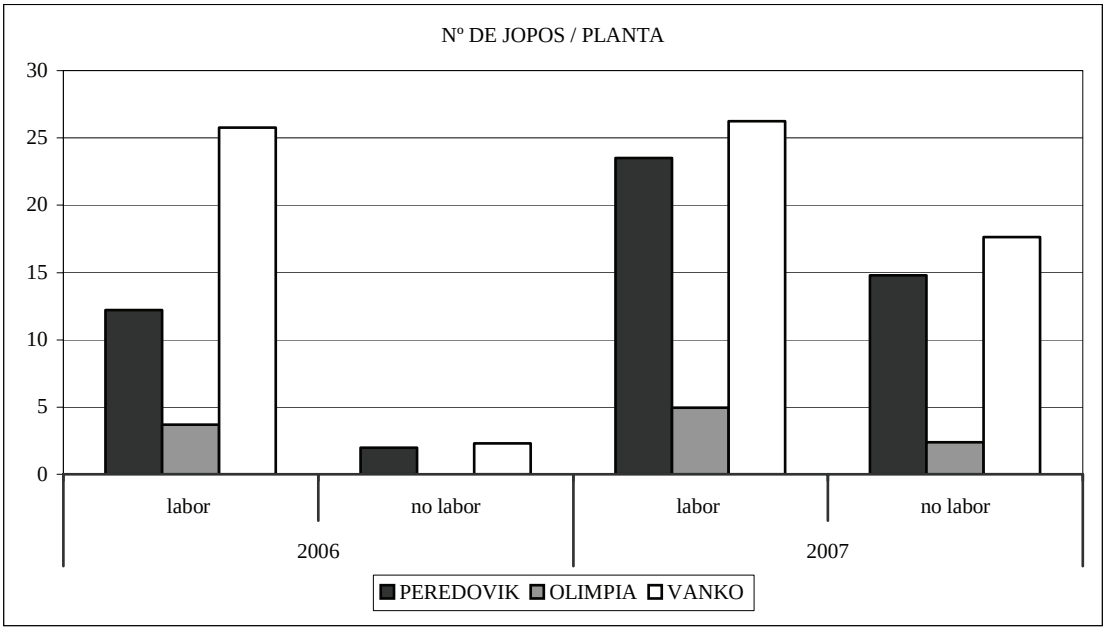

Fig. 3. Evolución del número de jopos por planta para las tres variedades y los dos sistemas de manejo del suelo.

El análisis del rendimiento medio obtenido por cada variedad en los dos años de ensayos y en los dos sistemas de manejo del suelo (Fig. 4), muestra que la siembra directa mejora la producción media en todos los casos, sin diferencias significativas en la variedad Olimpia y con diferencias significativas en la producción en las variedades Peredovik y Vanko.

Los buenos resultados de la variedad Olimpia, pueden ser debidos, por un lado a que el porcentaje de plantas afectadas por el jopo es menor en el tratamiento de siembra directa que en el tratamiento de laboreo tradicional, lo que explicaría su mayor producción en este tratamiento, y por otro a su tolerancia al jopo en suelos con altas infestaciones donde variedades susceptibles ven muy mermada su producción.

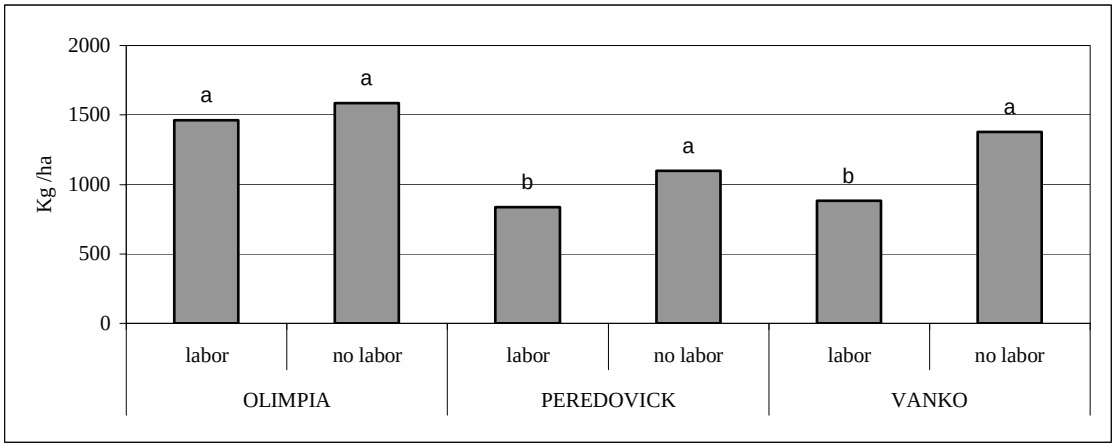

Fig. 4. Rendimiento medio en los dos años de las variedades usadas en el ensayo según el sistema de manejo del suelo.

El sistema de siembra directa es más efectivo reduciendo la infestación con variedades no tolerantes o susceptibles, ya que al eliminar la redistribución del suelo, supone una barrera para la incorporación del jopo a los horizontes inferiores donde se desarrollan las raíces del girasol.

En el análisis de los rendimientos medios de los dos años (Fig. 5), se observa que las tres variedades, independientemente del sistema de manejo de suelo utilizado, han disminuido su producción en el año 2007 de forma significativa con respecto a las obtenidas en el año 2006. Así, Olimpia ha disminuido su producción en un 19%, Peredovik en un 31% y la variedad Vanko en un 52%.

Esta disminución general de producción parece estar en contradicción con la precipitación del año 2007 (Fig. 1), que ha sido superior en 150 mm a la registrada en el año 2006.

Una posible explicación es la reiteración del monocultivo de girasol durante tres año en la misma parcela que favorece el empobrecimiento de los horizontes del suelo que exploran las raíces de la planta y aumenta la densidad inóculo de jopo.

Esto explicaría que en la variedad Vanko, que es muy susceptible a la raza “F” de jopo, sea la más afectada en la disminución de su rendimiento productivo y Olimpia, que es la más tolerante, sea la que mantenga una mayor producción.

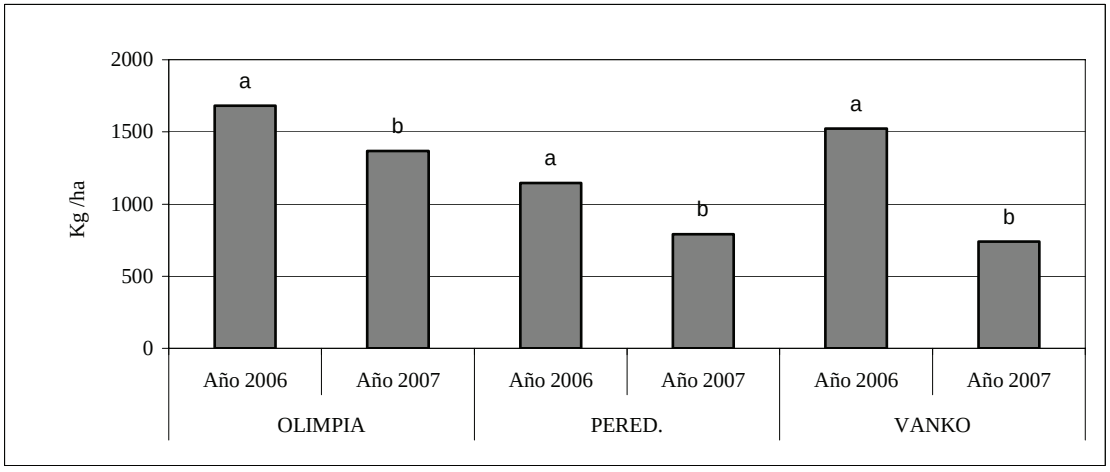

Fig. 5. Rendimiento medio de las tres variedades en los dos años de ensayos.

CONCLUSIONES

Los resultados obtenidos de los experimentos realizados en estos dos años de ensayos con objeto de estudiar la influencia de la siembra directa en la expansión del jopo, parecen indicar que:

1.- Las infestaciones de jopo (% plantas con jopo y numero de jopos por planta) son significativamente menores en el sistema de siembra directa que en el de laboreo tradicional, pero estas diferencias disminuyen notablemente cuando se repite el cultivo de girasol en la misma parcela durante dos o más años.

2.- Las producciones obtenidas son significativamente superiores en el sistema de siembra directa, salvo cuando se siembran variedades tolerantes, que aunque siguen manteniendo las diferencias en producción a favor del sistema de siembra directa, éstas no son significativas.

Por ello se aconseja introducir en el manejo del cultivo la rotación. Esta operación permite disminuir los riesgos y detener el ciclo de enfermedades, plagas y malezas, al renovarse anualmente el horizonte superficial, del suelo. Además, desde el punto de vista de la fertilidad química del suelo, una rotación de cultivos bien planificada favorece un uso más equilibrado de los nutrientes. En siembra directa las rotaciones también tienen un efecto favorable sobre la estructura de los suelos, debido a que las raíces de los cultivos implantados exploran diferentes estratos del perfil, generando una mejor distribución y estabilidad de los agregados.

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Changes in seed oil content of sunflower (*Helianthus annuus* L.) as affected by harvesting date

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ABSTRACT

The paper studied the effect of harvesting date on seed oil accumulation in three sunflower genotypes. Harvesting began seven days after pollination and continued at three to four day intervals until ten harvests were carried out in all. Parallel to this, seed moisture content was determined as well. The trial's locations were India and Serbia. In both locations, the genotypes ranked the same for oil content, but all three produced considerably higher values in Serbia. The trial locations differed as to how the minimum and maximum air temperatures fluctuated in them. Regression analysis revealed that in all three genotypes in both locations the highest oil contents were produced with seed moisture at harvesting at around 30%.

Key words: air temperature – moisture content – oil content – sunflower.

INTRODUCTION

Oil is the main reserve substance in sunflower seeds. Seed oil accumulation begins relatively early, several days after pollination, as soon as the space for oil storage forms. Seed oil percentage depends more on weather conditions in the first part of the seed filling stage, while absolute seed weight depends more on the conditions in the second. Higher temperatures in the early stages of seed fill lead to a higher seed oil content (Škorić et al., 1988). The question is, when does oil accumulation end? This is important because of the need for an earlier harvest, especially when chemical desiccation of sunflower is planned. The moment when seed oil content peaks could be expresseed as days after the end of pollination, but the length of this period depends greatly on weather conditions. In Russia, for example, seed oil content may reach its peak anywhere between 30 and 60 days after flowering (Šepetina and Rogoževa, 1971). Seed moisture content is a better indicator of the maximum oil content. Depending on the author, peak seed oil levels are achieved when seed moisture is at 60% (Role et al., 1976), 45% (Dedio, 1985), 33-50% (Chervet and Vear, 1989), 26-30% (Miklič, 2001), etc. Such diverse findings may be a result of differences in weather conditions or of different genotypes used in the trials.

The objective of this paper was to determine how the same set of sunflower genotypes behaves in different agroecological conditions with respect to oil accumulation rate.

MATERIALS AND METHODS

The trial was first carried out in India (Hyderabad, Andra Pradesh) in 1999 and then in Serbia (Rimski Šančevi) the following year. The usual crop tending measures were applied and a randomized block design with three replications was used. The following sunflower genotypes were studied:

1. Ha-Ns-26
2. Ocms-98
3. Ocms-74

The harvesting of sunflower heads began seven days after the end of flowering and continued therafter at three to four day intervals until a total of ten harvesting dates was reached. Three heads were taken from each replicate. The seed moisture content was determined right after harvesting using the common method of drying the seed in a dryer at 105°C to a constant weight. The seed oil content was expressed in relative terms and was determined by leaving the seed to dry naturally and then using nuclear magnetic resonance (NMR) to measure oil levels. Data on the minimum and maximum daily air temperatures during ripening were taken from the local weather stations.

Data were processed with the MSTATC statistical package and the results were interpreted using two-factor ANOVA and regression analysis.

RESULTS AND DISCUSSION

In India, the Ocms-98 genotype had the highest and Ocms-74 the lowest average seed oil content (Table 1). Differences between the genotypes were highly significant. The highest average oil content was recorded on the last harvesting date, with an average seed moisture (ASM) of 10.75%. From the sixth harvesting date on (at 43.27% ASM), there was no significant increase in seed oil content observed.

Table 1. Seed oil content (%) as affected by harvesting date in three sunflower genotypes in India

Genotype	Harvesting date										Average
	1	2	3	4	5	6	7	8	9	10	
Ha-Ns-26	6.9	15.0	29.1	34.5	36.9	37.3	36.3	36.8	35.7	37.3	30.6
Ocms-98	13.3	22.8	31.4	40.0	38.0	37.8	39.9	38.0	38.1	37.1	33.6
Ocms-74	5.5	1.4	4.7	24.5	20.9	28.5	31.8	33.5	34.1	35.8	22.1
Average	8.5	13.1	21.7	33.0	31.9	34.6	36.0	36.1	36.0	36.7	28.8

LSD	Genotype	Harvesting date	Genotype x Date
5%	1.68	3.08	5.33
1%	2.24	4.09	7.09

The Ha-26 genotype had the highest oil contents on the sixth and tenth harvesting dates (at 34.66 and 11.00% ASM), although from the fourth date (52.14% ASM) onwards, there was no statistically significant increase in the oil content. In Ocms-98, the highest seed oil content was found on the fourth harvesting date (51.14% ASM) and there were no significant changes in this parameter from then on. In Ocms-74, the highest seed oil content was observed on the tenth harvesting date (10.27% ASM), with no significant increases from the seventh date (41.00% ASM) onward.

In Serbia, the highest average seed oil content was found in Ocms-98 and the lowest in Ocms-74 (Table 2). Differences between the genotypes were either significant or highly significant. The highest average seed oil content was recorded on the ninth harvesting date (at 19.18% ASM). From the fourth date (50.11% ASM) forth, the value of this parameter did not increase significantly.

Table 2. Seed oil content (%) as affected by harvesting date in three sunflower genotypes in Serbia

Genotype	Harvesting date										Average
	1	2	3	4	5	6	7	8	9	10	
Ha-Ns-26	32.5	34.9	40.2	37.6	36.1	40.5	37.9	36.5	44.9	46.4	38.8
Ocms-98	29.6	33.8	41.1	43.5	51.2	51.4	48.1	52.6	50.8	50.2	45.2
Ocms-74	15.6	28.3	32.9	39.8	40.9	39.4	41.2	40.6	44.0	41.8	36.5
Average	25.9	32.3	38.1	40.3	42.7	43.8	42.4	43.2	46.6	46.1	40.2

LSD	Genotype	Harvesting date	Genotype x Date
5%	1.85	3.38	5.85
1%	2.46	4.49	7.78

The highest seed oil content of Ha-Ns-26 was achieved on the tenth harvesting date (8.87% ASM), with no significant increase being recorded after the ninth date (12.45% ASM). In Ocms-98, the highest oil content was recorded on the eighth date (33.61% ASM), and there was no significant increase in this parameter from the fifth date (54.59% ASM) on. Ocms-74 had the highest oil content on the ninth harvesting date (22.93% ASM) and no significant increase after the fourth date (50.10% ASM).

A strong relationship between seed moisture content and seed oil content at harvesting was found. The regression curves below show increasing oil content with decreasing seed moisture. In most cases, maximum oil levels were achieved with seed moisture at about 30% (at any time the coefficient of determination was around 0.9 or higher) (Fig. 1.). The coefficients of determination were high, ranging from 0.62 to 0.96.

Minimum and maximum daily temperatures at ripening varied a lot between the two locations (Fig. 2).

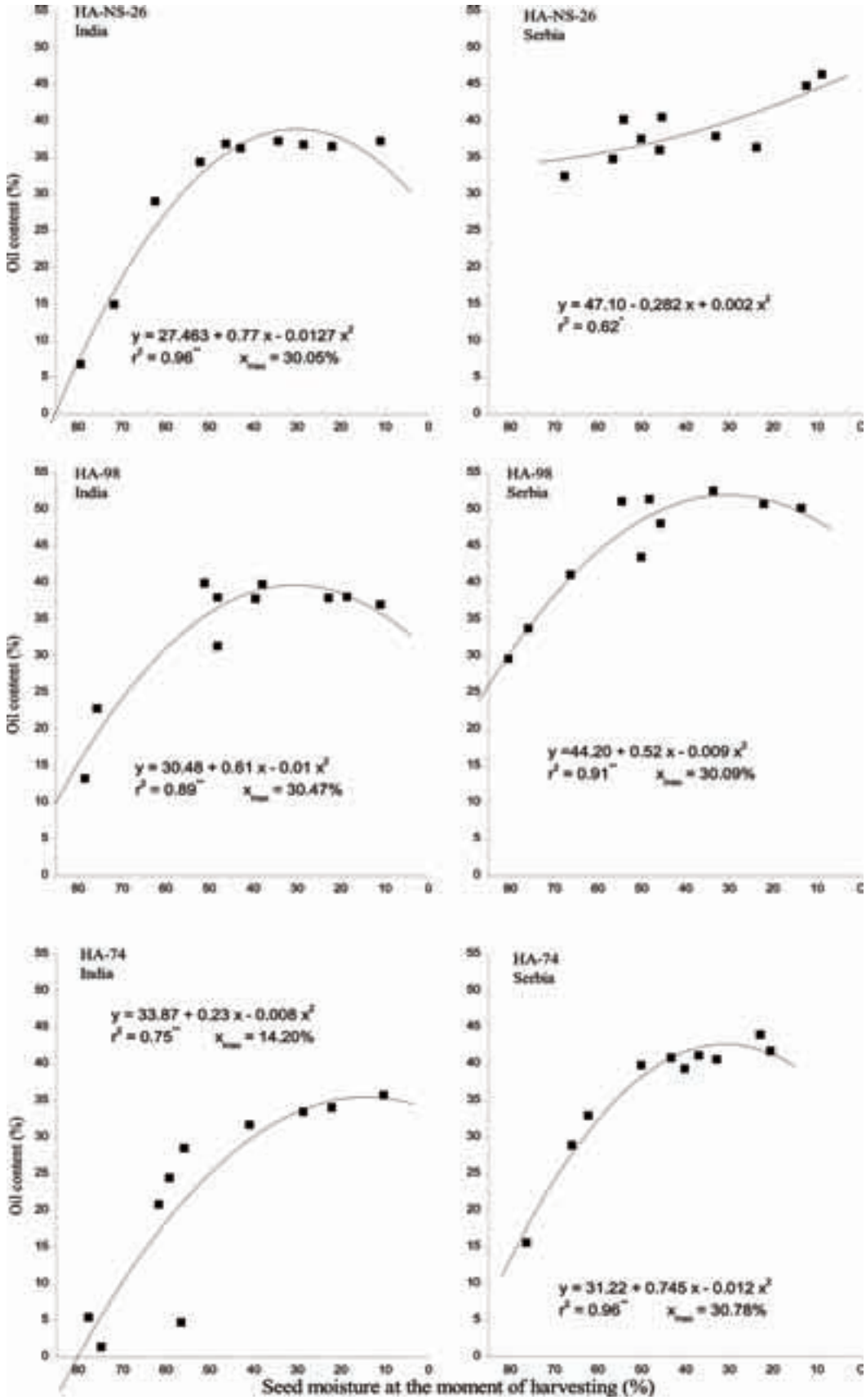

Fig. 1. Seed oil content as affected by seed moisture content at harvesting

Minimum and maximum temperatures varied a lot less in India than in Serbia. In India, the maximum temperatures were initially below 30°C and then they kept increasing slightly for much of the rest of the season, whereas the minimum temperatures increased steadily and significantly from the beginning. In Serbia, the maximum and minimum temperatures were considerably higher in the early stages of ripening, after which they kept decreasing steadily, albeit with large fluctuations. There was no significant precipitation in either location during the period. Day length was considerably greater in Serbia.

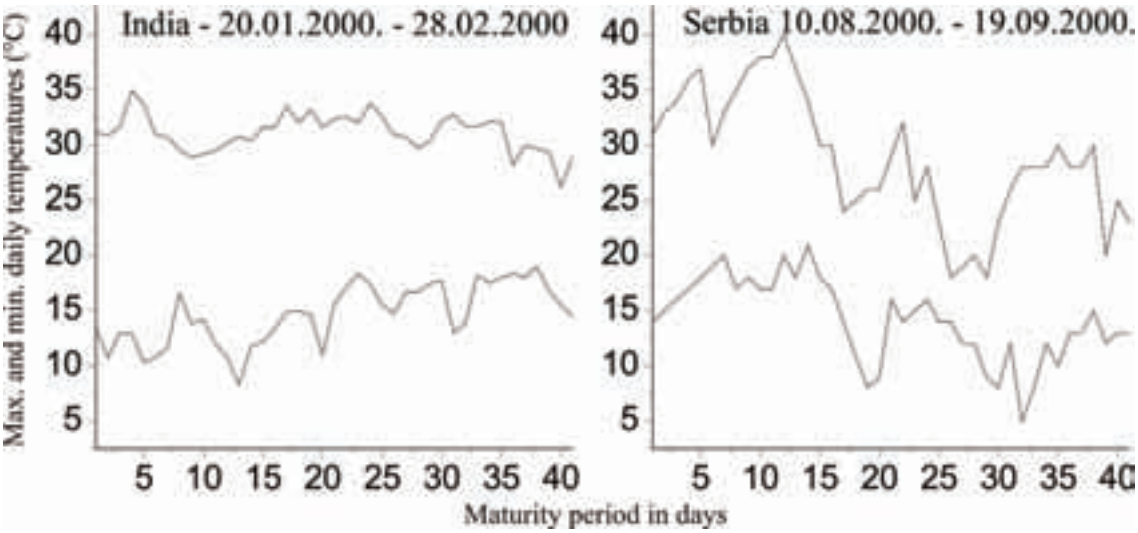

Fig. 2. Minimum and maximum temperatures during maturation

In both locations, therefore, the maximum oil content was reached with seed moisture at about 30%, which is in agreement with the results of Miklič (2001). In most cases, however, statistically significant increases in oil content were already absent with seed moisture at around 50%, which is in agreement with the findings of Chervet and Vear (1989). Considerably higher oil contents were obtained in Serbia than in India. Given that the same set of genotypes was used in both locations, this could be attributed to weather conditions. In Serbia, these conditions were better suited to producing higher oil levels, because minimum and maximum air temperatures at seed fill were higher than in India. Higher temperatures in the first part of the seed filling stage will result in a higher oil content (Škorić et al., 1988). In some cases, minor drops in seed oil content were observed in the closing days of maturation. Rodrigues Pereira (1978) attributes this to the transfer of oil from the kernel to the husk and to dissimilation of accumulated reserves in the absence of inflowing assimilates once the connection between seed and the mother plant has ceased.

CONCLUSIONS

The Ocms-98 genotype had the highest oil content in both locations.

The highest average oil content at both sites was recorded on late harvesting dates, but in most cases no significant differences were recorded once the seed moisture content dropped down to 50% or thereabouts.

Regression analysis showed that in the majority of cases the oil content reached its theoretical maximum with a seed moisture level of 30%.

Considerably higher oil levels were achieved in Serbia than in India, most likely as a result of the more favorable weather conditions in the former.

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Sunflower yield and root system development under water stress in tropical conditions

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ABSTRACT

Field experiments were carried out in Limeira, SP, in 2001, 2002 and 2003, to evaluate the sunflower cv. M 742 root system and yield under water stress conditions. A modified absence and presence method was used to evaluate the root system. The experimental design was a randomized block with four replications. The treatments were: (i) always irrigated, (ii) irrigated in critical periods, and (iii) rainfed. Sunflower plants developing under severe water stress showed higher root number, which also grew deeper than in plants under slight or no water stress. Rainfed sunflower showed two to four times more roots at 30 to 80 cm deep than the irrigated treatments. Sunflower cv. M 742 showed a grain yield reduction of about 30% under hard water stress. Under moderate water stress, with water supplementation at budding and grain filling, sunflower showed 17.2% grain yield reduction in relation to no water stress.

Key words: rainfed – root development – root system evaluation – seed yield.

INTRODUCTION

Temperature and water stress are the most important factors for the sunflower crop development and yield, although this plant adapts well to water stress periods (Choné, 1983). The plant is highly sensitive to soil acidity, mainly when a low pH occurs in subsurface levels; in those cases the taproot bends and there is less secondary root development which leads to smaller plants and less grain yield (Ungaro et al., 1985).

There are many studies about sunflower water stress under temperate and semi-arid conditions. Olalde et al. (2001), under sub-humid and semi-arid climate conditions, found different patterns of sunflower development, yield and yield components. These authors suggested that the greater soil humidity led to greater soil nutrient absorption, which resulted in greater grain and oil yield under warm sub-humid climate conditions. The data agreed with those obtained by Asri et al. (2000), who observed that the water supplementation during grain filling period increased the grain yield up to 1500 kg/ha and produced a greater oil content in the seeds. Singh and Singh (2000) found that water stress during flowering and grain filling period negatively influenced the final seed yield.

Bona et al. (2000) argue that sunflower is more tolerant to water stress than other plant species and that the deep root system is the main factor responsible for this trait. The root system is responsible for fixing, absorption, storage and nutrient and water translocation. The root density or volume usually shows a direct influence on the plant growth (Gomes, 1996). The sunflower roots develop fast at the beginning of the plant life cycle, when it is more important than the aerial part. At this time, the roots represent 20 to 25% of the total dry matter; but this proportion drops progressively to 15% at the end of the life cycle (Merrien and Milan, 1992).

Bona et al. (2000) report that sunflower is tolerant to water stress in comparison to other crops due to morphological and physiological characteristics, to its deep root system and also to some metabolic modifications that can be induced by less water availability in the soil. The transpiration index can be used as a metabolic sign which is strongly linked to the plant's physiological process. Under temperate conditions, these authors verified that one of the morphological effects of water stress was the leaf area reduction, which can cause a potential photosynthesis reduction. Foliar development reduction was observed before the transpiration decrease, which demonstrates that sunflower plant is able to tolerate the water stress by limiting foliar development without any transpiration reduction. Sunflower plant subjected to progressive water stress is able to adapt itself and be more efficient than a plant under late stress.

The aim of the study was to evaluate the sunflower grain yield and root development under different water stress regimes.

MATERIALS AND METHODS

Field experiments were carried out at Campo de Pesquisa Hidroagrícola do Pinhal, in 2001 and 2002, and at Horto Municipal Florestal, in 2003, both in Limeira, SP, using the sunflower cultivar M 742, in a winter sowing. Liming was performed only in the first year. The soil was fertilized with 300 kg/ha of 4-20-20 in 2001 and 2002; in 2003, 375 kg/ha of 4-14-8 was used. Twenty days after emergence 40 kg N/ha and 2 kg B/ha was applied, according to Quaggio and Ungaro (1996). Normal spraying irrigation was used. The experimental design was a randomized block with three treatments and four replications. The treatments were: (i) *Always irrigated*; (ii) *Irrigated in critical periods*; (iii) *Rainfed*.

One trench with 1.0 m depth and 1.0 m width was dug between plant rows to expose the sunflower root system in each replication and for the three years of observation. A thin layer of soil (1-2cm) was carefully removed from the wall along the whole trench. The root evaluation was made by the presence and absence methodology, according to Bohm (1979), modified by the authors.

A wire-wood frame with a grid of 0.2 x 0.2 m was pressed against the trench wall and the presence and absence of roots in each grid were recorded. As this method underestimates the root system because it does not consider the number and diameter of the roots, a method modification was tested, consisting of counting the number of roots in each grid.

For the evaluation of the total root number in each layer interval, the number of roots in each grid of a specific layer was summed and added to the results obtained in each replication of the same treatment for the three years. For the seed yield determinations, samples were taken at plant physiological maturation from an area of 10.8 m^{-2} in each replication during the three years and for all treatments.

Data of number of roots were transformed to log (x+1) before analysis of variance. Turkey test at the p=0.05 level was used for mean comparisons.

RESULTS AND DISCUSSION

Sunflower root distribution in the soil profile is dependent on soil conditions. According to Reichardt (1981), the main factors for little deep rooting in tropical soils are: the low pH, high exchangeable aluminum, compaction, inadequate aeration, and low retention and diffusion of water. In the present study, the soil texture in Campo de Pesquisa had less sand than Horto Florestal (Table 1) and, consequently, showed higher water retention in the 1-m profile. Soil pH was 5.2; 5.4; 5.8; 6.1 in Campo de Pesquisa, and 5.8; 5.9; 5.9 and 5.8 in Horto Florestal at 0-0.25 , 0.25-0.50 , 0.5-0.75 , 0.75-1.0 m depth, so both soils presented good conditions for normal sunflower development.

Table 1. Soil chemical and physical characterization

Hidroagrícola do Pinhal								
Soil depth	MO	P	K	Ca	V%	B	DS	Soil type
	g/dm^3	$mmol/dm^3$			%	mg/dm^3	g/cm^3	
0-25	28	6	2.8	41	59	0.22	1.34	Lime
25-50	17	2	1.7	26	56	0.20	1.29	Lime
50-75	11	1	0.8	27	63	0.07	1.16	Lime
75-100	8	1	0.6	24	62	0.06	1.22	Lime
Horto Florestal								
0-25	26	217	3.6	46	78	0.28	1.76	Lime
25-50	16	117	1.1	36	76	0.18	1.76	Lime
50-75	8	48	1.1	23	69	0.15	1.78	Clay to lime
75-100	8	22	1.1	20	69	0.15	1.78	Clay to lime

DS= Soil Density

Fig. 1 shows rain precipitation during 2001, 2002, and 2003 between June and October, 2003 being the dryest year. In 2001 and 2003 the rain occurred at the beginning of flowering and in the seed filling, which should have diminished the water stress symptoms in the *Rainfed* treatment. With the soil drying process, the upper layers are the first to dry. The plant exhibits a predominantly superficial root system under no water stress (Table 3), as shown in the *Always irrigated* treatment, and a larger number of roots in the *Rainfed* treatment in the deep layers in comparison to the irrigated treatments. The root growing towards humid soil of deeper layers can be considered as a sunflower defense against water stress.

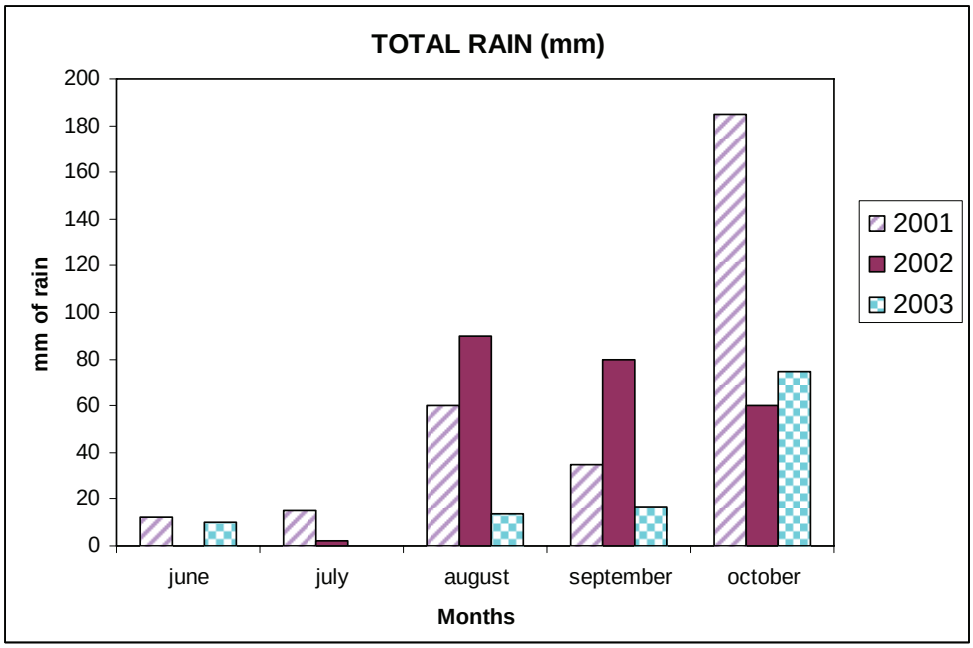

Fig. 1 Total month water precipitation for the years of 2001, 2002, and 2003.

Table 2 shows a significant difference between the average roots per grid of *Rainfed* treatment in relation to *Always irrigated and Irrigated in critical periods* by Turkey test at 5% significance. There was an increase in the number of roots per grid in the *Rainfed* treatment.

Table 2. Average number of roots/grid in the irrigation treatments, observed in the three years.

Treatments	Average roots/grid		
	2001	2002	2003
Always irrigated	7.54 b	13.70 b	6.39 b
Irrigated in critical phases	8.16 b	7.20 c	6.03 b
Rainfed	13.01 a	22.51 a	8.34 a
CV%	28.96	24.43	23.09

[1]Means followed by the same letter in column did not differ from Tukey at 5%.

According to Taiz and Zeiger (2004) moderate water deficits negatively influence the development of the root system. The ratio between root biomass and shoot apex seems to be governed by a functional balance between the root water absorption and shoot photosynthesis. This functional balance can be altered if the water supply decreases. The foliar expansion is affected by water shortage early on, but the photosynthesis activity is less affected. The inhibition of foliar expansion reduces carbon and energy consumption and a high proportion of vegetable assimilates can be distributed to the underground system to support the future growth of the roots. Those factors lead to a root growing priority to humid soils, as shown in the *Rainfed* treatment. With the increasing water shortage, the upper soil layers are the first to dry up.

Table 3 shows the total root number obtained by the sum of roots observed in each grid disposed in each layer of the treatments *Always irrigated*, *Irrigated in critical periods* and *Rainfed* using the adapted presence and absence methodology. Greatest total root number was observed in the *Rainfed* treatment. The total root number at each layer was affected by the water regime; *Rainfed* also showed between 2 to 4 times more roots in the deeper layers, between 30 and 80cm, than those of the irrigated treatments. In the *Irrigated in critical periods* and *Always irrigated* the roots were mainly in the more superficial layers.

Table 3 also shows a more superficial root system when the soil moisture is high, as verified in the treatment *Always irrigated*; when the upper layers dry, there is a root proliferation in the deeper layers in the *Rainfed* treatments. This root growing towards the humid soil according to Merrien and Milan (1992) and Connor and Hall (1997) can be considered a natural sunflower defense against water stress.

Table 3. Total root number found in each treatment in the different soil layers in the four replications.

Soil depth	*Always irrigated*	*Irrigated in critical phases*	*Rainfed*
00 – 10	6,763	7,201	6,814
10 – 20	2,267	1,947	2,148
20 – 30	415	215	382
30 – 40	154	36	250
40 – 50	39	46	157
50 – 60	43	76	114
60 – 70	66	18	65
70 – 80	6	3	22
80 – 90	5	0	3
90 –100	0	0	0
Total	9,542	9,758	9,955

Table 4 shows the grain yield obtained in each treatment and year. It is interesting to observe that the data shows the same yield level in the three years and in the two soil types, although 2003 was much drier with only 48mm of rain during the whole sunflower cycle, while 2001 and 2002 presented 90mm and 180 mm, respectively. The better soil characteristics of 2003 must have positively influenced the grain yield. The higher water stress in the *Rainfed* treatment resulted in a 30% yield reduction in relation to the treatment with no water stress. The moderate water stress presented by *Irrigated in critical phases* treatment reduced grain yield by about 17%.

Table 4. Results of the grain yield obtained in the three treatments, in 2001, 2002, and 2003, and the percentage of yield reduction in relation to *Always irrigated* treatment.

Treatment	2001	2002	2003	Average	%reduction
Always irrigated	1732 a	1604 a	1860 a	1732 a	0
Irrigated in critical phases	1483 b	1425 ab	1541 b	1483 b	17.2
Rainfed	1122 c	1112 b	1131 c	1121 c	29.8
Average	1446	1380	1511		

Means followed by the same letter in column did not differ from Turkey at 5%.

CONCLUSIONS

- The irrigation treatments showed no differences in relation to root number and distribution in the soil profile while *Rainfed* treatment developed two to four times more roots at 30 to 80cm deep;
- Under strong water stress the sunflower plant increases the number and the depth of the roots in order to minimize the lack of water;
- Sunflower cv. M 742, under hard water stress showed a grain yield reduction of about 30%;
- Under moderate water stress, with water supplementation at budding and grain filling, sunflower showed 17.2% grain yield reduction in relation to no water stress;

ACKNOWLEDGEMENTS

The research was partially supported by grants of FAPESP

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Insecticide residues cross-contamination of oilseeds during storage

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ABSTRACT

Pesticide residues are found in oilseeds and crude oils: they are mainly organophosphate insecticides (pirimiphos-methyl, dichlorvos, malathion) used in empty storage facilities and for application to stored cereal grains. Even if pests are found in stored oilseeds, French regulations do not permit the use of these insecticides on stored oilseeds, as they have an affinity for these lipophilic substances. These residues arise from cross-contamination from storage bins and facilities, and not from illegal use. This uptake of insecticide residues from their storage environment by oilseeds can lead to levels that exceed regulatory limits. An investigation of 11 grain storage companies allowed us to follow the course of 27 sunflower seeds batches, from reception at the storage facilities to outloading. Samples from each of these batches, made at outloading, were analysed by ITERG (French Technical Institute for Oil Industry), looking for insecticide residues. Traceability of sunflower seeds established by storers allowed us to identify cross-contamination sources. Substances discovered were dichlorvos, pirimiphos-methyl and malathion (and one case with chlorpyriphos-methyl). Pirimiphos-methyl was most commonly detected, but most cases of non-accordance with regulatory levels were caused by dichlorvos and malathion. Main cross-contamination hazard resulted from treatment of cereals at outloading, just before sunflower seeds were outloaded, especially when these cereal treatments were frequent on that elevator. Other situations led to cross-contamination, but generally at lower levels: outloading of sunflower seeds after outloading of treated cereal, sunflower seeds stored in bin that contained previously treated cereals, empty bins and handling equipment treated before receipt of sunflower seeds.

Key words: cross-contamination – insecticide – oilseeds – pesticide residues – storage.

INTRODUCTION

Post-harvest insecticide residues are frequently found on oilseeds at low levels, although no insecticide is allowed to be applied directly to oilseeds during storage. Consequently, maximum residue levels (MRLs) allowed by European regulation are very low (mostly at the lower limit of analytical determination): 0.01 mg/kg for dichlorvos (still authorised during this study, but forbidden now), and 0.05 mg/kg for pirimiphos-methyl. No MRL exists for malathion, so it should not be found beyond the analytical limit of quantification. These insecticide treatments are authorised on stored cereals and corn as a grain protectant, and on empty storage and handling equipment as a control agent for residual insect populations in empty granaries. Dichlorvos, malathion and pirimiphos-methyl were the substances most employed during this study (storage season 2006-2007, regulations changed later).

So, we can hypothesise that cross-contamination phenomena can exist, between these various kinds of seeds, cereals and oilseeds, sharing the same grain handling and storage system. This phenomenon has already been demonstrated in Canada on rapeseed (Watter and Nowicki, 1982, 1985; White, 1983), when empty bins were treated with organophosphorous insecticides (bromophos, malathion, fenitrothion). Canadian storers were warned that treating bins before storing rapeseed could lead to residues above the maximum allowable limits.

Uptake of pirimiphos-methyl by a single-layer of rapeseed or wheat on galvanized-steel surfaces was demonstrated in a laboratory study (Dauguet et al., 2006, 2007). It was shown that, for small bins (less than 50 tons), it could lead to residue quantities above regulatory limits. But in big elevators, insecticide uptakes by seeds can also occur at other stages: conveyor belts, handling of oilseeds after cereals had been treated in the same circuits, outloading bin, etc. Therefore we cannot rule out either risk for grain storage companies.

In order to improve our knowledge about this post-harvest insecticide cross-contamination, especially in big elevators, an investigation was carried out with the collaboration of several French grain storage companies. Real cases were observed, with an accurate traceability of sunflower seeds lots all along their route inside storage facilities (from receipt to outloading) to find where the insecticides were taken up by the oilseeds.

Results presented in this article were obtained in the first year of the investigation, concentrating on sunflower seeds during the storage season 2006-2007. This investigation will continue on rapeseed during the next storage season.

MATERIALS AND METHODS

The process adopted for this survey was:

- Identifying with storage operators sunflower lots that could be "traced" (recording of each step from receipt to outloading):11 grain storage companies agreed to collaborate, and allowed us to follow 27 sunflower seed bins. These companies were situated throughout the French sunflower crop area.
- Making a mean sample from each batch representative of sunflower seeds arriving at the storage facilities ("first sample") and preserving it. These samples were analysed only when residues were found in the final sample, in order to know if contamination occurred before receipt of grain. These "first samples" were analysed for 4 batches of seeds.
- Making a mean sample representative of outloaded sunflower seeds, "final sample", when the traced lot is commercialized (from one to eight months after harvesting). These "final samples" were always analysed. In one case, we had 2 samples for one sunflower batch, so that we analysed 28 final samples. The sampling method used was based on a standard method (moving seeds, for contaminant with heterogeneous distribution determination, prEN ISO 24333:2006): 25 elementary samples for 500 tons evenly distributed during the outloading (one elementary sample each 20 tons). This method was usually well observed by the commercial operators.
- Filling in a questionnaire called "traceability" which recorded each step from receipt to outloading. Operators had to indicate if treatments were applied on empty bins or handling equipment, or if cereals were treated at their receipt or outloading and if these cereals used the same conveyer circuit inside the storage facilities just before the sunflower seeds.
- Determination of insecticide residues in all the "final samples": the analytical laboratory of ITERG conducted these determinations, using the "common method" developed three years ago by a group of about twenty French laboratories (public and private) coordinated by CETIOM and ITERG: Soxhlet extraction of oil with hexane (NF EN ISO 659) was followed by analysis of organophosphorous residues by gas chromatography with NPD detection.

RESULTS

Twenty-eight samples were analyzed (Table 1). The insecticides used on cereals and for storage facilities treatment were detected: dichlorvos, pirimiphos-methyl, malathion and chlorpyriphos-methyl (only one case). Most commonly detected substance was pirimiphos-methyl: detected in 61% of samples and quantified in 39% of samples. But, malathion and dichlorvos were more frequently above MRL: 21% and 18% of cases, respectively.

On the whole, final samples were slightly contaminated as half of them contained less than 12 µg/kg of insecticide residues (sum of residue median), and 90% of them contained less than 120 µg/kg (sum of residues 9th decile).

Table 1. Analytical results (expressed in µg/kg) on the 28 final samples[1]

	LQ	MRL	Mean	Median	Standard deviation	9th decile	Maxi	% samples ≥ LD	% samples ≥ LQ	% samples > MRL
Dichlorvos	10	10	21	0	79	27	422	32%	29%	21%
Pirimiphos-methyl	10	50	19	5	55	29	295	61%	39%	4%
Chlorpyriphos méthyl	10	50	0	0			10	4%	4%	0%
Malathion	10	-	8	0	25	17	125	18%	18%	18%
Sum of residues			48	12	102	120	427			

[1]LQ: limit of quantification; LD: limit of detection; MRL: maximum residues limits in sunflower seeds; Sum of residues: a value of 5 µg/kg is given when a substance is detected but below the limit of quantification, and zero value if under the limit of detection.

Analytical results for each substance (Fig. 1, Fig. 2, Fig. 3): Pirimiphos-methyl - Only one sample had a very high level (T12: 295 µg/kg). The other samples were always below the MRL: 4 between 20 and 50 µg/kg, 12 between 10 and 20 µg/kg, and 11 below the limit of quantification; Dichlorvos - Only one sample had a very high level (T9: 422 µg/kg). Three other samples were between 20 and 50 µg/kg.

Five samples were near the MRL or below it. It was not detected in 19 samples; Malathion - Only one sample had a high level (T28: 125 µg/kg). Four samples were between 10 and 50 µg/kg, 12 between 10 and 20 µg/kg. It was not detected in 23 samples.

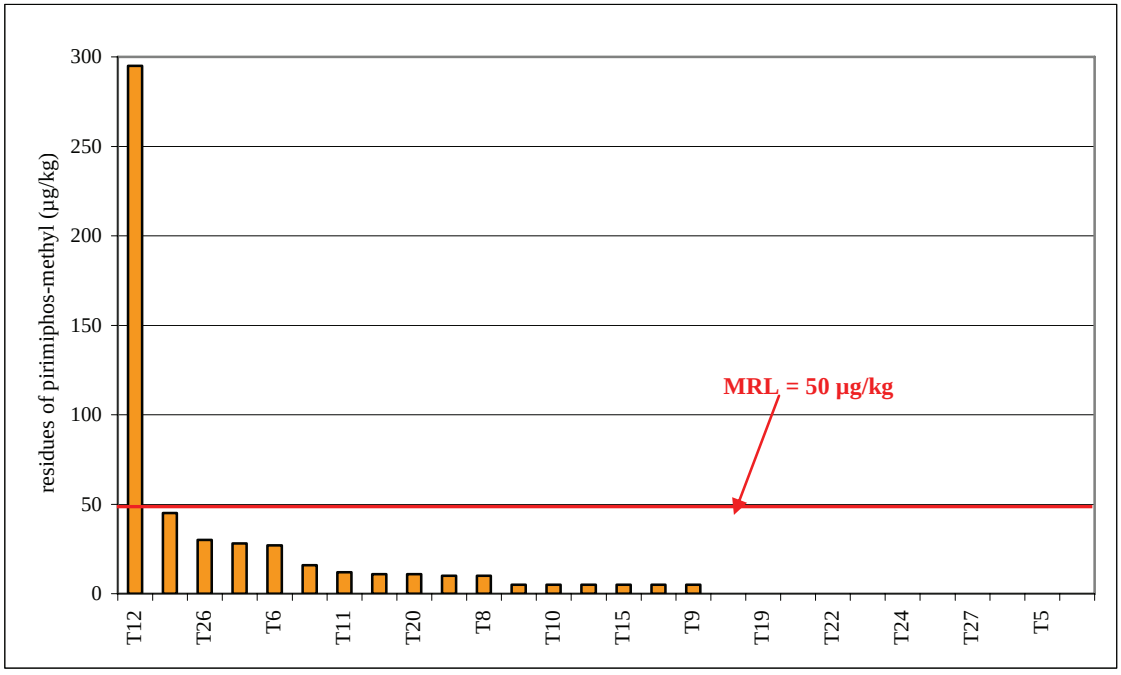

Fig. 1. Individual analytical results for pirimiphos-methyl

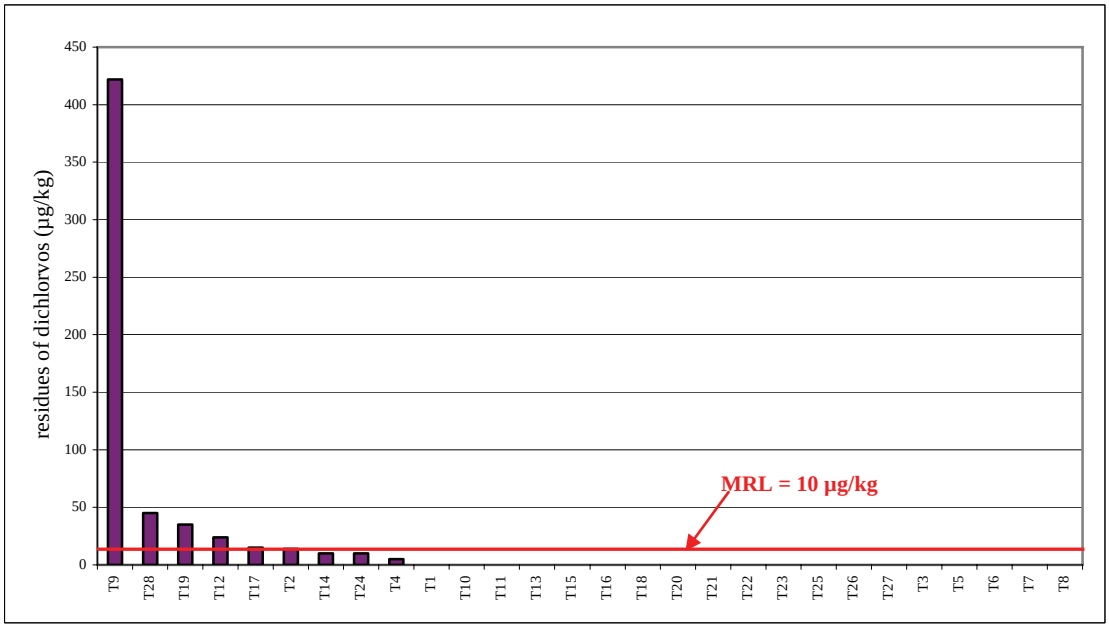

Fig. 2. Individual analytical results for dichlorvos

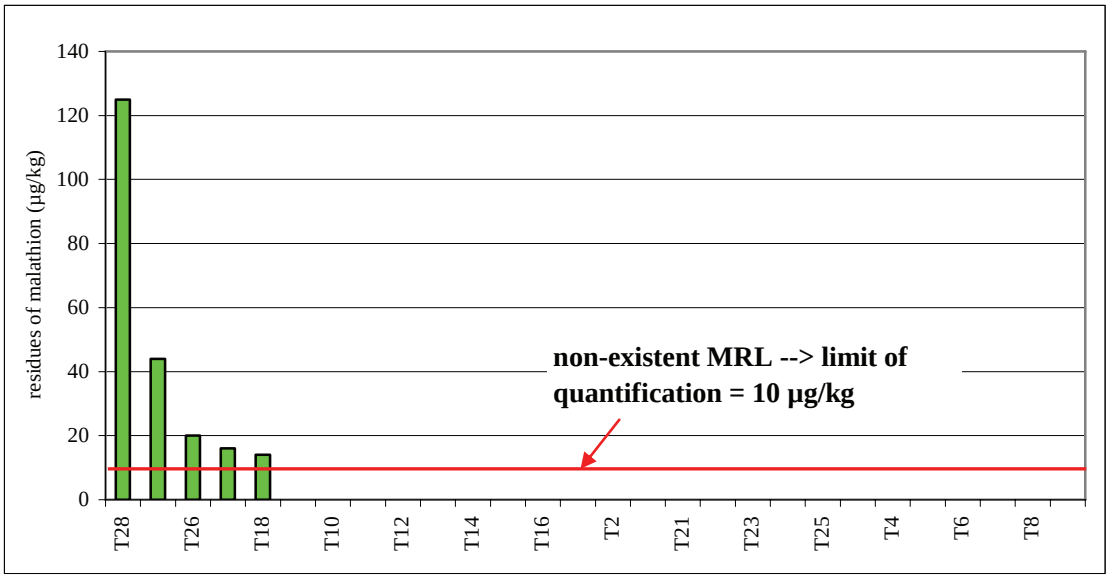

Fig. 3. Individual analytical results for malathione

In order to explain the contamination, when large residues were detected, the "first samples" (taken at receipt of the sunflower seeds at the storage facilities) were analyzed for the batches T4, T12, T19 and T28. The insecticide residues on each "first sample" were too low to explain the residues found at the end, in the "final samples". So, the explanation had to be found in the route of the sunflower seeds inside the elevator.

Four cases leading to cross-contamination were identified:

- K1: treatment of cereals at outloading, just before outloading of sunflower seeds
- K2: outloading of cereals, treated at their receipt, just before outloading of sunflower seeds
- K3: storage of treated cereals in the same bin just before storage of sunflower seeds
- K4: treatment of empty bin and of handling equipment before receiving sunflower seeds

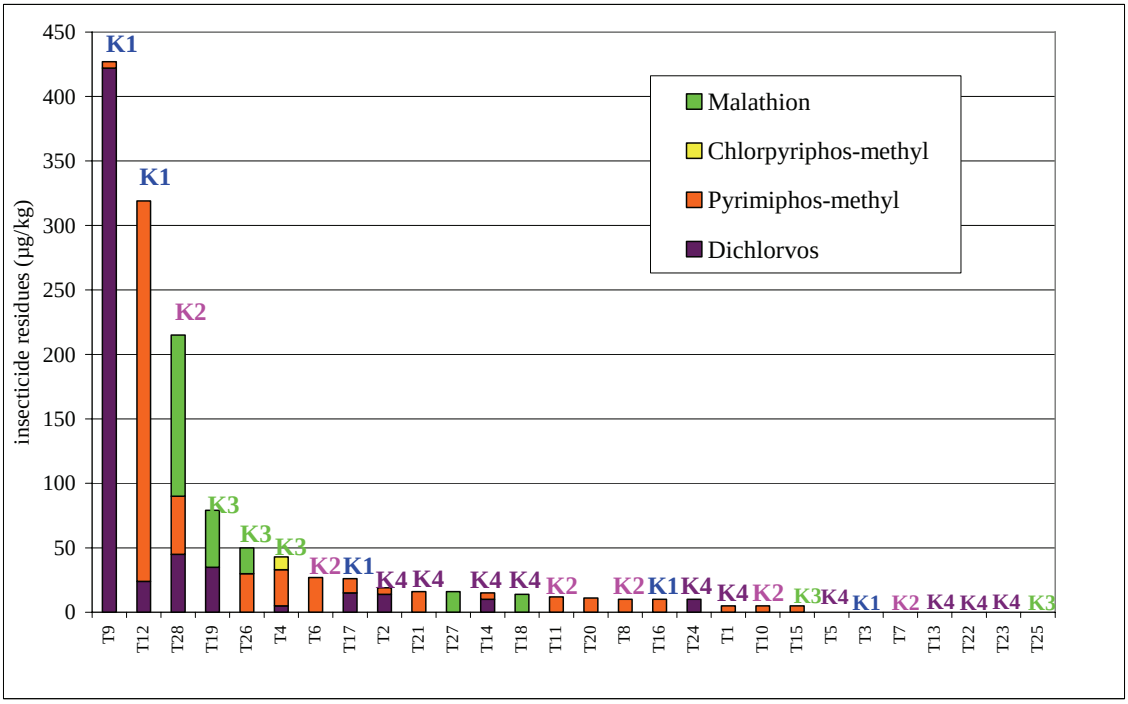

Fig. 4. Distribution of the four cases (K1, K2, K3, K4) for each sunflower lot, and sum of insecticide residues (µg/kg) for each sunflower final sample

It would seem that the biggest cross-contamination occurred with the case K1. Studying the circumstances of K1, the risk was higher when treatment of cereals at outloading was frequent or systematic. In the worst case (T9), the sunflower seeds batch was transported by lorry over a period of two weeks. Cereals were also sent during this period, and treated before outloading, and using the same outloading circuit as the sunflower (conveying belts, outloading bin). The other cases, K2, K3, K4, may also lead to a slighter cross-contamination. For one lot, there were two cases of a cross-contamination risk, and this could only have worsened the contamination.

CONCLUSIONS

Our study in real situations showed that cross-contamination of oilseeds by post-harvest insecticide residues exists, and can sometimes lead to residues above the regulatory limits.

The highest risk of contamination appeared when cereals were systematically treated at outloading, just before outloading of oilseeds, using the same conveyor circuits. The other identified cases may also lead to a slighter contamination. But, silo operators should concentrate on the accumulation of several risky cases, which could worsen the contamination.

Other sources of insecticide residues can occur in storage facilities, but we could not check them in this investigation. They include leak of insecticide from the application equipment, use of sampling equipment contaminated by pesticides. Another situation that we did not meet in our study was cross-contamination or accidental treatment on-farm of oilseeds subsequently delivered to a commercial store.

This investigation will be continued with rapeseed. This will allow us to check if rapeseed can be affected by the same cross-contamination as sunflower. The new work will be carried out in the new regulatory context in which dichlorvos and malathion are forbidden for cereal treatment. Thus, storage operators will certainly have new grain protection strategies, which could lead to a different cross-contamination risk for oilseeds.

ACKNOWLEDGEMENTS

This work was funded by ONIDOL (French oilseed chain organization) and FEDIOL (European oil and protein meal industry federation), with additional technical support from FNCG (French national oil industry federation), ITERG (French technical institute for oil industry), FNA (Fédération du Négoce Agricole), Coop de France-Métiers du grain, SAIPOL and CARGILL.

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Evaluating irrigation performance of sunflower in an irrigation scheme of Southern Spain

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ABSTRACT

Current water resource scarcity implies a change in the water management in the Spanish irrigation schemes. Assessment of irrigation performance over long time periods is a prerequisite for improving water use in the agricultural lands to respond to the water scarcity. We carried out a comprehensive assessment of the irrigation performance by documenting the water use of fields cultivated with sunflower at the Genil–Cabra Irrigation Scheme (GCIS), located in Andalusia, Southern Spain, from 1991 to 2007. We have used a model that simulates water balance for every field and three performance indicators to assess the performance of irrigation water use and management in the GCIS. Among the performance indicators, the average ratio of measured irrigation supply to the simulated optimum demand (ARIS) for sunflower ranged between 0.09 and 0.34, indicating that the crop was under water stress. The average Irrigation Water Productivity (IWP) provided low values for this crop, although in the last years it has reached higher values due to the recent increase of international market prices, with similar values to maize or wheat. Remote sensing techniques, based on using a satellite-based energy balance called METRIC, were used to obtain actual evapotranspiration maps during the 2004/05 irrigation season. Seasonal ET variability was found to be large between sunflower fields and also within sunflower fields in the GCIS. The current policies of subsidies in sunflower force the farmers to obtain a maximum profit from the irrigation water rather than a maximum yield, a scenario that must be considered when planning programs of water conservation.

Key words: deficit irrigation – evapotranspiration – irrigation management – water allocation – water supply.

INTRODUCTION

Sunflower occupies a large surface of the area cultivated in Andalusia. The high volume of crop water demand and a reduced water supply force farmers to use different strategies from irrigation water management. With the constant increase in water demand from other sectors of society, assessing water management is indispensable for proposing improvements in irrigation management and for quantifying water productivity (Molden and Sakthivadivel, 1999). There are different performance indicators for irrigation water use that require some information related with the irrigation applied by the farmers. However a detailed water-use record from each plot is often not available, which makes it impossible to carry out a good analysis of water management. Records of irrigation water use are required to obtain additional information, helping to estimate all water-balance components in fields, and which can be evaluated using a simulation model, hereafter named LORMOD, which can be employed to assess the actual performance and water management (Lorite et al., 2004a).

Most of the consumption of irrigation water corresponds to evapotranspiration (ET), the loss of water from the earth's surface through the combined processes of evaporation and transpiration. Therefore, the spatial and temporal quantification of ET is essential in agricultural water management. As a recent remote sensing technique, accurate ET estimations have been obtained using a satellite-based energy balance (Bastiaanssen et al., 1998; Allen et al., 2007). METRIC (*Mapping EvapoTranspiration with high Resolution and Internalized Calibration*) is an ET estimation model (Allen et al., 2007) based on the SEBAL (*Surface Energy Balance Algorithms for Land*) model of Bastiaanssen et al. (1998).

The objective of this work was to conduct a comprehensive assessment of the irrigation performance of sunflower, compared with maize and wheat, in an area using on-farm water-use information and a simulation model, as well as an ET estimation model (METRIC). The area selected was the Genil–Cabra Irrigation Scheme (GCIS) located in Andalusia, Southern Spain. This irrigation scheme was chosen because it disposes of accurate information on water use and on the cropping patterns of individual plots since the start of its operations (1990/1991) until present.

MATERIALS AND METHODS

The study area was located within the Genil-Cabra Irrigation Scheme (GCIS), in Cordoba province, Southern Spain (37º 31' N, 4º 51' W). The climate in this area is typically Mediterranean with an annual average precipitation of 606 mm and a rainless summer. The average air temperature ranges from 10 ºC for the coldest month to over 27 ºC for the warmest.

The study was carried out during 16 irrigation seasons (1991/1992 to 2006/2007). Daily meteorological data to estimate Penman–Monteith ASCE reference evapotranspiration (ET_0) and rainfall were obtained from a meteorological station located within the GCIS. Information about the cumulative water-meter for each plot was obtained by individual readings four/five times per irrigation season. Likewise, the information about irrigation practices, water supply and sowing dates was provided by the irrigation scheme manager or directly from farmers (Lorite et al., 2004a). Only the plots with a single crop were selected for this study.

A water-balance model was developed by Lorite et al. (2004a) to simulate water use in the GCIS. LORMOD is composed of sub-models that calculate the different water-balance components and estimate the effects of water stress on crop yield. It calculates the soil water balance components for each computation unit on a daily basis, generates optimum irrigation schedules and compares the optimum schedules for each field against the actual irrigation schedules, which were simulated by basing them on water-meter readings.

For each field, to assess the evolution of irrigation management and benchmarking, the Annual Relative Irrigation Supply (ARIS) was chosen, defined by Malano and Burton (2001) as:

$$\mathrm{ARIS} = \frac{\text{Annual volume of irrigation water inflow}}{\text{Annual volume of crop irrigation demand}} \quad (1)$$

Another indicator computed here was the Crop Yield Ratio (CYR; Bos et al. 1994), that relates the actual crop yield to the intended yield, defined as the attainable crop yield with optimum economic irrigation, defined as:

$$\mathrm{CYR} = \frac{\text{Actual crop yield}}{\text{Intended crop yield}} \quad (2)$$

To evaluate the productivity of the water used in irrigation in this area, the indicator considered was the Irrigation Water Productivity (IWP; Lorite et al., 2004a), defined as:

$$\mathrm{IWP} = \frac{\text{Increase in Annual Value of Agricultural Production due to Irrigation}}{\text{Annual Volume of Irrigation Water Inflow}} \ (€/m^3) \quad (3)$$

In this indicator, the numerator is computed as the difference between actual crop yields under irrigation minus rainfed yields. It is assumed that management does not change much as the grower shifts from rainfed to irrigated conditions, which is probably the case for the GCIS.

Satellite-based energy balance estimation of crop ET (METRIC)

Eleven Landsat 5 TM images were processed using the METRIC energy balance computation procedure of Allen et al. (2007) to obtain daily ET for each image date. The model METRIC estimates ET as a residual of the energy balance at the surface:

$$LE = R_n - G - H \quad (4)$$

where LE is the latent energy consumed by ET, R_n is net radiation, G is sensitive heat flux into the soil, and H is sensitive heat flux to the air. Details of the METRIC model are given in Allen et al. (2007) and Tasumi et al. (2005).

We define a crop coefficient, $K_{c\ act}$, as the ratio between actual ET estimated by METRIC, and the grass reference ET (ET_o) calculated following the ASCE standardized Penman-Monteith method (ASCE-EWRI, 2005). This $K_{c\ act}$ differs from the standard K_c (Allen et al., 1998) in that our actual ET estimate is usually below the maximum ET due to agronomic factors. Weather data for calculating ET_0 were provided by five automatic weather stations located close to the GCIS. These weather stations are part of the Agroclimatic Information Network of Andalusia (Gavilán et al., 2006).

RESULTS AND DISCUSSION

Throughout the period of study it was observed that the water applied to sunflower was different from year to year, due to variations in the precipitation and water availability (Fig. 1). Thus, the average annual rainfall in the periods 1991-1995 and 1999-2005 was smaller than 500 mm. Nevertheless, in the first period the water supply was very restricted (≈ 700 m^3 ha^{-1}), whereas in second the average water supply was three times bigger (≈ 2600 m^3 ha^{-1}) than in the first period. These policies importantly affected the volume of available water, that was little or null during the period 1992-1995 and increased significantly in the period from 1999 to 2005. In 1998/1999 irrigation season, the low precipitation forced all farmers, including those who did not usually apply water to sunflower, to increase the irrigation applied, favoured by the available water supply of that year, which was higher than average (Lorite et al., 2004a). For this reason, the variation between the fields in the annual volume of water applied, quantified by the coefficient of variation, diminished considerably in 1998/1999 irrigation season.

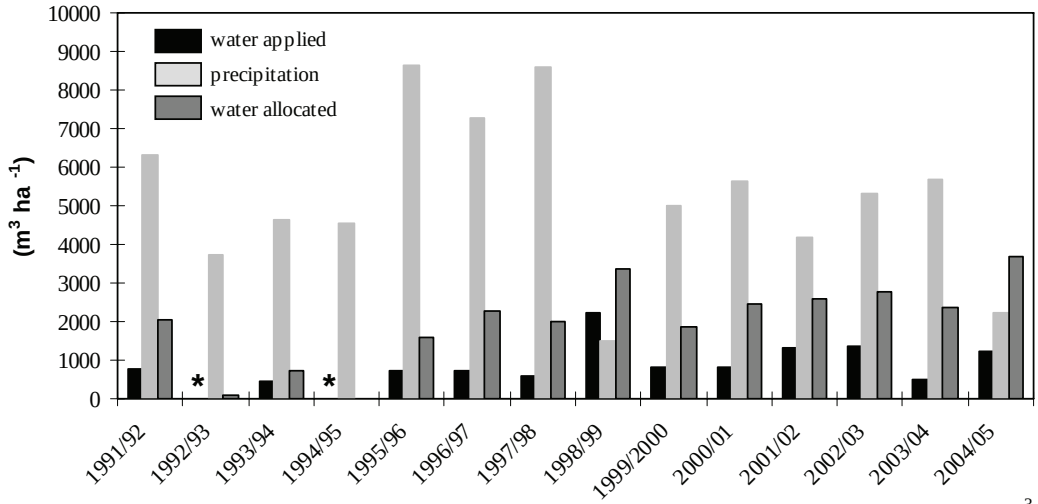

Fig. 1. Evolution of average irrigation water use, irrigation water allocation, and precipitation (m^3 ha^{-1}) from 1991/1992 until 2004/2005 irrigation seasons. * During 1992/1993 and 1994/1995 irrigation depth applied was practically null.

The limited water supply available during the period 1991-1995 caused an increase in sunflower and wheat areas (Fig. 2). However, during the following years the absence of restrictions caused a reduction in those areas, whereas there was a continuous increase in the one devoted to maize.

Performance indicators of irrigation water use

The average ARIS for sunflower and for wheat was very low during the whole period (0.23 and 0.26 respectively; Fig. 3A) with respect to maize, where the ARIS experimented a constant increase up to values close to 1, which represented an optimum irrigation. The low values of ARIS for sunflower and wheat suggest that these crops received below 30% of the maximum potential evapotranspiration. In dry seasons (e.g. 1998/1999), the values of ARIS increased for sunflower and wheat, although this increase does not signify a linear relation between ARIS and the amount of precipitation, as shown by García-Vila

et al. (2008). However, these authors observed that an increase in precipitation caused a clear decreasing of ARIS in cotton, sugar beet and garlic. Low values of the ARIS in sunflower show that most of the farmers consider that this crop could be cultivated as a rainfed crop, or with a small irrigation supply. On the other hand, the low ARIS values observed in the 16 seasons of study (smaller than 0.25; Fig. 3A) suggest that the crop remained under a constant water stress. One of the reasons for this water management is that the current policies of subsidies in sunflower are based on the cultivated area and not on yield. Therefore, for farmers it is more beneficial to obtain a maximum profit from the irrigation water rather than at maximum yield (Lorite et al., 2004b). The ARIS for sunflower showed a high variability, with an average variation coefficient of 1.25, although in the driest year of the period (1998/1999) it descended to 0.88 (Fig. 3B).

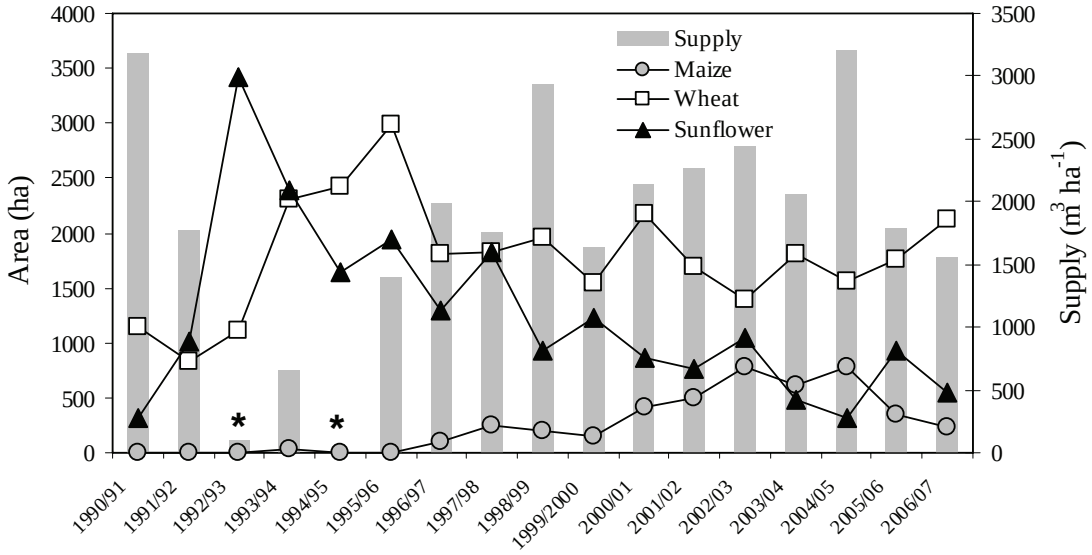

Fig. 2. Evolution of the area cultivated with maize, wheat and sunflower (ha) and irrigation water allocated (m^3 ha^{-1}) in GCIS from 1991/1992 until 2006/2007 irrigation season. *During 1992/1993 and 1994/1995 irrigation depth applied was practically null.

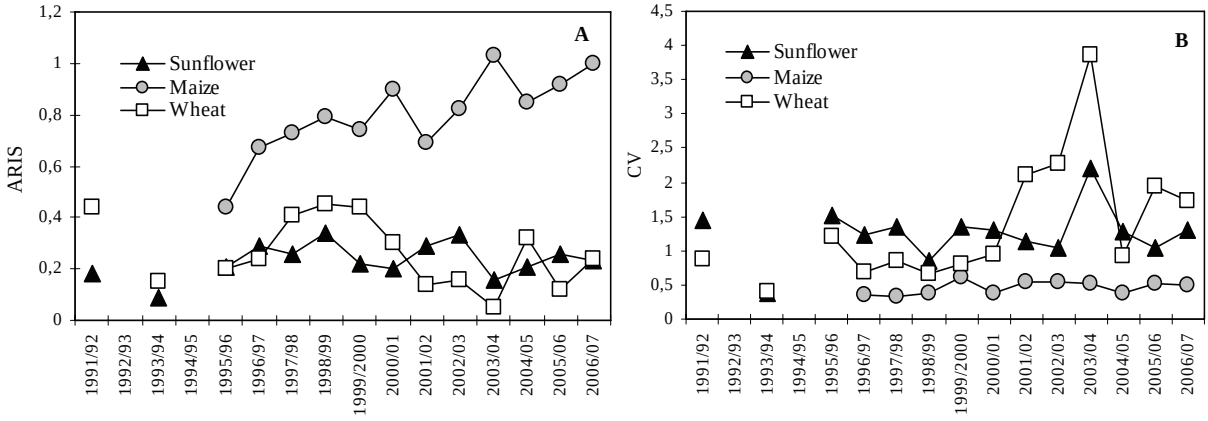

Fig. 3. Evolution of ARIS (A) and coefficient of variation (B) values for sunflower, wheat and maize from 1991/1992 to 2006/2007 irrigation season.

CYR values for sunflower ranged between 20% and 70% depending on the annual rainfall, although the average was 40%, evidencing that the production obtained during the whole irrigation season was smaller than 50% of that attainable, as observed by Lorite et al. (2004b). This confirms that the farmers are not interested in obtaining the maximum yield, and prefer to allocate the available water to other crops such as cotton or maize.

The IWP for sunflower, wheat and maize is presented in Fig. 4A. The average values were low in the three crops (sunflower, 0.19 €/m^3; wheat, 0.26 €/m^3; maize, 0.24 €/m^3), indicating that the application of irrigation here did not generate an increment in gross income, compared with rainfed production. However, in the last few years sunflower values have reached higher values due to its increase in international market prices. Comparing Fig. 3A and 4A, sunflower obtained similar values of IWP compared with maize, but with a significantly lower irrigation consumption. Thus, a deficit in irrigation for sunflower could be considered as a correct alternative compared with other crops such as maize.

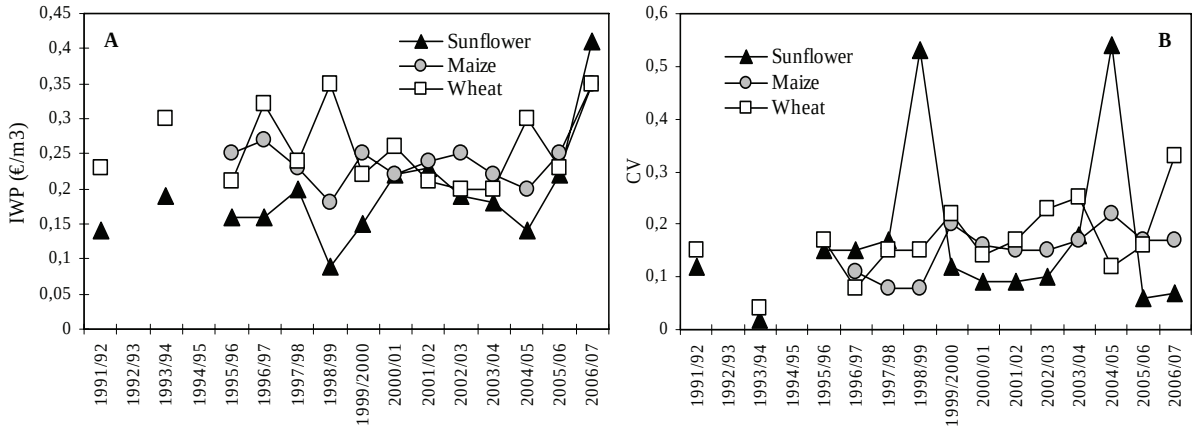

Fig. 4 Evolution of IWP values and coefficient of variation for sunflower, wheat and maize from 1991/1992 to 2006/2007 irrigation seasons.

Seasonal ET variability and crop coefficients for sunflower in the GCIS

The seasonal ET estimated with METRIC for all the plots in the GCIS for the 2004/05 irrigation season ranged from more than 1000 mm for well-irrigated fields, to almost zero for non-agricultural areas (Santos et al., 2008). Crop coefficients for individual fields were estimated as the ratio between METRIC ET and ET_0. Fig. 5A shows real crop coefficient values for different sunflower fields within GCIS, obtained with ET estimated by METRIC.

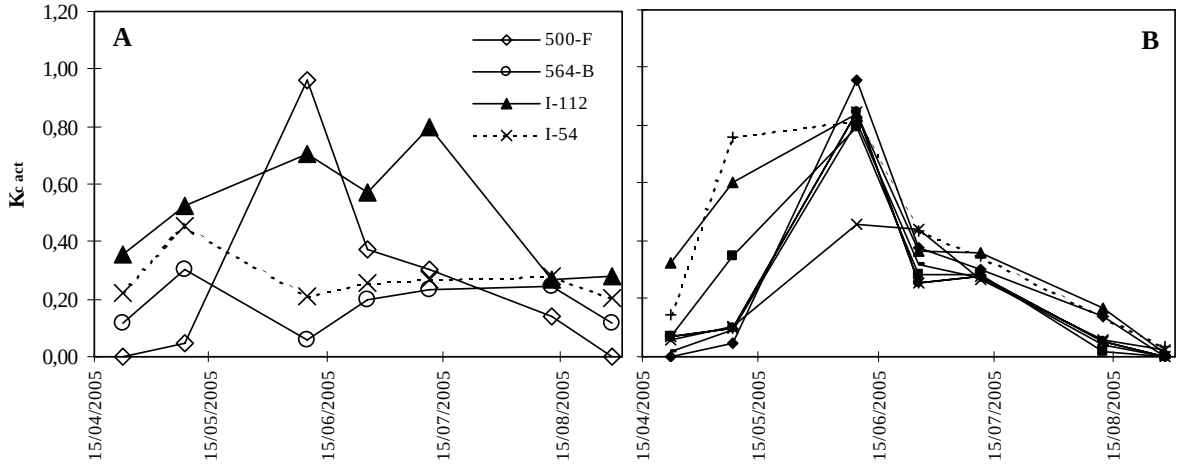

Fig. 5. Real crop coefficient curves for four sunflower fields within GCIS (A) and for one sunflower field having enough size to contain more than one thermal pixel with valid METRIC ET estimates (B).

ET variability was high in sunflower, with a variation coefficient of 0.28, while average ET was low (378 mm). This high variability in actual ET can be explained by the plot to plot variability in irrigation and crop management in the GCIS, as characterized previously by Lorite et al. (2004b).

In the plots with enough size to contain more than one thermal pixel with valid METRIC ET estimates, the ET variability within fields was assessed (Fig. 5B). The variation coefficient within fields

for sunflower was 0.13, which means higher variations than 160 mm (44% of seasonal ET) within a sunflower field, caused by emergence problems, very limited irrigation applied, etc.

In conclusion**,** the study of performance indicators for sunflower at the GCIS showed that this crop is frequently under a clear water stress, and this irrigation management has an impact on yield, which is usually lower than expected. The performance indicators analysed indicated a high variability during the irrigation seasons and between different sunflower fields. The high variability was confirmed with remote sensing techniques using the METRIC model to obtain actual ET measures.

In spite of the low level of inputs provided to the crop, irrigation productivity for sunflower in the last few years has provided similar values than for other crops such as maize, but with very low irrigation requirements.

ACKNOWLEDGEMENTS

We wish to express our gratitude to Mr. J. Berlanga (District Manager), Mrs. S. Carmona (District Technician) for their support. We also wish to thank the farmers of the Genil–Cabra Irrigation Scheme for the information provided.

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Size reduction of ornamental sunflowers by the application of daminozide

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ABSTRACT

The expansion of floriculture in Brazil in the last years is due to the development and structuring of new markets, professionalization of the components of the production chain and the more extensive purchasing of flowers and ornamental plants. The sunflower (*Helianthus annuus* L.), a species used for oil production, for bird feed and as silage for livestock, has gained a place and distinction among ornamental plants for cut flowers. In order to make ornamental sunflowers suitable for commercial production, technologies are needed that can adapt the species to greenhouse cultivation. Sunflowers are naturally tall plants, which is unsuitable for ornamental purposes. In order to facilitate their production in a protected environment as well as in the field, inhibitors of gibberellin synthesis can be utilized to reduce the size of sunflower plants. The aim of this study was the reduction in size of the ornamental sunflower hybrid BRS Oasis by the application of daminozide (B-Nine 850 PS™) at fifteen days after planting, testing different concentrations. The concentrations evaluated were 4,000, 6,000 and 8,000 $mg.L^{-1}$, which were compared to a control using water. The results obtained demonstrated that the size of the plants treated with the three concentrations of daminozide was smaller than that of the control. Therefore, for economical reasons, the use of 4,000 $mg.L^{-1}$ of daminozide is suggested.

Key words: B-Nine – floriculture – gibberellin inhibitor – *Helianthus annuus* – plant growth regulator – ornamental plant.

INTRODUCTION

The Brazilian market for flowers and ornamental plants has shown a substantial growth in demand and has been expanding in the last few years with the improved quality of products and an increased commercialized volume. It has responded positively to the offering of new products, thereby stimulating research into breeding and cultivation treatments. Sunflowers have a great potential as an ornamental plant because of their short growing cycle and easy propagation, but mainly because they have attractive inflorescences that are much sought after as cut flowers (Dasoju et al., 1998; Anefalos and Guilhoto, 2003). In the local market, although there is no official classification, ornamental sunflower inflorescences of 8-11 cm in diameter are commercialized as small flowers, those of 12-16 cm in diameter as medium flowers, and those more than 16 cm in diameter as large flowers (NAIR MIE NOMI, 2007).

The ornamental sunflower BRS Oasis, developed by Empresa Brasileira de Pesquisa Agropecuaria (EMBRAPA), has inflorescences with ornamental characteristics (Oliveira and Castiglioni, 2003), which allow it to be used as a cut flower. However, its size, which can reach up to 3 m in height, is unsuitable for its eventual purpose. To have a production and offering of ornamental sunflowers as cut flowers during the whole year in regions with a temperate climate, where frost occurs, it is necessary to grow the plants in a protected environment or greenhouse, demanding the development of techniques to reduce the size of the plants.

Inhibitors of gibberellin synthesis are widely used in floriculture to reduce the size of various ornamental plants (Whipker, 2001). One the most common agents for this purpose is daminozide (N-dimethylaminosuccinamic acid) (Weaver, 1972; Fahl et al., 1985). The ideal concentration of the plant growth regulator depends on the plant species, variety, number of applications made and size of the plant at the time of application (Lopes, 1977). Daminozide has been recommended for the size reduction of ornamental plants at concentrations varying from 2,000 to 8,000 $mg.L^{-1}$ (Hertwig, 1977; Nell et al., 1980).

The aim of this study was to reduce the size of ornamental sunflower plants, variety BRS Oasis, by the application of daminozide (B-Nine 850 PS™) at fifteen days after planting, testing different concentrations.

MATERIALS AND METHODS

This investigation was carried out in the period of February to May of 2006 in the municipality of Fazenda Rio Grande (PR, Brazil), located 25º37'32"S and 49º15'29" W and having an altitude of 910 m. The effect of the application of the plant growth regulator daminozide (B-Nine 850 PS™) was studied at concentrations of 4,000, 6,000 and 8,000 mg.L^{-1}, in the BRS Oasis hybrid of the ornamental sunflower, *Helianthus annuus* L., which is single-headed with male sterility, and has yellow disc florets and brownish ray florets. In the control treatment, water was applied under the same conditions in which the plant growth regulator was applied. The application of the regulator was effected at fifteen days after planting, when the plants showed two pairs of definitive leaves.

A complete randomized block design was used with four treatments and four replications, where ten plants were studied per parcel. The variables analyzed were: plant height, determined from the level of the soil to the point on the stem of the inflorescence; the stem diameter, determined at fifty centimeters below the inflorescence; and the head diameter. These variables were evaluated when 50% of the plants were with completely expanded ray florets and all the disc florets visible, corresponding to phenological stage R5.5 (Schneiter and Miller, 1981). The data obtained were submitted to analysis of variance. Initially, the variances of the treatments were determined with respect to their homogeneity by Bartlett's test. All variances were shown to be homogeneous, where the transformation of the data was not necessary and the means of the treatments were evaluated using the F test. When the results revealed the existence of significant differences between the means of the treatments, these were compared by Tukey's test at a significance level of 5%.

RESULTS AND DISCUSSION

The evaluations were carried out at 62 days after planting. There was a significant difference among the treatments for all the variables analyzed (Table 1). Although there was a significant difference in the height of the plants treated with the three concentrations of daminozide versus the control, no difference was seen between concentrations. The results agree with those of various authors who obtained a significant reduction in size of ornamental plants utilizing concentrations between 4,000 and 8,000 mg.L^{-1} (Cathey, 1975; Nell et al., 1980; El-Keltawi et al., 1996). Similarly, these findings agree with authors who obtained significant size reductions in ornamental plants utilizing an application of daminozide in *Ruellia colorata* L. at a concentration of 4,000 mg.L^{-1}, achieving a decrease in stem height of 13.85% (Carlucci, 1991) and in *Viola* × *wittrockiana* L applying daminozide at a concentration of 5000 mg.L^{-1}, obtaining a decrease of 18.94% in stem height (Gliožeris et al., 2007).

Table 1. Stem height (SH), stem diameter (SD) and head diameter (HD) of the ornamental sunflower BRS Oasis, after application of daminozide at different concentrations 15 days after planting (Fazenda Rio Grande, PR) in May 2006**.**

Concentration of daminozide (mg.L^{-1})	SH[1] (m)	SD[1] (cm)	HD[1] (cm)
0	2.300 a	1.274 a	9.20 a
4000	2.010 b	1.185 b	8.33 b
6000	1.986 b	1.144 b	8.21 b
8000	1.861 b	1.119 b	8.13 b
DMS	0.12	0.50	0.4

[1]Means followed by different letter in the column differ statistically, based on Tukey's test at the 5% level of probability.

Height diameter (Table 1) at all the concentrations tested was smaller than that of the control. Although there are no published reports on the effect of reducing stem diameter on the quality and life of post-harvest flowers of ornamental sunflower, this has been determined for chrysanthemum (*Dendranthema grandiflora* Tzevelev) (Nardi et al., 2001).

The head diameter (Table 1) at all the concentrations tested was smaller than that of the control. Considering that the ornamental sunflower market pays differently for flowers of smaller diameter, this could be a problem. However, according to the informal local standard for classification of ornamental

sunflowers (NAIR MIE NOMI, 2007) the diminution of the diameter of the flowers would permit them to be classified as medium inflorescences.

Considering that the reduction in the size of the plants was similar at the three concentrations of daminozide tested, it is suggested that, after the evaluation of the post-harvest quality of the flowers, 4,000 mg L^{-1} of daminozide be utilized for economic reasons.

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Participatory on-farm sunflower variety evaluation in northern and eastern Uganda

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ABSTRACT

Sunflower genotypes were evaluated on farmers' fields between 2005 to 2007 growing seasons in eastern and northern parts of Uganda. The evaluation covered five districts. Researchers worked with extension and private sector staff whereby the latter identified the farmers and also helped in monitoring the trials. Each genotype was planted in a single plot of 5m in length with 4 to 6 rows per farmer field. For each variety, the plot was divided into two blocks so that one block received N60 P30 kg/ha using urea and single superphosphate as source of nitrogen and phosphorous, respectively; meanwhile, the other block had no fertilizer application to compare the effect of fertilizer application on the different genotypes. The main data recorded included seed yield (kg/ha), plant height, head diameter, 1000 seed weight, uniformity, vigour, lodging and maturity. Results showed that the better genotypes were sunflower hybrids from South Africa which included Pan 7351 from Pannar Seed Company; AGSUN 8251 and AGSUN 5383 from AGRICOL Seed Company; DKF68-22 and DK4040 from Monsanto Seed Company. It was observed that plots where fertilizers were applied improved their seed yield, plant height and head diameter significantly. The highest seed yield was by DKF68-22 with 3,556 kg/ha from Bunambutye, in Sironko district. In most areas, hybrids such as DK4040, DKF68-22, and AGSUN 8251 performed better than PAN7351, which was officially released in Uganda in 2003. Because of their good performance from these trials on farmers' fields, those three new varieties were also officially released for commercial production in Uganda in 2007.

Key words: farmer participation – sunflower– variety evaluation

INTRODUCTION

The cultivated sunflower (*Helianthus annuus* L.) ranks with soybean, rapeseed and groundnut as one of the four most important annual crops in the world grown mainly for edible oil. Sunflower is grown in around 20 million hectares in the world. Average grain yield in the world is around 1.1 t/ha, varying from 0.5 to 3.6 t/ha (Krizmanic et al., 2006).

In Uganda, sunflower has become the most important oilseed crop. The other oilseed crops are sesame, soybean, groundnut, and oil palm. It is mainly grown in the eastern and northern parts of the country, where it has become an integral part of the farming system. The crop is primarily cultivated for its grain, which is used for oil extraction and production of animal feeds. The extracted oil is generally used as a cooking medium and in the manufacture of soap.

Uganda's interest in sunflower production dates back to the late 1940's (Bua and Molo, 1985). However, research activities were minimal and varietal evaluation was on imported hybrids and open-pollinated varieties. Up to the late 1990's, evaluation of sunflower varieties and hybrids were done on trial verification centers (TVCs) located across regions in the country. These trial centers, owned by the government, although good for understanding the performance of the different varieties across locations, do not present the attitude and criteria of selecting varieties by farmers. Selection of varieties by farmers themselves leads to a wider acceptability of that variety. Researchers in most cases rely on results from on-station research when evaluating variety performance. The problem is that on-station conditions rarely mirror the farmer's production constraints, that include demands of manual cultivation, input shortages and limited labor supply and yet they have to grow the crops (Laker-ojok, 1994). The Vegetable Oil development Project (VODP) impact assessment report (VODP, 2007) indicated that the reasons for choice of type of sunflower variety demanded by farmers in Uganda were higher yields (60%), tolerance to weather and diseases (18%), high oil content (13%) and fast maturity period (9%).

The objective of this paper is to present the results and the methodology used in the participatory evaluation of the sunflower varieties planted together with the farmers in their own fields.

MATERIALS AND METHODS

The evaluation trial undertaken on farmers' fields was carried out between 2005 to 2007 growing seasons in eastern and northern parts of Uganda. A collaborative approach was undertaken whereby agricultural extension staff and private seed sector staff working in the localities of the farms identified the farmers or farmer groups who were active in cultivating sunflower in their area. Each variety was planted in single plots of 4 or 6 rows at a length of 5 m. In some cases, two blocks were organized so that one block was applied with fertilizer at a rate of N60 P30 kg/ha using urea and single super phosphate as source of nitrogen and phosphorous, respectively, whereas the remaining block had no fertilizer application. Spacing followed was 75 x 30 cm with one plant per hole.

Farmers participated in planting together with the extension staff of the area and the researchers involved. Weeding and thinning were the responsibility of the farmer. Planting was done in at least four districts per season and in at least two farmer fields per district. Farmers also participated in selecting criteria for identifying better varieties before the actual yield was recorded. The main districts covered were Lira, Apac in northern Uganda, Soroti, Kumi, Bukedea and Sironko in eastern Uganda. Data recorded were: head diameter, plant height, uniformity, lodging, vigour of the plot, leaf spot disease, 1000 seed weight, and yield per plot converted to yield per hectare. To avoid damage by birds, the locals were encouraged to scare the birds. Head diameter, plant height, uniformity, lodging and vigour were recorded during physiological maturity, while leaf spot was recorded two weeks after flowering. 1000 seed count and yield were recorded in the laboratory after seed cleaning. Ten to fifteen plants were measured for plant height and head diameter.

The scales used for scoring sunflower data if no actual measurement taken were:

Plant height:	1= Very short	9= Very tall
Lodging:	1= No lodging	9= Completely lodged
Head diameter:	1= Very big head	9= Very small head
Vigour:	1= Very vigorous	9= Very poor vigour
Maturity:	1= Very early	9= Very late
Uniformity:	1= Very uniform	9= Very variable
Leaf spot disease	1= immune	9= completely diseased

RESULTS AND DISCUSSION

In the evaluation undertaken in the second season of 2005 in Apac district, the best hybrid was PAN7351 in the plots where no fertilizer or some fertilizer was applied (Table 1). Where the fertilizer was applied, it had yields of 2,067 kg/ha. Other hybrids that also did well were DK4040, DKF68-22 and AGSUN 8251, which originated from South Africa. All these hybrids have already been released officially for commercial production in Uganda. PAN 7351 was also the tallest hybrid (183 cm) except for AGSUN 8251 which was the tallest under the fertilized condition with 186 cm. Where fertilizer was applied, there was a significant yield increase in some hybrids, especially the high yielding hybrids. Fertilizer increased plant height and head diameter (Table 1).

In Lira district, the hybrid DKF68-22 recorded 2,333 kg/ha as the highest yielder where no fertilizer was applied and AGSUN 8251 recorded the highest yield with 2,600 kg/ha where fertilizer was applied. These were indicators of good hybrids to compete with PAN 7351. Fertilizer also increased plant height, head diameter and vigour of the plants.

Table 1. Performance of yield and other components of ten sunflower hybrids/varieties as affected by fertilization at Loro (Apac District) in the second season of 2005.

Genotype	Country of origin	Seed yield (kg/ha)[1]		Plant Height (cm)		Head diameter (cm)		Vigour[2]		Uniformity[3]		Lodging[4]	
		N0[5]	N1	N0	N1	N0	N1	N0	N1	N0	N1	N0	N1
PAN 7351	South Africa	1,600 (1)	2,067 (1)	183.5	183.5	14.0	14.1	4.5	4.5	3.0	4.0	2.0	2.0
DK 4040	South Africa	1,233 (5)	1,567 (4)	143.5	160.5	13.8	14.8	4.5	3.5	2.0	2.5	1.5	1.5
DK 68-22	South Africa	1,567 (2)	1,800 (3)	167.8	166.5	15.0	14.3	5.0	4.5	2.5	2.0	2.0	1.5
NSH 160	India	1,100 (6)	1,367 (6)	139.8	156.8	14.6	15.8	5.5	5.0	3.5	2.5	4.0	4.0
S 3503	India	967 (8)	967 (9)	163.5	170.3	16.9	17.6	5.5	4.0	2.5	3.0	4.0	2.0
AGSUN 4683	South Africa	633 (10)	767 (10)	134.5	148.8	12.9	15.3	6.5	4.0	3.5	3.0	2.0	2.5
AGSUN 5551	South Africa	1,400 (4)	1,567 (4)	148.3	176.0	14.5	15.6	4.5	3.0	2.5	1.5	2.0	1.5
ASUN 8251	South Africa	1,467 (3)	1,867 (2)	137.3	186.4	13.3	16.4	4.5	3.0	2.0	2.0	1.5	1.5
8998	Kenya	734 (9)	1,067 (8)	125.8	160.0	12.2	15.2	6.0	5.0	3.5	4.0	4.0	2.5
SUNFOLA	Uganda	1,000 (7)	1,267 (7)	162.3	175.5	12.6	13.6	5.5	45	4.0	3.5	4.5	2.5
Mean		**1,170**	**1,430**	**150.6**	**168.4**	**14.0**	**15.4**	**5.2**	**4.1**	**2.9**	**2.8**	**2.8**	**2.2**

[1]Numbers in brackets indicate the ranking of the genotypes at each treament
[2]From 1= Very vigorous to 9= Very poor vigour
[3]From 1= Very uniform to 9= Very variable
[4]From 1= No lodging to 9= completely lodged
[5]N0=no fertlization, N1=fertilization with N60 P30 kg/ha

Table 2. Performance of yield and other components of ten sunflower hybrids/varieties as affected by fertilization at Bar Apwo (Lira District) in the second season of 2005.

Genotype	Country of origin	Seed yield (kg/ha)[1]		Plant Height (cm)		Head diameter (cm)		Vigour[2]		Uniformity[3]		Lodging[4]	
		N0[5]	N1	N0	N1	N0	N1	N0	N1	N0	N1	N0	N1
PAN 7351	South Africa	2,267 (2)	2,267 (3)	224.0	223.4	18.8	18.4	4	3	4	3	2	1
DK 4040	South Africa	2,267 (2)	2,267 (3)	198.0	217.0	16.8	17.0	3	3	3	3	1	1
DK 68-22	South Africa	2,331 (1)	2,400 (2)	209.0	218.0	13.6	18.0	4	4	3	2	2	1
NSH 160	India	2,067 (5)	1,667 (7)	154.0	174.0	13.0	16.2	5	5	2	2	1	2
S 3503	India	1,733 (7)	1,933 (6)	190.0	204.0	14.4	14.4	5	4	3	2	1	1
AGSUN 4683	South Africa	1,000 (10)	1,000 (10)	157.0	168.0	15.4	13.2	5	6	3	3	1	2
AGSUN 5551	South Africa	2,133 (4)	2,100 (5)	164.0	186.0	12.2	13.4	5	4	2	2	1	2
ASUN 8251	South Africa	1,067 (9)	2,600 (1)	158.0	185.0	14.4	16.6	5	3	2	2	1	1
8998	Kenya	2,000 (6)	1,333 (9)	137.0	149.0	13.4	9.2	6	6	4	3	1	2
SUNFOLA	Uganda	1,200 (8)	1,400 (8)	168.0	192.0	16.4	14.6	5	5	4	5	2	2
Mean		**1,807**	**1,897**	**175.9**	**191.6**	**14.9**	**15.7**	**4.6**	**4.3**	**3.8**	**2.7**	**1.2**	**1.4**

[1]Numbers in brackets indicate the ranking of the genotypes at each treament
[2]From 1= Very vigorous to 9= Very poor vigour
[3]From 1= Very uniform to 9= Very variable
[4]From 1= No lodging to 9= completely lodged
[5]N0=no fertlization, N1=fertilization with N60 P30 kg/ha

During the first season of 2006 (Table 3) at Kasoka in Bukedea district, DKF68-22 had the highest yield of 2,067 kg/ha and it had also good vigour and head diameter. The hybrids that showed very good plant vigour were PAN7351, DK4040, DKF68-22 and AGSUN 5383, recording a value of one.

Table 3. Evaluation of sunflower hybrids/varieties at Kasoka (Bukedea District) during the first season of 2006.

	Yield (kg/ha)			Plant height (cm)		Head diameter (cm)		Vigour[3]		Maturity[4]	
Hybrid/Variety	N0[1]	N1	Mean[2]	N0	N1	N0	N1	N0	N1	N0	N1
PAN 7351	1,822	1,200	1,511 (3)	160	166	27.9	23.0	2	1	5	6
DK 4040	1,222	1,311	1,267 (8)	135	152	22.4	24.0	1	1	5	6
DKF 68-22	2,000	2,133	2,067 (1)	166	167	20.6	21.7	3	1	5	5
NSH 160	800	1,600	1,200 (10)	148	149	19.3	19.4	4	4	4	4
S 3503	1,267	1,644	1,456 (6)	147	172	19.3	21.5	4	3	4	4
8998	711	1,111	911 (15)	130	161	18.0	19.7	4	3	4	4
AGUSUN 5282	1,044	1,311	1,178 (11)	163	199	17.0	18.6	3	1	6	6
AGSUN 5383	1,333	1,644	1,489 (4)	153	179	15.4	19.9	3	3	4	4
AUSIGOLD 4	1,644	1,244	1,444 (7)	170	171	21.2	19.1	3	4	4	4
AGSUN 8251	1,689	1,244	1,467 (5)	193	161	17.0	18.2	4	4	4	4
Hysun 33	1,711	1,556	1,634 (2)	172	173	18.0	18.2	3	4	6	5
Hysun 39	622	844	733 (16)	166	172	20.0	17.8	4	4	5	6
Hysun 44	533	489	511 (17)	151	152	17.6	18.9	5	4	6	5
Sunrise 1	978	1,511	1,245 (9)	154	173	13.0	19.3	4	5	4	4
Sunrise2	889	1,022	956 (14)	157	168	12.6	15.9	5	5	4	3
Sunrise 3	978	978	978 (13)	147	148	13.2	19.4	4	4	4	4
Sunfola	889	1,200	1,045 (12)	162	190	13.0	18.4	5	4	4	4
Mean	**1,184**	**1,297**	**1241**	**157**	**167**	**18.0**	**19.6**	**4**	**3**	**5**	**5**

[1]N0=no fertlization, N1=fertilization with N60 P30 kg/ha
[2]Numbers in brackets indicate the ranking of the genotypes at each treament
[3]From 1= Very vigorous to 9= Very poor vigour
[4]From 1= Very early to 9= Very late

In Table 4, evaluation of sunflower was undertaken at Bunambutye in Sironko district in the first season of 2006. This site recorded the highest seed yield. The genotype DKF68-22 again had the highest seed yield of 3,556 kg/ha followed by Sunrise 1 with 3,333 kg/ha. Due to high soil fertility in this area, plant height, head diameter, and 1,000 seed weight were high for most genotypes. Plant heights of over 270 cm and head diameter of over 28 cm were recorded in this area.

Table 4. Evaluation of sunflower hybrids/varieties at Bunambutye (Sironko District) during the first season of 2006.

	Seed yield (kg/ha)			Plant height (cm)		Head diameter(cm)		1000-seed weight (g)		Vigour[1]		Maturity[2]			
Hybrid/Variety	N0[3]	N1	Mean[4]	N_0	N_1	N_0	N_1	N_0	N_1	N0	N1	N0	N1	DF[5]	DM[5]
PAN 7351	2,444	2,556	2,667(4)	224	232	20.9	21.3	67.8	66.0	3	1	4	3	58	93
DK 4040	2,000	2,111	2,222(8)	180	203	22.9	23.2	81.4	80.4	2	2	3	2	55	98
DK F 68-22	3,556	3,556	3,556(1)	229	227	20.4	19.3	58.6	58.8	1	1	1	1	59	100
NSH 160	2,222	2,222	2,222(8)	204	207	20.5	19.1	70.2	64.6	5	5	3	5	47	83
S 3503	2,667	2,667	2,667(4)	230	227	23.3	21.5	64.6	59.0	3	3	2	2	53	89
8998	1,556	1,556	1,556(13)	188	177	20.6	20.2	69.2	71.0	5	6	4	5	52	89
Sunf. SAARI	1,778	1,889	2,000(11)	238	252	21.1	21.3	62.0	68.4	4	2	4	7	51	86
Sunf. UOSPA	1,778	1,667	1,556(13**)**	231	280	22.4	20.6	69.8	75.8	3	2	4	3	55	89
Hysun 33	2,667	2,889	3,111(3)	247	247	20.2	19.1	64.2	69.8	2	1	2	3	59	97
Hysun 39	2,222	2,445	2,667(4)	262	280	20.2	21.9	69.0	59.0	1	1	3	2	60	99
Hysun 44	2,222	2,045	1,867(12)	251	219	24.2	28.8	49.6	62.6	1	1	4	2	63	102
Sunrise 1	3,778	3,556	3,333(2)	265	241	20.0	20.7	74.0	71.8	3	3	2	3	55	96
Sunrise2	2,667	2,572	2,477(7)	212	198	18.7	19.7	60.4	64.6	5	4	5	4	49	86
Sunrise 3	2,667	2,445	2,222(8)	220	227	21.8	22.0	57.8	55.4	2	2	2	2	54	93
Mean	**2,441**	**2,445**	**2,461**	**230**	**227**	**21.3**	**21.2**	**66.2**	**65.6**	**2.4**	**2.9**	**3.1**	**3.1**	**55**	**93**

[1]From 1= Very vigorous to 9= Very poor vigour
[2]From 1= Very early to 9= Very late
[3]N0=no fertlization, N1=fertilization with N60 P30 kg/ha
[4]Numbers in brackets indicate the ranking of the genotypes at each treatment
[5]DF=Days to flowering; DM=Days to maturity

Table 5. Seed yield (kg/ha) for sunflower on-farm variety trials across locations during the first season of 2007[1].

	Ocamon yang (Lira)	Adeko kwok (Lira)	Atik (Apac)	Atana (Apac)	Bar-Apwo (Lira)	Kasoka (Bukedea)			Nyero (Kumi)		
						N0[2]	N1[2]	Mean	N0[2]	N1[2]	Mean
DKF 68-22	1,200(8)	1,600(4)	1,000(9)	1,200(4)	2,667(1)	1,867	2,067	1,967(6)	2,200	1,677	1,934(3)
Alexandra	1,467(6)	800(9)	800(12)	666(10)	1,533(3)	1,800	2,000	1,900(7)	2,000	2,067	2,034(2)
Arena	1,667(3)	1,667(3)	1,600(5)	1,333(3)	1,533(3)	2,000	2,000	2,000(4)	2,067	1,667	1,867(5)
NKMY	267(15)	400(10)	1,133(7)	867(8)	867(11)	667	533	600(11)	733	1,000	867(10)
NKAR	733(11)	333(11)	1,000(9)	800(9)	600(13)	1,200	867	1,034(9)	1,600	1,533	1,567(7)
AGSUN 4672	400(14)	333(11)	-	333(12)	267(14)	533	400	467(12)	733	400	567(12)
AGSUN 5282	1,267(7)	1,267(5)	1,800(4)	1,200(4)	1,200(6)	2,467	2,200	2,334(1)	1,333	1,467	1,400(8)
AGSUN 5383	1,800(2)	1,267(5)	2,000(2)	1,467(2)	933(10)	2,467	800	2,200(2)	2,000	1,667	1,834(6)
AGSUN 5551	1,000(9)	1,200(7)	1,600(5)	1,133(6)	1,133(7)	2,600	1,467	2,000(4)	1,733	2,533	2,133(1)
AGSUN 8251	1,867(1)	2,600(1)	2,333(1)	1,533(1)	1,467(5)	2,533	2,000	2,167(3)	1,733	2,067	1,900(4)
AGSUN 8751	733(11)	200(13)	1,000(9)	467(11)	-	2,333	1,200	1,000(10)	933	467	700(11)
Sunrise	467(13)	1,000(8)	-	-	1,600(2)	800	-	-	-	-	-
Hysun 33	1,600(4)	-	-	-	1,133(7)	-	-	-	-	-	-
PAN 7351	1,000(9)	1,867(2)	2,000(2)	1,133(6)	867(11)	-	-	-	-	-	-
8998	1,533(5)	-	1,067(8)	200(13)	1,000(9)	1,333	1,200	1,267(8)	1,200	1,133	1,167(9)
Mean	**1,133**	**1,118**	**1,444**	**949**	**1,200**	**1,678**	**1,478**	**1,578**	**1,522**	**1,472**	**1,498**

[1]Numbers in brackets indicate the ranking of the genotypes at each treatment
[2]N0=no fertlization, N1=fertilization with N60 P30 kg/ha

During the first season of 2007 (Table 5), yield data were recorded and compared across locations in eastern and northern parts of Uganda. In Lira and Apac, which are located in northern Uganda, AGSUN 8251 had the highest yields in four locations while hybrid DKF68-22, Arena, and AGSUN 5383 were considered stable across locations. Kasoka (Bukedea) on-farm trial had the highest mean yield of 1,578 kg/ha followed by Nyero (Kumi district) with 1,497 kg/ha. As a result of this on-farm trial and other trials evaluated on government trial centers across locations, three hybrids were officially released in Uganda for commercial production on top of PAN 7351, which was earlier released in 2003. These new hybrids released are: DKF68-22, DK4040 and AGSUN 8251.

ACKNOWLEDGEMENTS

The project was supported by the Vegetable Oil Development Project (VODP) and the World Bank in conjunction with Uganda Government under Agricultural Research and Training Programme Phase 2 (ARTP2). We are indebted to the extension staff of Apac, Lira, Kumi, Bukedea and Sironko districts for the support they gave us in identifying the farmers and monitoring the trials which became a success. We are also grateful to the sunflower staff who contributed in data collection and compilation.

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Initial growth of sunflower in soils with high concentrations of boron and heavy metals

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ABSTRACT

Phytoremediation studies have been conducted in an area contaminated by heavy metals, located in Piracicaba - SP, Brazil. This area was contaminated accidentally by the addition of auto scrap shredding to the soil and was limed later to reduce heavy metal mobility in the environment. Previous characterization showed that it also presents high concentration of boron, which has limited the initial plant development of some species. As sunflower plants require a high boron supply and the literature describes its use in the phytoremediation of soils contaminated with heavy metals under some conditions, the aim of this work was to evaluate its potential for the remediation of this area. In the present study, the results of preliminary tests are presented, aiming at the evaluation of sunflower plant germination and its initial development when cultivated in the contaminated soil described. Two sunflower hybrids were sown in soils treated with different rates of boron and in the soil from the contaminated area in study. The results showed that sunflower plants had a normal initial development, even in the soil from the contaminated area. Therefore, sunflower is a promising crop and further studies will be developed to evaluate the sunflower efficiency in phytoextraction or phytostabilization of heavy metals in areas where boron contamination also occurs, as is the case in the study area.

Key words: boron – contamination – *Helianthus annuus* L. – phytoremediation – phytotoxicity.

INTRODUCTION

Boron is an important micronutrient, but when found in soils at high phytoavailable concentrations it can cause phytotoxicity. Many crops are very sensitive to boron toxicity, showing severe symptoms, such as yellowing of the leaf tips and stunted growth. High concentrations of B may occur naturally in the soil or in groundwater, or can be added to the soil from mining, fertilisers, or irrigation water (Nable et al., 1997). Another anthropogenic source of boron in soils is the use of wastes as fertilizers. Some industrial residues, such as from steelmaking, should be highlighted, once they frequently contain boron and heavy metals in their composition.

Nable et al. (1997) consider that B concentrations in soil higher than 5 mg dm^{-3} are toxic to the plants. However, this value could vary as a function of plant species, sampling time, soil characteristics, among others. Studies carried out in Brazil, testing different soils and species, showed that toxics levels of Boron vary from 1.8 to 8.3 mg kg^{-1} (Mariano et al., 2000; Fageria, 2000; Lima et al., 2007).

In Piracicaba city, located in São Paulo state, Brazil, there is an area which was contaminated accidentally by the addition of auto scrap shredding to the soil and was limed later to reduce heavy metal mobility in the environment. The environmental protection agency of the state isolated the area due to its high heavy metal concentration, and allowed researchers to run remediation studies using the soil from this area. The soil presents the following concentrations of heavy metals (mg kg^{-1}): 8 of Cd; 268 of Pb; 160 of Cu; 103 of Cr; 47 of Ni and 2454 of Zn. So, we are concentrating our efforts in the phytoremediation of Zn and Pb. However, previous studies showed that the high concentrations of available boron found (4 to 14 mg kg^{-1}) were limiting plant development and/or causing the death of some plant species.

Sunflower plants (*Helianthus annuus* L.), when compared with other species, require a large supply of boron. That is why this species is frequently used as an indicator plant for boron deficiency (Schuster and Stephenson, 1940). In addition, sunflower is able to absorb heavy metals selectively (Tan, 2000), presenting potential to be used to phytoremediate (phytoextract and/or phytostabilize) contaminated areas.

Based on the following statements: (i) sunflower plants require large boron supply; (ii) the major factor that limits initial plant development in the contaminated area was the high concentration of boron

in the soil; (iii) sunflower plants present potential to phytoremediate areas contaminated with heavy metals; we decided to evaluate the initial development of two sunflower hybrids cultivated in a test soil with increasing rates of boron and in the soil from the contaminated area in Piracicaba city.

MATERIALS AND METHODS

Two hybrids of sunflower, Helio 250 and Helio 358, were sown in pots containing 500 g of soil, in a greenhouse located in Embrapa Environment Unit, Jaguariúna city, SP, Brazil. The soil samples used corresponded to subsurface soil samples (B horizon) of a typical oxisol and surface soil samples (A horizon) of the area contaminated (CA) with heavy metals and boron, located in Piracicaba (Cambisol). The experimental design was completely randomized with three replicates, and the treatments were arranged in a 2x6 factorial design, that is, two sunflower hybrids and six boron rates.

The evaluated variables were plant height, dry matter, shoot boron concentration and soil boron concentration (extracted by hot water). The data were submitted to variance analysis by the SISVAR software and the means of the treatments were compared by Tukey test (5%).

The experiment was performed based on the following treatments, corresponding to B rates added to the oxisol, for each hybrid evaluated: Control – Co (oxisol, no fertilization); Boron 0 – B0 (oxisol + mineral fertilization, no boron added); Boron 2 – B2 (oxisol + mineral fertilization + 2 kg ha^{-1} of B); Boron 4 – B4 (oxisol + mineral fertilization + 4 kg ha^{-1} of B); Boron 8 – B8 (oxisol + mineral fertilization + 8 kg ha^{-1} of B) and Contaminated Area – AC (soil from contaminated area, no fertilization). Boron was added in the form of boric acid.

After filling the pots with soil, lime was added to the oxisol treatments, in order to raise the base saturation to 70%, as indicated by Ambrosano et al. (1996). The soil from the contaminated area presented a pH of 7.4, so it was not necessary to lime it. Then, all the pots were incubated for fifteen days, and soil humidity was maintained at 70% of the soil water retention capacity.

The mineral fertilization consisted of 63 mg dm^{-3} of N (NH_4NO_3), 150 mg dm^{-3} of P ($Na_2HPO_4.2H_2O$), 120 mg dm^{-3} of K (KCl), 30 mg dm^{-3} of S ($MgSO_4.7H_2O$), 1 mg dm^{-3} of Cu ($CuSO_4$), 5 mg dm^{-3} of Zn ($ZnSO_4.7H_2O$) and 5 mg dm^{-3} of Mn ($MnCl_2.4H_2O$).

After the incubation period, mineral fertilization and boron addition were performed according to each treatment and ten seeds were sown per pot. During germination and the initial development of the sunflower plants, soil humidity was also maintained at 70% of the soil water retention capacity.

Twenty five days after sowing, plant shoots were harvest, washed, and dried (60 $^{\circ}C$). Dry matter was quantified, and the samples were ground (2 mm) and analyzed for boron concentration (US-EPA, SW-846, method 3050B, with determination by ICP-AES). Soil samples from each pot were collected, dried (60 $^{\circ}C$), ground (2 mm) and homogenized to be analyzed for concentration of boron extracted by hot water (Berger and Truog, 1939).

RESULTS AND DISCUSSION

For the evaluated variables (plant height, dry matter, shoots B concentration and soil B concentration), only dry matter production was statistically different when considering the sunflower hybrids. Helio 358 was more efficient (24.02 g per pot) than Helio 250 (20.09 g per pot) in dry matter production. For the other variables, differences were only observed for boron rates factor. Plants cultivated in the soil from the contaminated area presented the highest dry mass production in the study. Plant height was not different for the treatments containing increasing B rates, except for treatments with no boron added, which were lower than the others (Fig. 1).

Fertilizer recommendation of Boron in sunflower cultivation for the São Paulo State is 1 kg ha^{-1} of B when the soil presents 0 to 0.20 mg dm^{-3} of B extracted with hot water and 0.5 kg ha^{-1} of B when the soil presents 0.21 to 0.60 mg dm^{-3} (Ambrosano et al., 1996). The original concentration of boron in the oxisol soil used in the experiment was 0.30 mg dm^{-3}. The rates of B added to the soil (2, 4 and 8 kg ha^{-1}) were deliberately higher than the recommendation, since the aim was to evaluate sunflower tolerance to the excess of boron in the soil. Despite this, the available Boron concentration in the soil was not as high as expected, even for the highest rate added (B8), which was 1.91 mg kg^{-1} (Fig. 2). Boron availability depends on different attributes of the soil, such as pH, organic matter content, parent material, mineralogy (Gupta, 1993); and, consequently, it depends on its adsorption on soil colloids (Goldberg, 1993). Therefore, the low concentration of available boron observed could be the result of high adsorption of this element on the oxisol studied.

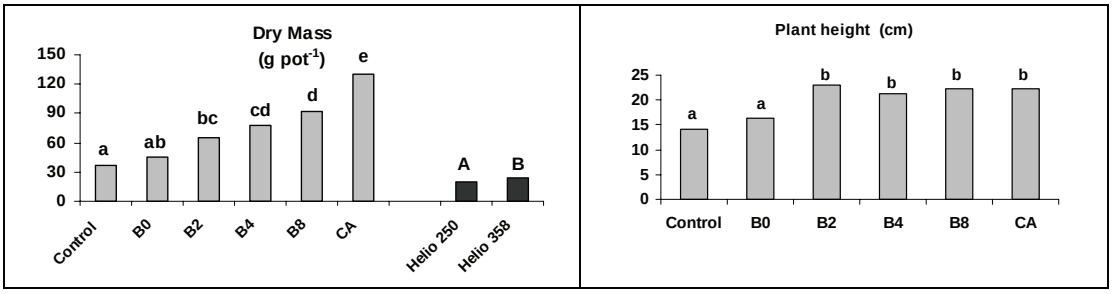

Fig. 1. Dry matter production and plant height of sunflower hybrids cultivated in soils treated with different boron rates[1].

[1]Control (oxisol with no fertilization); B0 (oxisol + mineral fertilization, no boron added); B2 (oxisol + mineral fertilization + 2 kg ha^{-1} of B), B4 (oxisol + mineral fertilization + 4 kg ha^{-1} of B), B8 (oxisol + mineral fertilization + 8 kg ha^{-1} of B); CA (soil from the contaminated area, no fertilization). Within each figure, values followed by the same letter (lower case: boron treatments; upper case: sunflower hybrids) are not statistically different (Tukey, 5%).

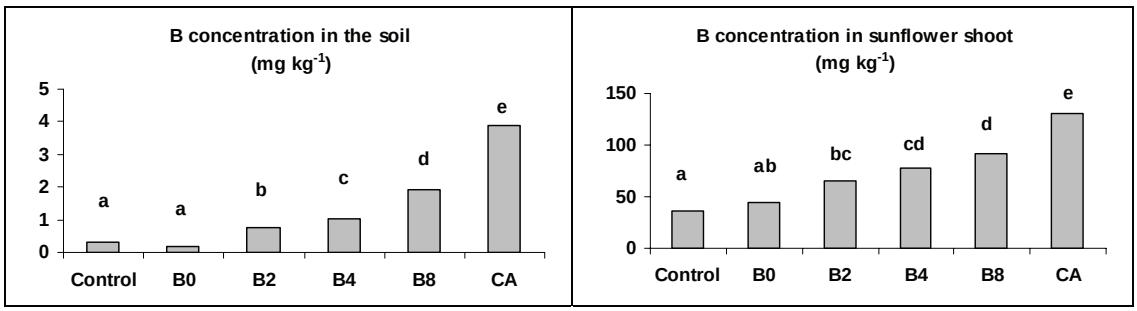

Fig. 2. Soil boron extracted with hot water and Boron concentration in the shoots of sunflower hybrids cultivated in soils with different boron rates[1].

[1]Control (oxisol with no fertilization); B0 (oxisol + mineral fertilization, no boron added); B2 (oxisol + mineral fertilization + 2 kg ha^{-1} of B), B4 (oxisol + mineral fertilization + 4 kg ha^{-1} of B), B8 (oxisol + mineral fertilization + 8 kg ha^{-1} of B); CA (soil from the contaminated area, no fertilization). Values followed by the same letter are not statistically different (Tukey, 5%).

In the treatment with soil from the contaminated area, available boron concentration was 3.90 mg kg^{-1} (Fig. 2). Although this concentration could be considered high, it was expected to be even higher, since other determinations performed with soil samples from the same area found boron concentrations extracted with hot water up to 14.87 mg kg^{-1} (Gonçalves et al., 2007a, b). This result reflects the high heterogeneity of the soil from the contaminated area.

Boron concentrations in the leaves from 15 to 20 mg kg^{-1} are considered adequate for plant nutrition (Malavolta, 2006). Specifically for sunflower, Sfredo et al. (1984) suggested that the suitable boron level in the leaves should be 40 mg kg^{-1}, based on studies carried out in the south region of Brazil (Paraná state). In the present study, boron levels in the shoots varied from 36 to 130 mg kg^{-1} (Fig. 2). Even for the plants cultivated in the soils that received the highest amount of boron, no toxicity symptoms were observed, neither was there any initial development reduction. This indicates that sunflower plants have a potential for being cultivated in soils contaminated with Boron, which is frequently found in soils contaminated with heavy metals.

It can be concluded that sunflower plants present a normal initial development when cultivated in soils that receive high amounts of boron. Therefore, sunflower is promising and should be tested as a phytoextractant and/or phytostabilizer of heavy metals in areas where boron contamination also occurs, as is the case of the study area.

ACKNOWLEDGEMENTS

To FAPESP for their financial support of this study.
To Helyanthus do Brasil Ltda. for donating the seeds used in the experiment.

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Impact des facteurs limitants du rendement du Tournesol (*Helianthus annuus* L.) en conditions réelles d'utilisation par les agriculteurs, en Midi-Pyrénées – Etude de cas

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ABSTRACT

Sunflower (*Helianthus annuus* L.) is largely cropped in France, particularly in Midi-Pyrénées (second producing area) and it is naturally adapted to South-west agro-climate conditions. The yield depends on plant/environment interactions in its growth cycle. The main limiting factors are drought stress and cryptogrammic diseases. The aim of this work was i) to identify which factors influence the sunflower yield limitations, and ii) to evaluate the importance of cryptogrammic diseases and deep tillage in these limitations. This study presents the results of an initial experiment carried out in 2007. Two types of tillage were compared: control and deep tillage. The results showed that *Phoma macdonaldii* was the main factor influencing yield, its components, and morphological data. Significant differences on tillage were observed in morphological data stem length. Moreover, connections between organs linked to stress were highlighted. The slight difference between the two types of tillage on yield can be attributed to three main facts, i) neither tillage treatment was discriminating enough, ii) the presence of *Phoma macdonaldii*, iii) the absence of drought conditions. Similar studies will be carried out from 2008 to 2010 in different agro-ecosystems, with the aim of establishing the importance of physical soil constraints on the limitation of sunflower yield in Midi-Pyrénées (France).

Key words: compaction – fatty acids – France – *Phoma macdonaldii* – tillage – yield.

RESUME

Le Tournesol (*Helianthus annuus* L.) est une culture largement cultivée en Midi-Pyrénées (seconde région productrice de France) et naturellement adaptée à ses conditions agro-climatiques. Le rendement est la résultante d'interactions tout au long du cycle cultural. De nombreux facteurs limitants existent, au premier rang desquels figurent les maladies cryptogamiques et le stress hydrique. Les objectifs de ce travail sont i) de définir quels facteurs agissent sur la limitation du rendement du tournesol et ii) dans quelles mesures la présence de maladies et le type de travail du sol y jouent un rôle. Ce travail présente les résultats d'une étude de cas préliminaire réalisée au cours de la saison 2007, en suivi de parcelles d'expérimentations, selon les pratiques agricoles usuelles. Sur cet essai étaient comparés deux types de travail du sol, un témoin, un travaillé en profondeur. Nos résultats montrent que le premier facteur impactant sur le rendement, ses composantes, et les données morphologiques recueillies, est *Phoma macdonaldii*. Des différences significatives entre les deux types de travail du sol ont été observées sur une donnée morphologique, la longueur de la tige. Par ailleurs des relations liées au stress entre différents organes (aériens et souterrains), ont aussi été mises en évidence. La faible différence entre les deux types de travail du sol sur le critère rendement peut être imputée à plusieurs faits: i) les deux traitements n'étaient pas suffisamment discriminants, ii) la présence de *Phoma macdonaldii*, iii) l'absence de conditions sèches. Des études similaires seront conduites de 2008 à 2010, dans différents agro-écosystèmes, dans le but d'établir l'importance des contraintes physique du sol sur la limitation du rendement en Midi Pyrénées.

Mots clés: acides gras - *Phoma macdonaldii* - rendement - tassement - travail du sol - tournesol.

INTRODUCTION

La culture du tournesol (*Helianthus annuus* L.) présente des atouts agronomiques et environnementaux dans les régions à faible disponibilité en eau comme le Sud-ouest de la France, tel que la tolérance aux stress thermiques et hydriques (Merrien et Milan, 1992). Le rendement obtenu est le résultat d'interactions entre la plante et son milieu tout au long du cycle cultural ; de nombreux facteurs limitants sont signalés au premier rang desquels figurent les maladies cryptogamiques et le stress hydrique (Alignan, 2006; Merrien et Milan, 1992). En France, le rendement moyen oscille entre 20 et 25 quintaux hectare, alors que

le potentiel des meilleures variétés avoisine 45 quintaux hectare dans les milieux les plus favorables (CETIOM, 2006). Ceci rend le tournesol peu compétitif vis-à-vis des autres grandes cultures, et ne s'explique pas par un défaut de progrès génétique (Vear et al., 2003). Le Phoma (*Phoma macdonaldii*) classé en 2004 seconde maladie plus importante après le mildiou (Alignan, 2006), est un facteur déterminant de la limitation du rendement du tournesol. De la même façon, le tassement sous-superficielle du sol et donc de la résistance qu'offre le sol à la pénétration des racines agit négativement dans l'élaboration du rendement pour les espèces à système pivotant (Andrade et al., 1993; Montagu et al., 2001; Diaz-Zorita, 2004; Sadras et al., 2005).

L'évolution des pratiques culturales depuis les vingt dernières années a engendré des impacts majeurs sur les sols cultivés (Le Bissonais et al., 2002). L'agrandissement des parcelles, la spécialisation des cultures, l'évolution des techniques culturales, entrainent une diminution de la qualité de la structure des sols et la baisse de la teneur en matière organique (Girard et al., 2005). Ces contraintes pourraient être liées au phénomène naturel de tassement (Andrade et al., 1993; Lampurlanès et Cantero-Martinez, 2003; Sadras et al., 2005). Les réductions du rendement attribuables aux évolutions du tassement des sols ont été décrites pour différentes cultures, dans différents types de sols, et dans différentes régions productrices à travers le monde (Diaz-Zorita, 2004; Tennant et Hall, 2001). Chez le tournesol, de fortes conditions de tassement sur des sols à texture fine réduisent l'expansion foliaire, la biomasse aérienne et le développement des racines (Andrade et al., 1993; Diaz-Zorita, 2004). La différence entre les rendements réels et potentiels pourrait être en partie expliquée par une plus faible efficience d'absorption hydrominérale, consécutive à une réduction du volume de sol exploré par les racines (Connor et al., 1992; Andrade et al., 1993; Diaz-Zorita, 2004; Goodman et Ennos, 1999). Ceci ayant pour conséquence la réduction de la quantité d'eau absorbée et de fait la diminution de l'absorption des éléments minéraux (N, P, K) et des oligoéléments (B) (CETIOM, 1983; Colomb et al., 1995).

Les objectifs de ce travail sont i) de définir quels facteurs agissent sur la limitation du rendement du tournesol, et ii) dans quelles mesures la présence de maladies et le type de travail du sol y jouent un rôle.

MATÉRIELS ET MÉTHODES

Le cultivar MELODY (Syngenta SEEDS SAS, Semences NK) a été suivi en parcelle d'expérimentation (dispositif split plot, quatre répétitions, parcelles de 12 rangs sur 10 mètres) au cours de la saison 2007, en conditions réelles d'utilisation agricole sur l'exploitation de l'E.I. Purpan: Ferme de Lamothe, (43°30'11.75''N ; 1°14'54.53''E). Le semis a eu lieu le 30 avril 2007 (semoir pneumatique, écartement 0.6m, 52700 plantes/hectare), en sol Limoneux Sablo-argileux (A: 22.4; L: 47.5; S: 27.3; pH: 6.2). Un covercrop a d'abord été passé sur l'ensemble de la parcelle, suivi d'un passage de décompacteur (profondeur: 0.5m, écartement: 0.6m). Un second passage de décompacteur a été passé perpendiculairement sur la zone correspondant au second traitement du futur essai. Un passage simple constituait le traitement un (T1), deux passages perpendiculaires le traitement deux (T2). La phénologie a été surveillée tout au long de la saison sur trois plantes consécutives dans chaque parcelle expérimentale. L'évolution des surfaces foliaires a été notée à cinq reprises à partir du stade 3.1, jusqu'au stade 5.4 (Hutley Bull, 1995). Pour chaque notation, l'Indice Foliaire (IF) a été estimé à partir de la mesure de la largeur des feuilles (Scheiner et Lavado, 1999). La floraison est intervenue le 17 juillet 2007, stade 4.3. La présence de *Phoma macdonaldii* a été remarquée dès le 24 juillet 2007, des notations de diamètre et de tâche de Phoma au collet ont été réalisées conformément au Tableau 1.

Tableau 1. Echelle de notation inspirée par l'échelle de notes «G2» sur Phoma (*Phoma lingam*) du colza (*Brassica napus*) (CETIOM, 2004).

Notation	Taille de la tache
1	Moins de ¼ de la circonférence
2	Entre ¼ et ½ de la circonférence
3	Entre ½ et ¾ de la circonférence
4	Entre ¾ et toute de la circonférence
5	Tâche encerclante

La parcelle a été récoltée le 11 septembre 2007. Les données de rendement ont été obtenues à partir de prélèvements de capitules réalisés dans chaque parcelle sur huit mètres consécutifs. Du fait de la contamination par *Phoma macdonaldii*, nous avons effectué une double récolte sur chaque répétition, la première étant qualifiée fortement touchée (note Phoma = 5): M1; la seconde de «saine»: M2. Afin

d'obtenir des données physiologiques et morphologiques précises, nous avons extrait dans chaque parcelle et pour chaque traitement, trois plantes entières successives. Les différents organes des plantes entières extraites ont été nettoyés, séparés et caractérisés. Le diamètre des capitules de tournesol a été mesuré avant égrenage. Les feuilles ont été mises en étuve (72h à 45°C), les tiges et les racines séchées à l'air. L'ensemble de l'appareil aérien et souterrain a été pesé et mesuré (diamètre et longueur). Le Poids de Mille Grains (PMG) a été obtenu. Les données de nombre et de poids des grains par capitule, des poids, longueurs et volumes spécifiques, du peuplement hectare par micro parcelle, ont été obtenues par recoupage des données précédentes. Par ailleurs le calcul des données de longueur et volume spécifiques de la tige et de la racine, a été emprunté au manuel de STICS (Brisson, 2002). Les données de qualité d'huile ont été obtenues par spectroscopie proche infrarouge (Ayerdi Gotor et al., 2007). En termes d'analyses statistiques, des modèles linéaires généraux et des régressions linéaires multiples ont été réalisées pour l'ensemble des données recueillies.

RÉSULTATS ET DISCUSSION

Durant la saison 2007 aucun symptôme de stress hydrique n'a été observé sur les plantes. Le rendement de l'essai était égale à 37.5 quintaux / hectares (peuplement récolte égal à 47,083 plantes/hectare).

Dans les conditions expérimentales, l'appareil foliaire présente une relation directe avec les composantes de rendements, mais aussi avec la partie aérienne de la plante (Merrien et Milan, 1992) (Tableau 2). Conformément aux observations de Sadras et al. (1993), le poids de l'appareil foliaire augmente à mesure que la longueur de la tige diminue (Fig. 1). De plus l'augmentation de la biomasse des feuilles présente une corrélation positive avec l'augmentation de la biomasse souterraine (appareil racinaire) (Fig 1.). Ces deux systèmes, le système racinaire et le système aérien (tige et feuilles), ont un impact direct sur les composantes de rendement. En effet, le PMG augmente en parallèle du poids de la tige, et dans une moindre mesure avec le diamètre de la racine (Fig. 1). Le type de travail du sol a eu un impact sur la longueur de tige, qui s'accroit avec un passage supplémentaire de décompacteur, (Tableau 2).

Tableau 2. Mesures morphologiques réalisées sur plantes entières prélevées au champ: Analyses de variance, moyenne des traitements[1]. Travail du sol: T; Présence de Phoma: M.

Mesures morphologiques	Moyenne T1	Moyenne T2	Moyenne M1	Moyenne M2	Moyenne des Traitements
Nombre de feuille	16.1	16.3	15.3 a**	17 b**	16.2 ± 0.45
Nombre de grains par capitule	1,153.9	1,234	1,080 a**	1,307.9 b**	1,193.9 ± 65.4
Longueur spécifique des racines	1.3	1.4	1.5 a**	1.2 b**	1.3 ± 0.1
Longueur tige	147.7 a**	153 b**	149	15.8	150.4 ± 1.5
Poids des grains par capitule	43.1	48.1	36.1 a***	55.1 b***	45.6 ± 3.4
Poids spécifique de la racine	29.2	23.4	19.2 a**	33.5 b**	26.3 ± 4.2
Poids de Mille Grains	36.1	38.1	32.6 a***	41.7 b***	37.1 ± 1.4
Volume de la racine	19.8	18.6	16.7 a*	21.7 b*	19.2 ± 1.7
Volume spécifique de la racine	0.8	0.9	0.9 a**	0.8 b**	0.9 ± 0.1
Biomasse feuille	17	16.6	14 a**	19.6 b**	16.8 ± 1.7
Biomasse Tige	50.5	52.8	45.7 a**	57.9 b**	51.6 ± 3.1
Longueur spécifique de la tige	3.2	3.1	3.5 a**	2.8 b**	3.1 ± 0.2

[1]a, b: groupes homogènes selon le Test de Student; *: Probabilité significatives à 0.05; **: Probabilité significatives à 0.01; ***: Probabilité significatives à 0.001.

Les conditions climatiques de la saison 2007 ont été particulièrement favorable au développement de *Phoma macdonaldii* (PROLEA, 2005; CETIOM, 2006). Des symptômes ont été observés après le stade 4.3, bien que le tournesol soit sensible depuis le stade cotylédon (Alignan, 2006). D'après le CETIOM (PROLEA, 2005), le taux de pieds secs tendrait à augmenter avec la surface foliaire. Une perte du poids de la biomasse foliaire de l'ordre de 16% a été observée, traduisant non pas une baisse du nombre de feuilles (non significatif), mais à un dessèchement précoce de ces dernières dû à la maladie (Tableau 2). De plus les infections issues de la tige (causant notamment les tâches encerclantes) sont plus agressives que celles issues des feuilles (PROLEA, 2005), ainsi nous avons pu constater une perte de 11% de la masse de la tige (Tableau 2). Les résultats présentés dans le Tableau 2 montrent que l'infection impacte négativement les organes directement liés aux fonctions de nutrition (racine, feuille) et de réserve (tige, capitule), ce qui limite la synthèse de ces même réserves ou leur acheminement vers la graine (Abou Al Fadil, 2006; Darvishzadeh, 2007). Les données dont nous disposons, nous amènent à penser que cette relation est probablement expliquée par ces deux hypothèses de manières successive ou simultanément.

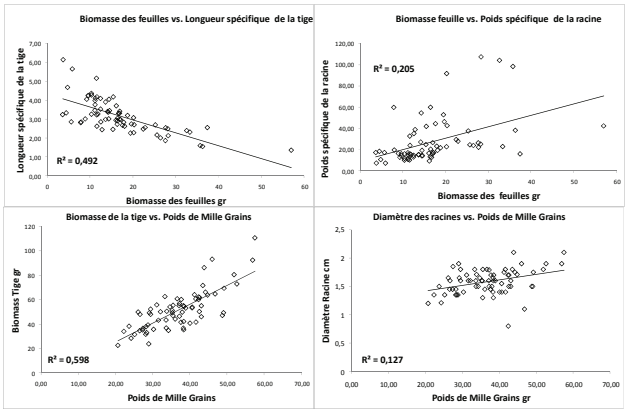

Fig. 1 Relations liées aux stress entre organes. Les corrélations sont significatives (régressions linéaires, P<0,001) entre les données morphologiques.

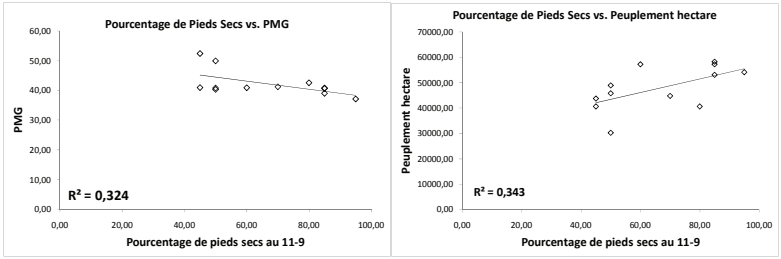

Fig. 2. Relation entre le pourcentage de pied sec (note Phoma = 5) par parcelle au 11septembre 2007, le PMG (régression linéaire, P=0.053) et le peuplement hectare (régression linéaire, P=0.045).

Les contaminations par le sol causent entre autre les nécroses au niveau du collet (zone de jonction entre la tige et la racine), celles-ci se traduisent alors par des nécroses encerclant la tige empêchant le transfert des assimilas (Alignan, 2006). Ainsi nous avons pu constater une perte de 27% du poids spécifique de la racine, de 13% du volume de celle-ci (Tableau 2). L'ensemble des symptômes, se traduit par une sénescence précoce de la plante ayant pour conséquence directe une perte de rendement. Le pourcentage de pieds secs par parcelle réalisé le 11 septembre 2007, nous montre que le PMG décroit à mesure que le taux augmente (Fig. 2). Dans les conditions expérimentales, le Phoma a pour conséquence une diminution de 12% du PMG. Nous avons aussi pu observer une diminution significative du nombre de grains par capitule de 10%. Ceci ayant pour conséquence une diminution significative de 49% du poids des grains par capitule, et plus globalement de 25% de perte de rendement sur l'ensemble de la parcelle expérimentale (Tableau 2 et 3). Ceci est en relation avec les observations réalisées par Abou Al Fadil (2006) et Darvishzadeh (2007). La diminution significative du nombre de grains par capitule nous amène à penser que l'infection se serait produite à partir du stade correspondant à la mise en place du nombre de grains par capitule et ce jusqu'à maturité (Alignan, 2006). De plus, le fait que nous ayons observé une augmentation du pourcentage de pieds secs avec le nombre de plantes à l'hectare, nous amène à émettre l'hypothèse de la présence d'une contamination secondaire (Fig. 2).

Tableau 3. Mesures et analyses réalisées sur des capitules prélevés sur huit mètres consécutifs: Analyses de variance, moyenne des traitements[1]. Travail du sol: T; Présence de Phoma: M.

Mesures au champ	Moyenne T1	Moyenne T2	Moyenne M1	Moyenne M2	Moyenne Des Traitements
Rendement	36.2	39.9	28.6 a**	47.5 b**	38.1 ± 4.2
PMG	42	42.4	36.4 a***	48.1 b***	42.2 ± 2.1
Poids spécifique	42.5	42.7	41.2 a***	43.9 b***	22.6 ± 0.4
Protéines	15.6	14.9	15.9 a*	14.6 b*	15.3 ± 0.5
Acide Palmitique	6.8 a*	6.9 b*	6.9 a***	6.7 b***	6.8 ± 0.04
Acide Stéarique	2.8	2.8	3 a*	2.6 b*	2.8 ± 0.1
Acide Oléique	23.1 a*	21.3 b*	20.8 a***	23.7 b***	22.2 ± 0.6
Acide Linoléique	68 a*	68.9 b*	70.1 a **	67.8 b**	68.5 ± 0.6

[1]a, b:groupes homogènes selon le Test de Student ; *: Probabilité significatives à 0.05; **: Probabilité significatives à 0.01; ***: Probabilité significatives à 0.001.

Dans le contexte expérimental les teneurs en acides palmitique, stéarique et linoléique sont plus élevées en présence de stress induit par le Phoma (M1), seule la teneur en acide oléique tend à diminuer dans le traitement M2. Cette diminution peut s'expliquer par la sénescence précoce de la plante (CETIOM, 1996). En effet, une plante de tournesol soumise à un stress dû à la maladie souffrira de prématurité, et de ce fait sera récoltée à surmaturité, lorsque ses congénères seront arrivées à leur maturité physiologique (CETIOM, 1996). Dans ce cas, Baldini et al. (2002) ont observé que la surmaturité entrainait une baisse de la teneur en acide oléique dans les graines récoltées. Les protéines étant les premières composantes à s'accumuler dans la graine de tournesol (Roche, 2005), elles souffriront moins du phénomène de prématuration. De la même manière, les acides palmitiques, stéarique, étant des précurseurs de l'acide oléique (Lagravère, 1999), ils auront tendance à moins souffrir de la sénescence précoce de la plante causée par le champignon, conformément à nos observations (Tableau 3). Le fait que la durée d'activité de la feuille est directement liée à la lipidogenèse, tend à confirmer ce fait (Merrien et Milan, 1992). Nous avons observé des différences sur la teneur des différents acides gras selon le type de travail du sol, bien que la teneur en huile ne soit pas en elle-même significative (Tableau 3). Nos résultats nous montrent une amélioration significative des teneurs en acide palmitique, stéarique et linoléique sur le traitement T2 (passage supplémentaire de décompacteur). Seule la teneur en acide oléique tendrait à s'améliorer sur un sol moins travaillé, et à décroitre sur T2. Les variations de la teneur en acide gras saturés et insaturés dues aux conditions environnementales restent encore peu connues et controversées. Flagella et al. (2006), ont mis en évidence que la teneur en acide oléique était positivement influencée par l'irrigation, et la teneur en acide linoléique négativement impactée par celle ci. Cependant, Baldini et al. (2002) ont observés un effet positif d'un léger stress hydrique, sur la teneur en acide oléique ; et de fait un effet négatif sur la teneur en acide linoléique. L'augmentation de la teneur en acide oléique sur T2, semblerait concordante avec les résultats de Baldini et al. (2002). Cependant en l'absence de stress hydrique réel, ces résultats seront à mettre en relation avec les résultats de nos travaux de 2008 à 2010.

Le rendement est le résultat d'une série d'interactions entre la plante et son milieu tout au long de son cycle. Il dépend dans les conditions de l'expérimentation, de la bonne capacité de la plante de tournesol à s'adapter à son milieu, à absorber l'eau et les nutriments nécessaires, à intercepter suffisamment de rayonnement solaire et à résister aux attaques de ravageurs et aux maladies. Si toutes ces conditions sont réunies, la plante sera à même d'optimiser son nombre de grains par capitule et son remplissage. Contrairement aux travaux de Diaz-Zorita (2004), une réduction du rendement liée au travail du sol n'a pas été observée. Les données précédentes nous amènent à penser que bien que le travail du sol a un effet sur la morphologie de la plante de tournesol, il ne se traduit pas directement sur les données de rendement au champ. Parallèlement dans les conditions expérimentales, nous avons constaté une baisse du rendement lié au Phoma. Cette relation directe s'établirait sur une de ses composantes principales, le PMG et sur le nombre de grains par capitule. Il existerait donc une relation entre le degré d'infection de la plante et la baisse du nombre et remplissage de ces grains. D'un point de vue strictement morphologique (dans l'attente de données physiologiques), l'expression du stress causé par le Phoma (hormis les symptômes caractéristiques) semble difficile à distinguer des symptômes de stress observés par Andrade (1993) et Diaz-Zorita (2004). La présence de cette maladie pourrait expliquer le fait qu'aucune différence significative n'ait été observée entre les deux types de travail du sol sur le critère rendement. Ceci pourrait aussi s'expliquer par le fait que ces deux traitements n'étaient pas suffisamment discriminants dans les conditions expérimentales. L'absence de contraintes liées à la sécheresse durant la saison 2007 peut aussi être une des raisons de l'absence de différence significative entre les deux types de travaux du sol sur la quasi-totalité des critères étudiés.

REMERCIEMENTS

Nos remerciements vont au personnel de la ferme de Lamothe pour son appui technique, ainsi qu'à, Mme. Mestries (CETIOM) et Mr. Debaeke (INRA - UMR 1248 –AGIR).

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Planting date effect on yield and yield components of sunflower in Miyaneh region

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ABSTRACT

An experiment was conducted at the research field of Islamic Azad University, Miyaneh, Iran, during the agricultural year 2005-2006, to study the effect of planting date on yield and yield components of sunflower varieties and hybrids. A factorial analysis, on the basis of a randomized complete block design with three replicates, was used. Factor A (planting date) consisted of three planting dates: April 9, April 30 and May 20 and factor B (variety) were the cultivars Sour, CMS26xR103, Azargol, and Armavirsky. Measured traits during study were plant height, head diameter, 1000 seed weight, oil percentage, oil yield, total biomass and seed yield. The results revealed that the effect of planting date on number of days to emergence, plant height, head diameter and oil yield were not significant, but the effect of planting dates on 1000 seed weight, total biomass and oil yield were significant. In contrast, the results showed that the effect of variety on all measured traits was significant. The interaction of planting date as well as variety on measured traits was significant, except for days to emergence, head diameter and oil yield. Second planting date (April 20) and Armavirsky cultivar were determined, respectively, as an appropriate planting date and variety.

Key words: biomass – *Helianthus annuus* L. – hybrids – yield – yield components.

INTRODUCTION

Oil crops are very important in human diet. In the last decade, Iranian yearly oil production was 85,000 t but its consumption was 992,000 t, so many oil products has to be imported every year (Anonymous, 2006). National oil production program emphasizes on applying optimum management methods besides genetic resources capacity. One of the most important decisions in plant cultivation is determining the best planting date for every cultivar. Attention is being paid to sunflower hybrids with uniform establishment, easier cultivation practices, higher yield, and higher tolerance to diseases. For these reasons, open pollinated cultivars are being substituted by hybrids. Research on both types of cultivars has been important. Miyaneh region, in North West of Iran, is a favorable area for sunflower cultivation. Its special geographical situation makes it possible to use different cultivars and planting dates and the introduction of new promising varieties. Plant yield is determined by the sun radiation received via canopy, so that a decrease in radiation received causes a decrease on seed and oil yields. Growers have moved sunflower cultivation from hot to temperate and cool climates. Delay in sowing increased the hull/achene ratio and decreased kernel oil percentage and total oil content (Alyari, 2000).

Khodabande (1989) showed that, at Zaria and Mehr, the best planting dates for combatting peregrine sparrows were between 1st September to 10th October. Thompson and Unger (1986) studied ten planting dates between 25th March and 1st August and showed that only planting dates until 19 July led to an increase in the head diameter and after that date head diameter decreased.

Goksoy et al. (2000) conducted a two year experiment on SUNBRED-265, H-1 and Vinimac 8931 cultivars in three densities (30, 47.5 and 95 thousand plant/ha) and three planting dates from March to April in upland dry farming. They showed that 95,000 plant/ha in mid March led to a higher thousand kernel weight and oil yield. Parmar and Kharwave (1992) showed that among four planting dates, 22 February, 4, 14, and 24 March, the planting in 14 March produced the highest yield. Alessi et al. (1977) showed that the best planting date for North Dakota was middle to end May and earlier or later planting dates led to a decrease in seed and oil yields.

MATERIALS AND METHODS

An experiment was conducted at the research field of Islamic Azad University, Miyaneh in the agricultural year 2005-2006 following a randomized complete block design with three replicates. The station, located on 25º, 37’ E and 43º, 47’ N. on Marton and Umburge divisions, has semi arid and semi warm conditions and dry summers and cold and wet winters. Average annual temperature is 13ºC, with a

minimum of 6.2ºC and a maximum of 19.8 ºC. Average precipitation at this location is 306 mm, with a minimum of 168 mm and a maximum of 500 mm. Frozen temperatures have been reported for 110 days. Soil texture is clayloam with 7.5 pH. Factor A (planting date) consisted of three levels: April 9, April 30 and May 20 and factor B (variety) consisted of four cultivars, Sour, CMS26xR103, Azargol, Armavirsky. After soil preparation, planting was done by hand and fertilization was done according to a previous soil analysis. Each plot was 5 x 2.4 m. Plant distances within row were of 25 cm and distance between rows was 60 cm. Weeds were controlled on time.

Measured traits were number of days to emergence, plant height, head diameter, 1000 seed weight, oil percentage, biomass, seed yield and oil yield.

RESULTS AND DISCUSSION

Results showed that there were significant differences among cultivars in days from planting to emergence (Table 1). It would seem that this period was affected by seed potential, soil nutrients, temperature and humidity more than by the planting date (Sindagi and Virupakshappa, 1990). Armavirsky emerged 4.78 days earlier than other cultivars (Table 2).

Table 1. Anova table of measured traits

SV[1]	df	Mean squares						
		Days to emergence	Plant height	Head diameter	1000 seed weight	Seed Yield	Oil Yield	Biomass
Rep	2	18.36**	72.33	33.58*	15.1**	249988	2800782	96.82
A	2	2.69	67.25	10.08	8.42**	2338233**	594281	1019**
B	3	19.58**	54.35	29.26*	27.67**	10803285**	3654338*	3912**
AxB	6	0.694	569*	17.12	0.29*	1394063**	546051	519**
e	22	1.028	224	10.92	0.1	497147	1199359	3.3
cv %		14.9	9.79	9.15	2.29	12.17	15.53	10.11

[1]Sources of variation (SV): A= Planting date B= Cultivars; df= degrees of freedom
*, ** significant at 5% and 1% respectively.

Table 2. Mean comparisons of measured traits[1]

Treatments	Days to emergence	Plant height (cm)	Head diameter (cm)	1000 seed weight (g)	Seed yield (kg/ha)	Oil yield (kg/ha)	Biomass (kg/ha)
Planting date							
Apr 19	7.333 a	160.42 a	22.42 a	43.25 b	3,816 ab	1,755 b	11,818 a
Apr 30	6.667 a	152.67 a	23.33 a	46.50 b	4,376 a	2,100 a	11,873 a
May 20	6.417 a	145.42 a	24.25 a	41.25 c	3,685 b	1,615 b	11,548 a
Cultivars							
SOUR	8.233 a	138.33 c	23.22 b	37.33 d	3,140 b	1,336 b	10,007 b
CMS26xR103	7.444 ab	128.33 c	18.89 c	41.00 c	2,744 bc	1,280 b	10,334 b
Azargol	6.778 b	162.22 b	24.11 ab	46.67 b	4,158 ab	2,065 a	11,433 b
Armavirsky	4.778 a	182.78 a	27.11 a	49.67 a	5,228 a	2,439 a	12,868 a

[1]Means with same letter show no difference at Duncan 5%

Plant height also differed among cultivars (Table 2). Armavirsky, with 182.3 cm and CMS26xR103 with 128 cm, showed the highest and the lowest height, respectively among cultivars. This difference was significant but differences between Sour and the others were not significant (Table 2). Plant height is a trait controlled genetically but environment can affect it. This was shown also by Ayin (1998), Hatami (1995), and Meinke et al. (1993).

Head diameter showed significant differences among cultivars (Table 2). Armavirsky and CMS26xR103 showed the highest and the lowest diameter, respectively. Head diameter has a great effect on yield, but there is an optimum value that maximizes seed yield. A larger head has more flowers that produce seeds. Cultivated sunflower cultivars have only one head and high yielding cultivars have a

bigger head (Alyari, 2000). Multiheaded plants may result from low density, early planting, soil higher N, and alternative drought stress. Garside (1998) showed that one of the Armavirsky traits with respect to other cultivars was its bigger head and more fertile flowers (Table 2).

Interaction of planting date x cultivar was significant for 1000 seed weight (Table 1). The 30th May planting date produced the highest value (48.67 g), and 21st May produced the lowest value (41.25 g) (Table 2). For cultivars, Armavirsky showed the highest value for 1000seed weight (48.67 g) and Sour with 37.33g the lowest. The high temperatures during the seed filling period in May plantings probably increased respiration and led to disturbance in seed filling resulting in an increase in the proportion of lighter seeds or hull. Goksoy et al. (2000) showed also that delay in planting led to decreasing 1000 seed weight.

Interaction of planting date x cultivar was also significant for yield (Table 1). Second planting date produced the highest yield but did not have a significant difference with respect to the first one (Table 2). Armavirsky produced 5,228 kg/ha and CMS26xR103 2,744 kg/ha, which were the highest and lowest seed yields observed (Table 2). Majid and Schneider (1987) showed that hybrid cultivars reaction to changing the planting date was lesser and Garside (1998), in a 4 planting dates (15, 30 March, 15 and 30 April) experiment and 4 cultivars, showed that effect of planting date on yield was significant.

The highest seed and oil yields were produced by Armavirsky on the second planting date. The reason for the decrease in seed yield in the later planting date was the decrease in 1000 seed weight. Thompson and Unger (1986) reported that yield is the most sensitive attribute to planting date changes. In their experiments, the 1st May planting produced the highest yield in all three years. Most reports showed that yield was higher at early and normal planting dates (Goksoy et al., 2000; Sindagi and Virupakshappa, 1990). Some reports also showed that no consideration of suitable planting date led to decreasing yields (Parmar and Kharwave, 1992).

Interaction of planting date x cultivar was significant on biomass (Table 1). Armavirsky, with 12,826 kg/ha and Sour, with 10,007 kg/ha produced the highest and the lowest biomass yield (Table 2). Planting date was also significant for this trait.

Effects of cultivar on oil yield were significant (Table 1). Armavirsky, with 2,439 kg/ha and CMS26xR103, with 1280 kg/ha, produced the highest and the lowest oil yield. The reason for the changes in oil yield in different cultivars is based on genetic potential, growth and developmental attributes and environmental conditions (Alyari, 2000). Factors such as higher biomass, head diameter and 1000 seed weight probably led to higher yield in Armavirsky. The higher temperatures during the seed filling period, especially 25 ºC in the flowering period, decreased oil yield. Miller et al. (1984) showed that the delay in planting from early May to early June decreased oil yield because the seed filling period coincided with hot days and short season. Alessi et al. (1997) reported that delayed planting resulted in a shortened growth period and, consequently the 1000 seed weight and the oil yield decreased.

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Some aspects of sunflower crop management in Romania

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ABSTRACT

The paper presents some research results regarding the technological management of sunflower, with the following conclusions: (i) sunflower should not be sown in the same field more than once every 6 years, avoiding the alternance with soybeans, due to the potential attack of *Sclerotinia*; (ii) the possibility of reducing soil tillage for fuel saving, without a yield decrease; (iii) the establishment of optimal sowing time in connection with soil temperature; (iv) optimal plant density varying between 40–50 thousands plants/ha. Although sunflower is a tolerant crop to dryness, the association between water deficit and high temperatures during the growing season caused very important yield losses and decreased the seed oil content.

Key words: plant density – Romania – *Sclerotinia* – soil tillage – sowing time – sunflower management.

INTRODUCTION

Sunflower is the main oleaginous crop in Romania, cultivated on an area that doubled in the 1990's, attaining 1 million hectares. The favorable market price sustained by the edible oil demands and by biofuel production, maintains the growth tendency of cultivated area. But this trend is limited by the crop rotation necessity and, also, by an increase in the rapeseed area enlargement.

Aiming to elaborate a crop management adapted to the agrobiological requirements of sunflower, a large number of experiments have been carried out, and their results have contributed to establishing some technological norms.

Research on the previous crop effect, the soil tillage, the sowing time, plant density, fertilizer application, the weeds and diseases control have been made in different countries, as well as in Romania (Blanchet et al., 1987; Sin et Ioniță, 1990; Vannozzi et al., 1990; Vrânceanu, 2000; Bonari et al., 1992; Sarno et al., 1992).

This paper presents some technological aspects resulting from the experiments carried out.

MATERIALS AND METHODS

This research work was carried out in the National Agricultural Research and Development Institute – Fundulea, under rainfed conditions, during a long period, on chernozem soil with a good fertility: organic matter (humus) - 2.5%; N – 0.18%; P_2O_5 – 28 ppm; K – 98 ppm; TON content – 30%; pH – 6.5. The annual mean rainfall is 560 mm, ununiformly distributed during the year and the annual mean temperature is 10.5 °C.

The experiments have included 2 – 6 years rotations and monoculture, soil tillage methods, different sowing treatments with dates and densities (3–6 plants/m^2). The size of an experimental plot varied between 100–500 m^2, and the number of replications between 3–5.

The experiment treatments are presented here, together with the research results.

RESULTS AND DISCUSSION

Crop rotation

The experiments aimed to establish the influence of previous crops and the minimum number of years for sunflower return in the same place, in connection with crop production and disease attacks.

The data presented in Table 1 emphasize the following aspects:

- the highest crop yield was obtained in the 6 years of rotation, demonstrating the necessity of sunflower growing in the same place not for a shorter time than 6 years;
- the 4 - year rotation favored the *Sclerotinia sclerotiorum* attack. Even the soybean in a crop rotation with sunflower increased the attack of *Sclerotinia*;

- the greatest *Sclerotinia* attack and the lowest yields were obtained in the case of sunflower cultivated after soybean and sunflower.

Table 1. Effect of the crop rotation on sunflower yield and *Sclerotinia* attack (4-year average)

Crop rotation	Yield q/ha	Difference q/ha	Difference %	*Sclerotinia* attack - %
Sunflower-Wheat-Sugar beet-Maize-Maize-Wheat (6-years rotation)	32.0	-	-	3.0
Sunflower-Wheat-Sugar beet-Maize (4-years rotation)	30.0	-2.0	7	12.1
Sunflower-Soybean-Wheat-Maize (4-years rotation)	27.9	-4.1	13	16.5
Sunflower-Soybean (2-years rotation)	23.0	-9.0	28	26.0
Sunflower-Sunflower	22.8	-9.2	29	23.4
LSD 5%		1.9		

Soil tillage

The research regarding soil tillage has taken into consideration soil fertility conservation and fuel consumption reduction. The experiment results showed that sunflower reacts weakly to soil tillage methods and to the loosening depth, the crop yield differences being non-significant (Table 2).

The lowest yield was obtained in the case of no-till soil.

The advantage of reduced soil tillage was represented by fuel saving.

Table 2. Relationship between tillage method, fuel consumption and sunflower yield (4-years average)

Soil tillage	Yield q/ha	Difference q/ha	Fuel consumption, %	
			Ground tillage	Total soil tillage+sowing
Plowing 20 cm	22.4	-	100	100
Plowing 30 cm	23.0	+0.6	130	120
Chiseling	21.9	-0,5	57	69
Paraplowing	21.6	-0.8	75	83
Disking	22.0	-0.4	20	41
No-till	20.3	-2.1	-	14
LSD 5%		2.2		

The fuel consumption by soil plowing at 20 cm depth (100%) diminished by 86% in the no-till and by 17–50% using tillage methods with chisel, paraplow and disk, instead of plow, without a yield reduction.

Sowing

This concerns the experiments including different sowing dates and plant densities:

The data presented in Fig. 1 show the yield variation in three different years, depending on planting date, connecting with the soil temperature at sowing depth.

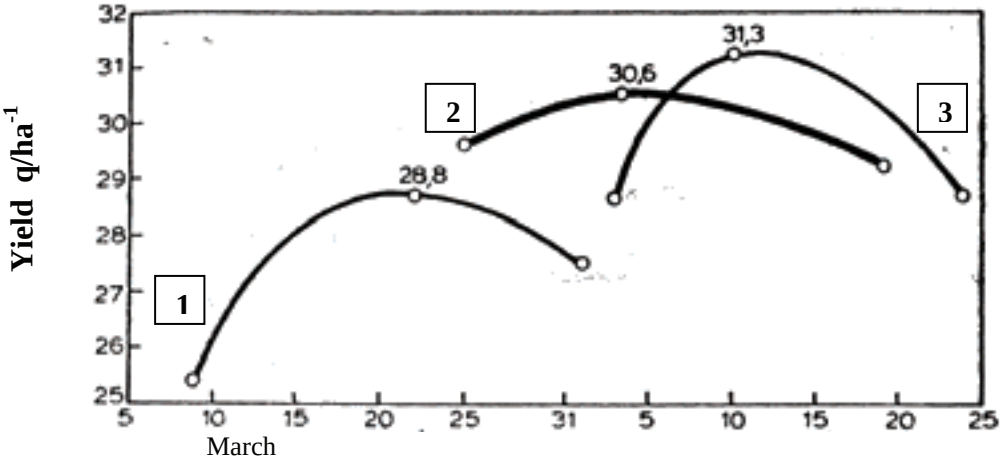
The highest yield was obtained when the soil temperature reached 7 °C. This relation was observed in early spring (1) and, also, in late spring (3), the optimal sowing data being marked by the occurrence of the respective soil temperatures. To sow earlier or later than this moment causes yield losses.

The plant density has been studied taking into account a variation from 30 to 60 thousand plants/ha.

The average results obtained for late hybrids are presented in Table 3.

The data pointed out a relation between the sowing density and the harvested plants, resulting in a decrease of 9.0–12.7%, with a growth tendency as the density increased.

The difference between both densities could be diminished by a better control of diseases and pests and by more careful cultivations, which could reduce the plant number.



1	2.7	6.9	8.1					
2	2.9	3.6	5.3	6.9	8.2	8.5	8.2	
				5.0	7.5	8.2	7.2	3

Soil temperature, in three different years (1, 2, 3)

Fig. 1. The effect of sowing time on sunflower yield.

Table 3. The effect of plant population on sunflower yield (10- year average)

Sowing density, 10^3 seeds/ha	Harvested plants/ha		Yield		Difference q/ha
	10^3	%	q/ha	%	
30	27.3	90.0	27.3	100	-
40	35.4	88.5	29.6	108	2.3
50	43.8	87.6	30.0	109	2.6
60	52.4	87.3	29.0	106	1.7
LSD 5%			2.0		

The sunflower yield varied non-significantly at a population of between 40 and 60 thousand plants/ha. The weak reaction to the variation in plant density is explained by a high capacity for compensation of crop yield components.

Relationship between water supply and sunflower yield

An analysis of rainfall regime during the years 2006 (wet year) and 2007 (with droughts) shows a net differentiation regarding plant growth, yield formation and its quality (Table 4).

Table 4. Relationship between climate conditions (rainfall amount and air temperature), yield level and seed oil in 2006 and 2007

	Wet year 2006	Dry year 2007	Difference
Rainfall, mm			
- Oct.2005-March 2006	301		
- Oct.2006-March 2007		122	179
- April-August	313	190	123
Mean temperature, °C			
- June	20.9	24.1	3.2
- July	22.9	26.9	4.0
- August	23.0	24.1	1.1
Seed yield, kg/ha	2400	550	1850
Seed oil content, %	50.8	42.5	8.3
Oil production, kg/ha	1219	234	985

The rainfall during October – March period and April – August growing period greatly varied with obvious differences between the two years of 179 mm and 123 mm, respectively.

The drought conditions of 2007 were stressed by the association of humidity shortage and high temperature, which was higher by 3 – 4 ^{0}C in 2007, as compared with the previous year.

This phenomenon negatively influenced the plant growth and yield formation, leading to a difference of 1850 kg/ha (77%).

The drought also reduced the seed oil content (8.3%), so that the oil production in 2007 was lower by 985 kg/ha, representing 19% from the oil production obtained in 2006.

CONCLUSIONS

Sunflower must be included in a 6 - year rotation, avoiding its alternance with soybean crop, due to the potential attack of *Sclerotinia*.

The soil tillage method has a weak influence on sunflower yield, offering the possibility to apply a reduced tillage, that ensures a way for fuel saving.

The optimal time for sunflower sowing is indicated by achieving the temperature of 7 °C, at sowing depth.

The optimal plant density for late hybrids is of 40 – 50 thousand plants/ha.

Although the sunflower is a tolerant crop to dryness, the association of water deficit with high temperatures during the growing season caused yield losses of up to 77% and decreased the seed oil content.

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Screening and drying conditions for early harvested sunflower

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ABSTRACT

Screening tests were held to examine the method to remove admixture from the sunflower grain before drying process. Moisture contents of samples were 34.4% and 20.0%. Screening sizes examined were 1.0-5.0 mm. According to the results obtained, it is difficult to fix a certain screen gap size to remove most admixtures because size of grain varies from year to year and grain and admixture have almost the same size. However, using the 2.0 mm gap screen, a certain ratio (26-75%) of admixture, which is smaller than the grain, can be removed, and this is good for drying process in order to reduce machine trouble and extra energy to dry the admixture. The drying conditions and their effects on quality of sunflower, especially on oil quality, were also analysed for POV (peroxide value), AV (acid value) and color of oil. The sunflower plant material was cv Harurinzo (Pioneer:63M80) and the initial moisture contents were of 15.8% w.b. and 31.5%. Each sample was dried in an oven with 45°C, 55°C , 65°C for 24 hours and a circulating dryer (capacity: 1 t) for 11 hours. Samples were expressed with a small expeller. The oil samples were analysed for POV and AV. POV of sunflower oil ranged from 1.9 meq/kg for 45°C drying with 15.8% initial moisture content to 6.8 meq/kg for 65°C with 31.5% initial moisture content. According to the results obtained, it is concluded that in order to avoid the degradation of oil quality, the initial moisture content should be low enough, and, if the harvested sunflower has a high moisture content, drying it at a lower temperature is better to maintain the quality of the oil.

Key words: acid value – drying – early harvesting – peroxide value – screening

INTRODUCTION

Recently, sunflower and rapeseed cultivated areas have increased rapidly in Japan because of the growing expectations of bio-fuels made from oil from those crops. But there are some problems in producing sunflower in Japan. One major reason is that farmers do not have enough experience of growing, harvesting and drying of sunflower.

The high admixture rate is a problem. In drying process much energy is lost in drying grain with so many admixtures. Moreover, with respect to the oil expressing process, a high admixture rate reduces the yield of the oil expressed and sometimes causes machine troubles. The admixture rate in a harvested crop occasionally reaches 20%. The high admixture rate is the result of harvesting sunflower under high moisture conditions, especially with high moisture in the stem and receptacle, with a harvester designed for rice or wheat.

In Japan, because of the high humidity in the climate, the drying process is inevitable. But farmers do not know the appropriate drying condition for sunflower, because of their lack of enough experience, and sometimes they degrade the quality of the sunflower grain through drying process. Therefore, it would be necessary to clarify the effect of the drying conditions on the quality of sunflower grain and its oil and to fix the drying conditions for this crop.

The size of the screen gap for removing the admixture from sunflower before its drying process was examined. Also, the drying conditions for keeping the oil quality high were examined with POV (peroxide value) and AV (acid value).

MATERIALS AND METHODS

Screening test

Experiments were held in 2006 and 2007. In 2006, the sample was harvested with 34.4% moisture content in Hikawa city, Shimane pref., Japan. The sample was sorted with the size grader before drying. The screen gaps examined were 2.0, 2.4, 2.8, 3.2, 3.6, and 4.0 mm. In 2007, the sample was harvested with 21.0% moisture content. The sample was sorted with the size grader before drying and the screen gaps examined were 2.0, 3.0, 4.0, and 5.0 mm. In both cases, the admixture was separated after drying with a winnower for experimental use and its weight was measured.

Material and initial moisture contents
The sunflower cultivar Harurinzo (Pioneer:63M80) was grown with usual cultivation practices from June to October of 2006 in the south of Ibaraki Prefecture, Japan. Samples were harvested twice. The moisture contents of each sampling were "*I*: 15.8%" and "*II*: 31.5%". Moisture contents were measured with the 10 g (grain) -105°C-24 hours method.

Drying settings
To fix the best drying temperature for each moisture contents samples were dried with the air of "*A*: unheated", "*B*: 45°C", "*C*: 55°C" and "*D*: 65°C." A circulating dryer (*E*) was used because this dryer is very popular with Japanese rice farmers and it is useful if it can be utilized for drying of sunflower.

Drying settings were shown in Table 1. For *A* the ventilation dryer, Issingo Kaneko Agricultural machinery co., Ltd, has 6.6 m^2 mesh deck and air flow upward by blower (0.75kW) without burner burning. The drying oven was Espec Convection Oven LC-123. The circulating dryer was Iseki GA100 (Capacity: 400-1200 kg of wheat).

Each sample from *A* to *D* was about 2 kg and packed in 30cm x 40cm plastic mesh bags. Sample *E* was dealt as bulk. After drying, each sample was preserved in 10°C refrigerator.

Table 1. Drying Settings

	Dryer	Air Temperature (°C)	Drying time (hr.)
A	Ventilation dryer	Unheated	24
B	Drying oven	45	24
C	Drying oven	55	24
D	Drying oven	65	24
E	Drying oven	Unheated-55 (changing)	11

Expression of oil sample
A small expeller (San-Seiki S100-200, Capacity: 3.5 kg/h) was used. Moisture contents of samples were 5-6% when expelled. Yield of oil was 20-30% of the input grain weight.

Evaluation of oil quality
Expelled oil samples were examined by POV (peroxide value), AV (acid value) and the color of oil. POV and AV are used as indices for oil as food constituent. The measurement of POV and AV were outsourced to Japan Institute of Oil, Fats and Other Foods Inspection Foundation and analysed using Standard Methods for the Analysis of Fats, Oils and Related Materials (Japan Oil Chemists' Society).

RESULTS AND DISCUSSION

Screening test
Results of the screening test are shown in Fig. 1 and Fig. 2. In 2006 (Fig. 1), the admixture rate was 14.4% of whole weight. The admixture classified into 0-2.0 mm was 10.8%, others were lower than 1.0% of whole sample. 75% of the total admixture was classified into 0-2.0 mm. For the grain, 55.7% of whole sample, 65.1% of grain, was classified into more than 4.0 mm class.

In 2007, (Fig. 2) the admixture rate was 20.4% of whole weight. The admixture classified into 0-2.0 mm was 5.4%, 2.0-3.0 mm 4.2%, 3.0-4.0 mm 7.1%, 4.0-5.0 mm 3.0% and 5.0 mm- 0.7%. 26.5% of the admixture was classified into 0-2.0 mm. For the grain 37.0% of whole sample, 45.5% of grain was classified into more than 3.0-4.0mm class. And 36.2% of whole sample, 45.4% of grain was classified into more than 4.0-5.0 mm class.

From the results it is difficult to fix a certain size of screen gap to remove most of the admixture from mixture of grain and admixture because the grain size is different every year due to environmental conditions. However, screening with 2.0 mm screen removes 26-75% of the admixture. This seems to be of use as a rough screening before drying process because it will reduce the energy used for drying and the risk of machine trouble from the dryer even if the screening process after drying is inevitable.

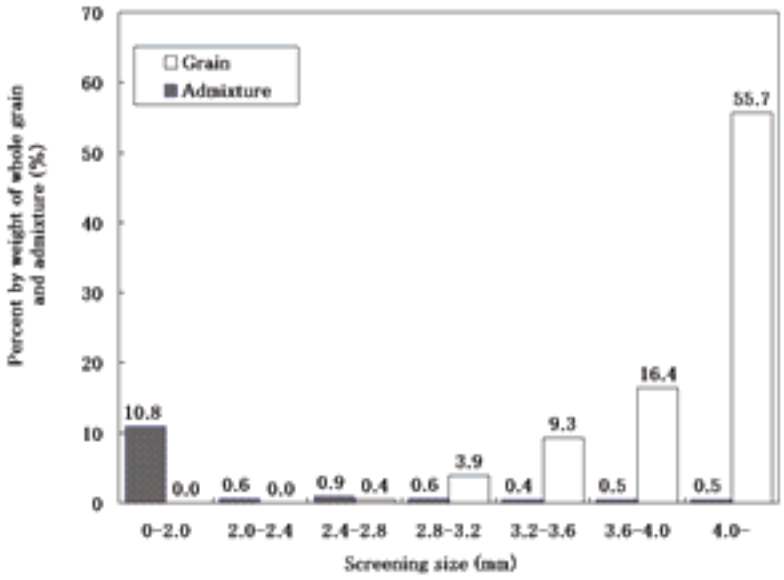

Fig. 1. Results of admixture screening test in 2006

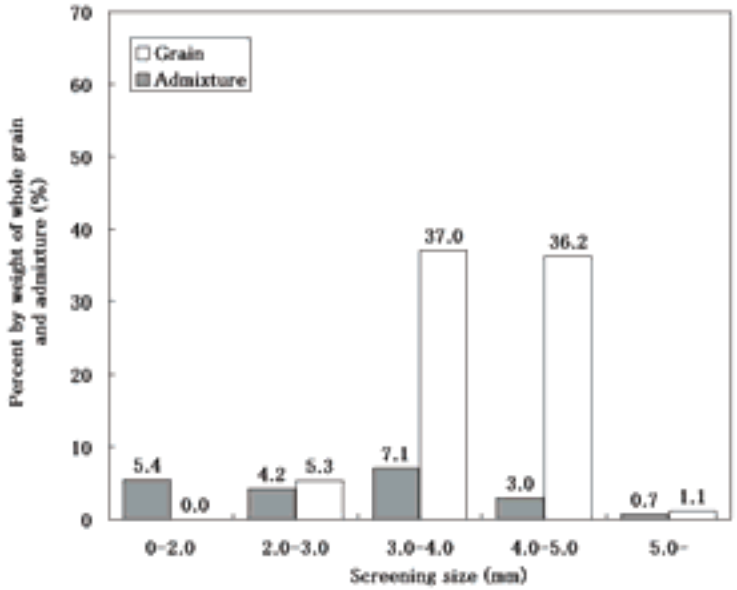

Fig. 2. Results of admixture screening test in 2007

Drying conditions

Fig. 3 shows POV of sunflower dried under each setting. When the initial moisture content was 15.8% (*I*), *D* setting had the highest POV followed by *C*, *B* and *A* which was the lowest. When the initial moisture content was 31.5% (*II*), *E* setting had the highest POV followed by *D*, *C*, *B* and *A*, which was the lowest.

The higher drying temperature resulted in a higher POV. That tendency was stronger for higher initial moisture content, 31.5% (*II*) than for 15.8% (*I*). The results also showed the effect of the initial moisture content on POV. POV of *II* (31.5%) was higher than that of *I* (15.8%) in all drying conditions from *A* to *D*.

It is concluded that to avoid the degradation of oil, firstly it is important to harvest enough dried grain below 16%. Secondly, if the harvested grain has a high moisture content of around 30% it should be dried with unheated or low temperature air.

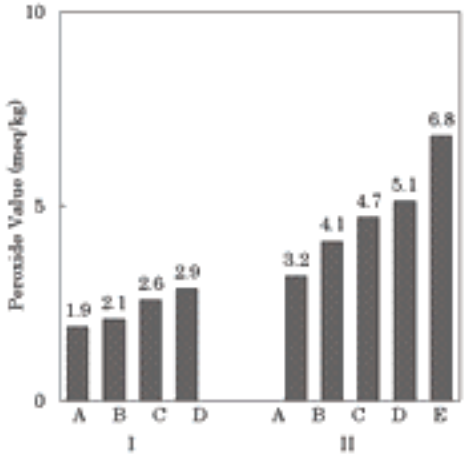

Fig. 3. POV of sunflower under each drying condition

The AV of sunflower oil of each sample varied from 0.3 to 0.7 and did not show any effects from the drying condition. Average of the AV of *I* was 0.3 and *II* 0.48. AV seems to have a proportional relationship with the initial moisture content.

The oil with an AV of over 1.0 was classified as being unsuitable for food, based on Japanese Standard. The AV of sunflower oil fulfilled the standard. The drying conditions did not have much effect on the deterioration of the AV. This implied that the higher initial moisture content of the grain caused a higher AV.

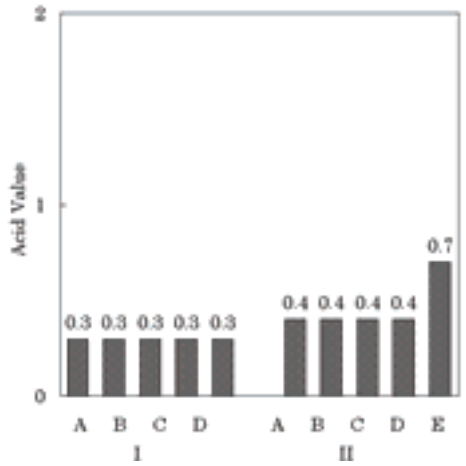

Fig. 4. AV of sunflower under each drying condition

CONCLUSIONS

The size of the screen gap in removing the admixture from sunflower before its drying process was examined. Also, the drying conditions for keeping the oil quality high were examined with POV (peroxide value) and AV (acid value). The conclusions of this study are follows:

1) From the results it was seen to be difficult to fix a certain size of screen gap to remove most of the admixture from mixture of grain and admixture because the grain size is different every year due to environmental conditions. However, screening with 2.0 mm screen removed 26-75% of the admixture. This would seem useful as a rough screening before drying process because it reduces drying energy and the risk of machine trouble from the dryer even if the screening process after drying is inevitable.

2) To avoid the degradation of oil, firstly it is important to harvest enough dried grain below 16%, and, secondly, if the harvested grain has a high moisture content of around 30% it should be dried with unheated or low temperature air.

3) The drying conditions did not have much effect on the deterioration of the AV. This implied that the higher initial moisture content of the grain gave a higher AV.

Physiological maturity in sunflower. Correspondence between the quantitative and the visual definition

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ABSTRACT

The identification of physiological maturity (PM) in sunflower (*Helianthus annuus* L.) by visual methods is highly subjective. In order to find an indirect method for objectively defining PM, this study was conducted to correlate, in two sunflower hybrids, Macón and MG60, quantitative color parameters in the receptacle (Hue and Chroma in the *HSB* color space and *L**, *a** and *b** in the *CIE* color space) with physiological markers such as fruit dry weight (FDW) and fruit water content (FWC). Fruits from each cultivar were sampled at 2-day intervals from first anthesis until harvest maturity (HM). Fruit dry weight and color changes of the receptacle base, using digital images, were followed over time until HM. Fruits attained their maximum dry weight when the capitulum color turned from dark green to pale green in MG60 and when it turned from dark green to buttery-yellow in Macón. The color parameters L*, a*, b*, Hue and Chroma were tested against the fruit dry weight, and several good correlations were found, but from a crop management point of view the Hue (r_{MG60}= 0.876; $r_{Macón}$= 0.794) appeared to be a valid color parameter to define visual PM.

Keywords: color correlation – color parameters – *Helianthus annuus* – physiological maturity – sunflower.

INTRODUCTION

Physiological maturity (PM; Schneiter and Miller, 1981), is an important reproductive stage of the sunflower crop. At PM fruit dry weight (FDW) has reached its maximum value with a water content (FWC; d.w.b.) of about 38% (Rondanini, 2007). In the decimal notation by Schneiter and Miller (1981), the most frequently used scale to define the developmental stages of sunflower, PM, also defined as phenostage R9, is externally observed when the phyllaries become brown and brittle and the receptacle base turns buttery yellow.

The time elapsed to attain PM varies according to genotypes and environmental conditions such as nitrogen and soil water availability, temperature and photoperiod (Connor and Hall, 1997). The same genotype can differ from between 7 and 10 days to reach PM in response to changes in the variables mentioned (Kaya et al., 2004). Therefore, although the scale by Schneiter and Miller (1981) is a useful tool to study many sunflower genotypes, it fails for others. In fact, in some "stay green" (SG) genotypes the base of the receptacle at PM is green or yellowish green; only the phyllaries can become slightly brown (Cukadar-Olmedo and Miller, 1997).

Changes in color of the sunflower receptacle when approaching PM are recorded at naked eye. This is why the method is highly subjective. The aim of this work was to determine the correspondence among chromaticity of the receptacle base, by analyzing digital images of the receptacle development from first anthesis (FA) until harvest maturity (HM), the phenostage scale developed by Schneiter and Miller (1981) and the evolution of FDW and FWC.

MATERIALS AND METHODS

Two short season sunflower hybrids: Macón (Syngenta, Argentina) and MG-60 (Dow-Agrosciences, Argentina) were used in the study. Plants were grown at the Chacra Experimental de Barrow (INTA-MAA, Tres Arroyos, Argentina; Lat. S. 38º20'; Long. W. 60º13') following conventional cultural practices.

Qualitative determination of phenological stages was made using the scale by Schneiter and Miller (1981). At FA (phenostage R5.1; Schneiter and Miller, 1981) twelve plants of each hybrid were selected and labeled. FDW and FWC (d.w.b.) were measured in 6 plants of each hybrid by taking samples of fruits from the capitulum's rim at 3-day intervals from FA to HM.

A biphasic fit of FDW vs. time (days from FA) was performed using the model: **y** = a+b.X (for X<c); **y** = b.c (for X>c), where **c** corresponds to the unknown break point of the two linear functions, this being the maximum grain weight of the fruit F(t), where PM is attained.

Simultaneously with fruit sampling, photographs of the receptacle base were taken from 8:00 a.m to 9:00 a.m. to the remaining 6 plants of each hybrid using a digital camera. A color reference scale was included in each image. Digital images were corrected for light intensity changes and analyzed to determine the parameters *L**, *a** and *b** within the CIE *L*a*b** color space, (CIE, 1986, 2001), using Photoshop CS2 software (Adobe Systems Inc.; San José, CA, USA).

*L**, *a** and *b** values were furthermore converted into the *HSB* color space (Adobe Systems Inc., 2000; MacEvoy, 2005), defining the parameters Hue (the attribute of color by means of which it is perceived to be red, yellow, green, blue, etc. Pure white, black, and gray possess no Hue) and Chroma (also called "saturation" and indicating the amount by which a color differs from gray, white or black, from neutral to fully saturated color). The values run from 0%, which is no color saturation, to 100%, which is the fullest saturation of a given Hue, using the algorithms:

$$\text{Hue} = h^* = \tan^{-1}(b^*/a^*), \text{ [when } a^*>0 \text{ y } b^*\geq 0]; \text{ Hue} = h^* = 180 + \tan^{-1}(b^*/a^*) \text{ [when } a^*<0]$$

$$\text{Chroma} = C^* = [a^{*2} + b^{*2}]^{1/2}$$

RESULTS AND DISCUSSION

Maximum FDW significantly differed ($p<0.01$) between genotypes, with 0.043 g/fruit, 31 days after FA in MG60 (Fig. 1A) and 0.045 g/fruit, 28 days after FA in Macón (Fig. 1B). Maximum FDW for both hybrids was attained with a FWC of 38.6% in MG60 (Fig. 1A) and 39.2% in Macón (Fig. 1B). These values showed no significant differences ($P<0.05$). However, Macón showed a higher average FWC (Fig. 1B), possibly as a consequence of green mass retention at PM.

The magnitudes of the *L*a** and *b** at the time of PM were: *L**: 68.3 (MG60) and 73.6 (Macón); *a**: -4.2 (MG60) and -6.2 (Macón); *b**: 48.4 (MG60) and 52.3 (Macón) (Fig 2. A and B, respectively).

The *L** magnitude showed important fluctuations (Fig. 2A-B) in response to variations in daily luminosity when digital images were taken. This probably masked the real magnitude of luminosity as maturity advanced (Shewfelt et al., 1988). However, it was observed that *L** magnitude decreases with capitulum maturity in response to opacity and darkening of the capitulum's tissue (Fig. 2 A-B).

A significant correlation between the FDW and colorimetric parameters *a** (0.752; 0.638), *b** (0.771; 0.670), Hue (0.876; 0.794) and Chroma (0.669; 0.593) for MG60 and Macón, respectively, were observed. Nevertheless, it was found that both Hue and Chroma were the best color parameters to be considered when working in a relationship between their changes with time of capitulum maturation and FDW.

In early developmental stages the presence of a high concentration of chlorophyll in the receptacle tissues is significantly related to the green color observed. So, as maturity advances, chlorophyll degradation, (Sexton and Woolhouse, 1985) and the predominance of xanthophylls and other carotenoid pigments (Sinecker et al., 2002) are the reason for the variation in color turning from green to yellow.

Magnitudes of *a** and *b** moved over time from minus *a** (green component; HunterLab., 2001) to plus *a** (yellow-red component; HunterLab., 2001) (Fig. 2A-B). The parameter *b** (yellow-blue component; HunterLab., 2001) always had positive values.

For both hybrids, results showed that the magnitude of *b** tends to increase up to the moment of the maximum value of FDW and then decreases (Fig 2 A-B) following the diminution of FWC, in response to plant senescence (Fig. 1A-B). The *a** value increases as capitulum maturity advances (Fig 2 A-B), allowing the *b** component (yellow) to stand out. Yellowing of the receptacle was characterized, as expected, by a constant increase in the value of *a** (less green) and a maximum magnitude of *b** (more yellow) (Fig. 2A-B).

Hue values decreased from 122.8 to 74.6 in MG60 (Fig. 1A) and from 115.4 to 71.1 in Macón (Fig. 1B). Chroma increased until 28 days after anthesis in MG60 (Fig. 1A) and in Macón (Fig. 1B), when both hybrids attained their maximum FDW. From that moment on Chroma magnitude started decreasing.

The maximum Chroma (maximum color saturation) in MG60 was attained 2 days before PM (Chroma=59; Fig. 1A); the Hue at that time was 98 showing a buttery yellow capitulum base and brown phyllaries. The maximum Chroma in Macón was attained at PM (Chroma=62; Fig. 1B) with a yellowish receptacle base and the phylllaries still green. Also, in this hybrid with a higher retention of green tissue, PM was attained 12 days before phenostage R9 was observed (Fig. 1B).

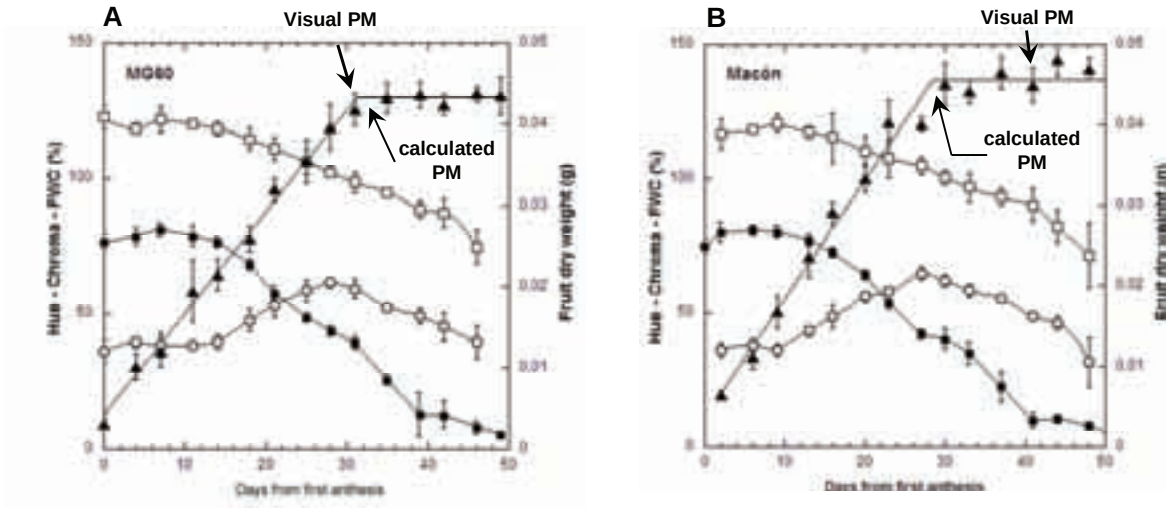

Figure 1: Evolution from first anthesis until harvest maturity of fruit dry weight (FDW), fruit water content (FWC) and the color parameters Hue Chroma in MG60 (A) and Macón (B). The visual PM (R9; Schneiter and Miller, 1981) and maximum FDW (calculated PM) in MG60 (A) was atta 31 days after anthesis. The maximum FDW in Macón (B) was attained 28 days after anthesis, while the visual PM was approximately 12 days l Then only in the genotype MG60 (B), the maximum FDW coupled the visual PM according to the morphological characteristics defined by Schn and Miller (1981). (□) Hue; (○): chroma; (▲): fruit dry weight (FDW); (■): Fruit water content (FWC). Vertical bars: ± 1SE.

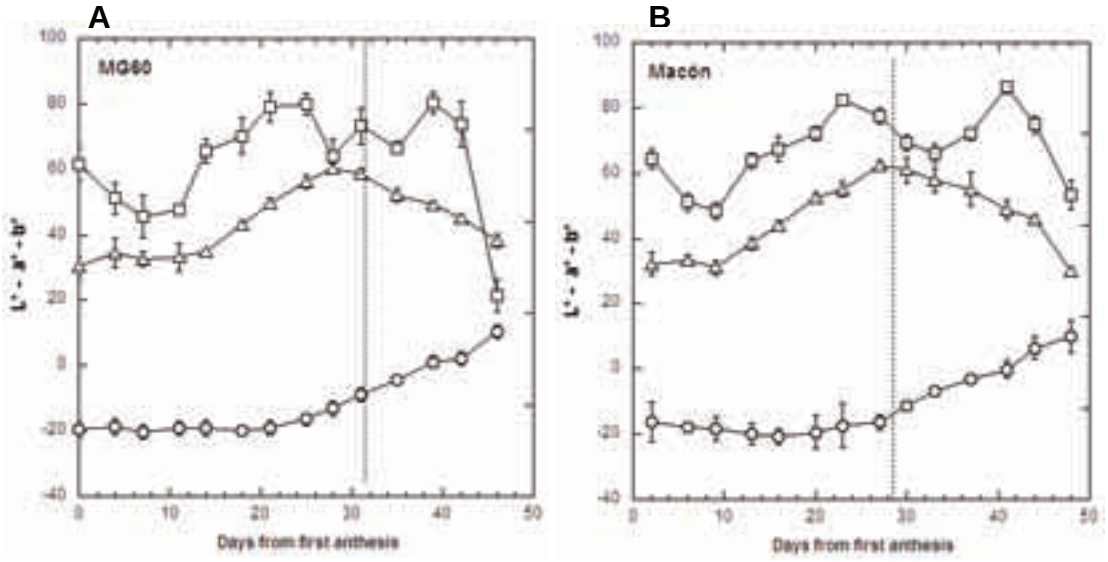

Fig. 2. Changes in the CIE *L*a*b** parameters in the base of capitula of the sunflower hybrids MG60 (A) and Macón (B) from first anthesis until HM. The vertical dashed line indicates the time when the maximum FDW, and hence PM was attained. (□) *L**; (○): *a**; (△): *b**. Vertical bars: ± 1SD.

The hybrid MG60 attained visual PM (R9; Schneiter and Miller, 1981) 31 days after first anthesis (Fig. 1A) while Macón, attained visual PM 40 days after FA (Fig. 1B). In MG60 visual PM (Hue= 98) and measured PM were reached at the same time (Fig. 1A). In Macón the maximum FDW was attained 12 days earlier than visual PM (Fig. 1B) indicating that fruits reached their maximum dry weight when the receptacle base was still green with a Hue of 103.

The linear variations in Hue, between 10 and 40 days after FA in both hybrids (Fig. 1A-B), showed the direct relationship between the receptacle color change and the advance of fruit maturity. The Hue is then best associated with the attainment of the visual PM, corresponding to phenostage R9, this value being nearly similar for both hybrids: Hue Macón=103; Hue MG60=98 (Fig. 1A-B). Therefore, the Hue of the receptacle base could be a useful parameter to express differences or similitudes between sunflower genotypes in the attainment of PM.

This work demonstrates that visual scales, which are generally widely subjective, are not always appropriate for determining maturity stages of crop plants, particularly sunflower. The brown phyllaries as a qualitative concept of PM cannot be applied to all genotypes. Using quantitative color parameters in genotypes grouped by maturity length and/or green mass retention could be a more precise approach to determine the correspondence between the measured PMs and their visual morphological characteristics.

ACKNOWLEDGEMENTS

This work was funded by grants to L.F.H. of the Agencia Nacional de Promoción Científica y Tecnológica and the Argentine Sunflower Association (ANPCyT-ASAGIR, PICTOS-13151), the Secretaría Gral. de Ciencia Tecnología (SeGCyT-UNS) and the Comisión de Investigaciones Científicas (CIC, La Plata) Argentina. The valuable technical suggestions made by Mr. J. J. Detzel, Diario La Nueva Provincia, Bahía Blanca, Argentina are greatly appreciated. Sunflower seed was kindly provided by Dow Agrosciences and Syngenta Seeds, both of Argentina.

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Influence of desiccation on germination and field emergence of sunflower

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ABSTRACT

In this paper we present results on the influence of desiccation on laboratory germination and field emergence of sunflower hybrid Favorit and its parental lines. As desiccants we used Reglone forte [diquat] (3 l/ha) and Harvade 25F [dimethipin] (2 l/ha), with water usage of 500 l/ha. Desiccation was done on August 20, 2006, and, after 20 days, the genotypes were harvested. In each trial variant (combination of genotype and treatment) laboratory germination was performed on four subsequent dates with 2-month intervals. With relation to the control, the Harvade 25F variant had a statistically significant higher germination of 4.38%, while the Reglone forte variant had a statistically significant higher germination in relation to both, control and Harvade 25F, of 7.32% and 2.95%, respectively. Regarding field emergence, differences among the treatments were small, statistically non-significant. Like in the laboratory germination test, Reglone forte treatment had the highest field emergence (78.55%) which was higher than the control and Harvade 25F in 2.94% and 2.86%, respectively.

Key words: desiccation – field emergence – Harvade 25F – laboratory germination – Reglone forte – sunflower.

INTRODUCTION

Chemical desiccation before harvesting is a very useful agro-technical measure, which is applied on different crops like cotton, rice, potato, alfalfa, soybean, oil rape and sunflower. Desiccation of sunflower is performed at technological maturity of plants, when seeds contain 24-50% of moisture (Degtyarenko, 1976; Palmer and Sanderson, 1976; Kosovac and Sudimac, 1980; Tombu, 1988; Miklič et al., 2001; Johnson et al., 2004; Radić, 2006). At that time the process of seed forming and filling has been completed, the seeds begin to lose their moisture and plants are still green.

Desiccation remarkably decreases moisture of seeds, leaves, heads and stalks, accelerates maturation and enables earlier harvest. Losses of seeds in combining as well as drying expenses, bird damage and presence of weeds on fields are reduced. It is possible to prepare the field earlier for the next crop and there are no harmful consequences for oil and byproducts quality (Hill et al., 1974; Kosovac and Sudimac, 1980). Desiccation has an especially positive influence in years with heavy rainfalls during the maturation period of sunflower when attacks of fungal diseases are very intensive.

Besides its positive influence on grain yield, desiccation also improves seed quality (Dembinski et al., 1974; Palmer and Sanderson, 1976; Miklič et al., 2004; Đukić et al., 2006). The objective of this research was to test desiccation influence on germination and field emergence of the sunflower hybrid Favorit and its parental lines by treating them with the desiccants Reglone forte (total herbicide) and Harvade 25F (growth regulator).

MATERIALS AND METHODS

Research was conducted in the experimental field and laboratory of the Agricultural Institute Osijek. Field trial was sown on April 22, 2006, with inter row space of 70 cm, in-row space of 23.5 cm, giving a plant population of around 61000 plants/ha. Main plot had surface of 14 m^2 (4 rows by 5 m length).

Genotypes in the research were the sunflower hybrid Favorit and its parental lines, developed at the Agricultural Institute Osijek. Treatments in the research were Reglone forte [diquat] (3 l/ha) and Harvade 25F [dimethipin] (2 l/ha), with water usage of 500 l/ha, and non-desiccated control treatment. Desiccation was done on August 20, 2006, and, after 20 days, the genotypes were harvested. In each trial variant (combination of genotype and treatment) laboratory germination was performed four times (October 23, 2006; December 21, 2006; February 22, 2007 and April 23, 2007). Seed vigor was calculated as the percentage of seeds that germinated after four days. Seed germination was calculated as the percentage of

seeds that germinated after 10 days (Official Gazette, 4/2005). For field emergence determination, seeds were sown in the field on April 13, 2007, and counting of emerged seedlings was carried out on May 11, 2007. The experimental data obtained were processed by SAS for Windows (SAS, 2003) software.

RESULTS

Seed samples of sunflower hybrid and parental lines were taken at the same time, before desiccation. Seed moisture of hybrid was 24%, female line 22.8%, and pollinator line 34.4%. The latter had distinctly the highest moisture. Twenty days after desiccation, the genotypes were harvested. Table 1 presents seed moisture of analyzed treatments and genotypes. As we expected, the highest seed moisture was found in the control (8.48%), then Harvade 25F, and a statistically significant lower moisture content than the control (1.28%) was found with the Reglone forte treatment. Among the genotypes, statistically significant differences were also found. The pollinator line had a statistically significant higher moisture content in relation to the hybrid (0.40%) and the female line (0.55%).

Table 1. Sunflower seed moisture at harvest of tested variants and genotypes

		Seed moisture (%)
Treatment	Control	8.48
	Reglone forte	7.20
	Harvade 25F	8.21
	LSD 0.05	0.36
Genotype	Favorit	7.88
	Female line	7.73
	Pollinator line	8.28
	LSD 0.05	0.36

Laboratory germination was estimated in the seed laboratory of the Agricultural Institute Osijek on four subsequent dates at 2-month intervals (Table 2). In the first count on October 23, 2006, seed vigor was very low (29.89%) as well as germination (53.83%). This could be explained by distinctive seed dormancy. In the second and third count, seed vigor and germination had almost the same values. In the fourth, final count on April 23, 2007, seed vigor was 91.39%, and germination 92.39%, respectively, which was statistically significantly greater ($P<0.05$) than in the previous count.

Table 2. Seed vigor and germination in subsequent germination tests

Germination test	Seed vigor (%)	Germination (%)
October 23, 2006	29.89	53.83
December 21, 2006	87.18	89.37
February 22, 2007	87.27	89.58
April 23, 2007	91.39	92.39
LSD 0.05	2.40	2.40

Among the treatments evaluated, we found statistically significant differences for seed vigor and germination (Table 3). In relation to control, the Harvade 25F variant had a statistically significant higher germination of 4.38%, while Reglone forte showed a statistically significant higher germination in relation to control and Harvade 25F of 7.32% and 2.95%, respectively. Among the genotypes, hybrid Favorit and female parental line did not exhibit any statistically significant differences in seed vigor and

germination, but the pollinator line had distinctly lower seed vigor and germination in relation to both hybrid Favorit and female parental line of almost 10%.

Table 3. Seed vigor and germination of tested variants and genotypes.

		Seed vigor (%)	Germination (%)
Treatment	Control	70.77	77.39
	Reglone forte	77.52	84.71
	Harvade 25F	73.51	81.77
	LSD 0.05	2.08	2.08
Genotype	Favorit	77.36	84.03
	Female line	77.05	84.74
	Pollinator line	67.39	75.10
	LSD 0.05	2.08	2.08

Among the treatments, differences in field emergence were small, statistically non-significant (Table 4). Again, Reglone forte treatment, as in the laboratory germination test, had the highest field emergence (78.55 %), which was higher than control and Harvade 25F for 2.94% and 2.86%, respectively.

Table 4. Field emergence of tested treatments and genotypes

		Field emergence (%)
Treatment	Control	75.61
	Reglone forte	78.55
	Harvade 25F	75.69
	LSD 0.05	ns
Genotype	Favorit	82.39
	Female line	77.45
	Pollinator line	70.02
	LSD 0.05	6.02

Among the genotypes, the highest field emergence was shown by hybrid Favorit (82.39%), which had a field emergence significantly higher (12.38%) than pollinator line and non-significantly higher (4.94%) in relation to female line. Also, the female line had statistically significant higher field emergence (7.43%) than the pollinator line.

DISCUSSION

Desiccation is a very important agro-technical measure, which has a positive influence on grain yield and seed quality in seed production. According to the research of Miklič et al. (2006), the highest germination occurs when moisture in harvest is below 32%, and in most cases between 22-23%. Desiccation accelerates moisture reduction in seed and plant parts, enabling earlier sunflower harvesting. After application of Reglone forte, moisture decreased at harvest level for 5-10 days (Dembinski et al., 1974; Kosovac and Sudimac, 1980), and with Harvade 25F application for 3-4 weeks (Ames and Walz, 1988).

In this research, when the seeds were harvested 20 days after the desiccant treatments seed moisture was distinctly reduced by 26.12% in the pollinator line, 16.12% in the hybrid, and 15.07% in the female line, enabling a considerably earlier harvest.

With the aim of estimating seed quality, after harvesting, laboratory germination of analyzed sunflower seed variants was tested. In the first count, seed vigor and germination were very low, which can be attributed to seed dormancy. Also, there was a large difference between seed vigor and

germination rate (23.94%). In further counts, seed vigor and germination increased, and the differences between them greatly declined.

Tested desiccants have shown different responses regarding seed vigor and germination of analyzed genotypes. Reglone forte showed statistically significant higher germination in relation to the control. These results are in accordance with those of Dembinski et al. (1974), Palmer and Sanderson (1976) and Miklič et al. (2004).

Harvade 25F is a growth regulator which shows good results in years when there have been many precipitations during maturation (Ames and Walz, 1988; Lebedev et al., 1997). Because this was not the case in this research, the results given with Harvade 25F were as expected. Germination of variants treated by Harvade 25F was significantly higher in relation to the control, but lower in relation to Reglone forte. Among the genotypes tested, almost the same values of seed vigor and germination were shown by the hybrid Favorit and female line, while for the pollinator line differed from both of them. In the field emergence test, Reglone forte had the highest statistically non-significant field emergence, while Harvade 25F and the control had almost the same behaviour.

On the basis of these results, a chemical desiccation had a favorable influence on seed maturity acceleration. Desiccant Reglone forte showed higher seed vigor and germination in laboratory germination and field emergence tests, hence its recommendation for use in seed production.

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Physiological traits for quantification of drought tolerance in sunflower

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ABSTRACT

Five Romanian sunflower hybrids were grown in the greenhouse under two watering regimes for each genotype: control variant – in which plants were maintained at 70% from TSWC (total soil water capacity)] and *stress treatment* in which sunflower seedlings were irrigated no more than 40% from TSWC. The results showed that water stress induced the decrease of leaf area, shoot size, chlorophyll content and yield. Some differences between the tested sunflower hybrids were recorded.

Key words: biomass – chlorophyll content – drought – leaf area – sunflower.

INTRODUCTION

Drought is probably the most important factor limiting crop yields worldwide, and in Romania, too. Because of its complexity, drought tolerance is probably the most difficult trait to improve through conventional plant breeding. The challenge is even greater for developing drought tolerant cultivars for Romanian environment where the occurrence, timing and severity of drought may fluctuate from year to year.

In Romania, NARDI at Fundulea has devoted considerable effort during the past ten years to improve drought tolerance in wheat, maize and sunflower. Extensive research has been conducted in the area of breeding, agronomy, and most recently, physiology.

The physiology work has focused on morpho-physiological traits induced by drought and associated with drought tolerance of plants, and the elaboration of screening methods for rapidly measuring of drought tolerance using plants in an early stage of vegetation.

Sunflower is a well adapted to drought crop, essentially because of its powerful water uptake due to its efficient root system (Belhassen, 1995).

The present paper reports the responses of five Romanian sunflower genotypes to water stress. The aim was to identify morpho-physiological traits that could be used as screening criteria in a breeding programme for drought tolerance, and which could be rapidly measured using plants in an early stage of vegetation.

MATERIALS AND METHODS

Seeds of five sunflower hybrids: Alex, Favorit, Justin, Romina and Splendor were germinated and then planted at a depth of 3–4 cm in PVC tubes (35 cm long and 11 cm diameter) and in Mitcherlich pots filled with a soil-sand mixture (1:1). The plants were grown in a greenhouse up to the four leaf stage for the experiment from PVC tubes, and up to harvest maturity for another experiment.

In both experiments each genotype was tested in five replicates and two watering regimes: control variant – [in which plants were maintained at 70% from TSWC (total soil water capacity)] and stress treatment (where sunflower seedlings were irrigated no more than 40% from TSWC).

The biomass of the above and below-ground parts was measured after drying them to the constant weight.

The chlorophyll concentration was assessed using a SPAD-502 chlorophyll meter (Minolta, Japan).

Leaf area was calculated with the formula: L x l x 0.66 where: L = leaf length; l = leaf width and 0.66 = correction coefficient for sunflower. The root volume was measured by water displacement from a filled beaker.

RESULTS AND DISCUSSION

Under water stress conditions the reduction in leaf area and height of plants was recorded. Leaf area was insignificantly reduced in sunflower seedlings grown for one week under drought conditions (from 0.4% for Romina up to 15% for Justin hybrid) and significantly reduced in all sunflower genotypes grown for two weeks under drought conditions (up to 50%). It is obvious that young plants are a little more sensitive than mature

ones when water stress acts for two weeks (Table 1). This response could be considered as a usual reaction of sunflower plants in order to reduce water use.

In all sunflower hybrids, the effect of drought treatment consisted of a significant decrease in height of plants, less in hybrid Alex and more in hybrid Justin (Table 2).

Table 1. The effect of water stress on leaf area of sunflower seedlings

Hybrids	Relative reduction of leaf area due to water stress (%)			
	Seedlings (stressed one week)	Seedlings (stressed two weeks)	Plants (stressed one week after flowering)	Plants (stressed two weeks after flowering)
Alex	8.5	55.2	23.8	48.0
Favorit	0.8	52.6	24.3	48.7
Justin	15	42.4	24.1	44.7
Romina	0.4	47.8	25.5	30.9
Splendor	4.3	50	24.2	36.3

Table 2. The effect of water stress on shoot size of sunflower seedlings

Hybrids	Variants	Height of plants	
		mm	%
Alex	Control	621	100
	Water stress (2 weeks)	457	73.6
Favorit	Control	641	100
	Water stress (2 weeks)	459	71.6
Justin	Control	600	100
	Water stress (2 weeks)	385	64.2
Romina	Control	659	100
	Water stress (2 weeks)	450	68.3
Splendor	Control	571	100
	Water stress (2 weeks)	413	72.3

The significant positive correlation between leaf area and plant height under water stress condition is obvious (r = 0.953***, Fig. 1).

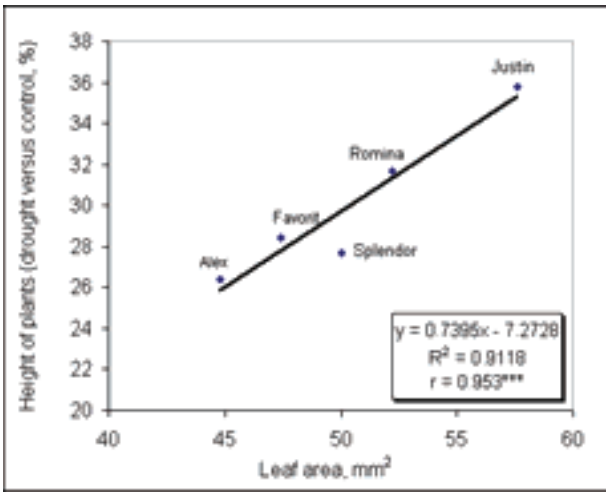

Fig. 1. Relationship between leaf area and height of plants.

The chlorophyll content is considered one of the most important indicators of vegetation stage and its degradation is normally considered a measure of drought resistance (Beard, 1973; Kim et al., 1989). The total chlorophyll content (expressed as SPAD units) was reduced under drought conditions, except for Favorit and Justin which presented the same SPAD units after first water stress period (Fig. 2).

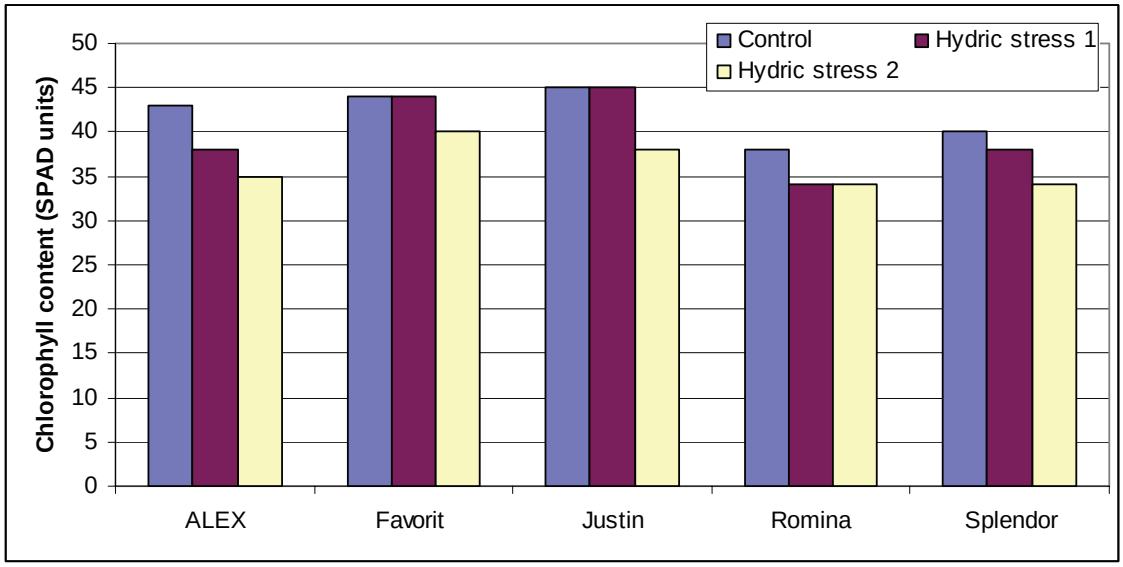

Fig. 2. Effect of water stress on chlorophyll content in sunflower leaves

Dry matter production of shoots, leaves and roots was significantly reduced under water stress conditions for all tested genotypes. Besides the genetic variability of tested sunflower hybrids, differences were recorded between the analyzed organs, too. It is obvious that leaves and shoots were more influenced by water stress than roots.

Thus, dry matter accumulation in roots of Favorit hybrid under drought was higher than under the control. Also, the values of the rest of the tested sunflower hybrids were up to 70%. Under water stress conditions, the root/shoot ratio increased. The increase in the Favorit hybrid was obvious (72%) and from 23 to 45.8% in Romina and Justin, respectively. These results show that the total root mass increases with drought stress (Table 3).

Table 3. The effect of water stress on biomass accumulation in seedling sunflower hybrids

Hybrids	Experimental variants	Biomass accumulation (g dry matter)						Roots/shoot	
		Leaves		Shoots		Roots			
		g	%	g	%	g	%	ratio	%
Alex	Control	2.87	100	4.37	100	2.35	100	0.32	100
	Water stress (2 weeks)	1.48	51.5	1.93	44.1	1.42	60.42	0.42	131.2
Favorit	Control	3.24	100	4.60	100	1.52	100	0.19	100
	Water stress (2 weeks)	1.86	57.4	2.87	62.2	1.58	103.9	0.33	173.6
Justin	Control	2.72	100	4.97	100	1.86	100	0.24	100
	Water stress (2 weeks)	1.70	62.5	2.91	58.5	1.60	86.02	0.35	145.8
Romina	Control	3.22	100	5.29	100	1.83	100	0.22	100
	Water stress (2 weeks)	2.22	68.9	2.5	47.2	1.29	70.49	0.27	123
Splendor	Control	3.12	100	4.88	100	1.87	100	0.23	100
	Water stress (2 weeks)	1.81	58.0	3.51	71.9	1.60	85.56	0.30	130.4

The shoot/root mass ratios consistently decrease under drought stress, which is a universal expression of adaptation (Blum, 1988). The increase in root/shoot ratio is mentioned in literature (Sharp and Davies, 1985; Sharp and Boyer, 1986). Previous reports underlined the genetic diversity of hybrid sunflower roots and the influence of soil environmental conditions on the rooting system (Terbea et al., 1995; Petcu et al., 1997; Agüera et al., 1997).

Our results show that during the first days of water stress the nutritive reserves of sunflower seedlings were conducted towards developing the roots, in order to facilitate deep soil moisture extraction. This happened in detriment of shoot development, and, in this case, a drift occurred in the main sink to survive. Concerning the root/shoot ratio, the response of mature plants to water stress is different from seedling response as the sink is different.

The root/shoot ratio of mature plants decreased under drought stress in Favorit and Splendor but increased in Alex, Justin and Romina hybrid. So, some differences between the tested genotypes in response of drought were noticed (Table 4).

Table 4. The effect of water stress on biomass accumulation in mature sunflower plants.

Hybrids	Experimental variants	Biomass accumulation (g dry matter)						Roots/shoot	
		Shoots		Leaves		Roots			
		g	%	g	%	g	%	ratio	%
Alex	Control	36.8	100	20.4	100	10.8	100	0.19	100
	Water stress (2 weeks)	21.6	58.7	18.6	91.18	9.4	87.1	0.23	123.8
Favorit	Control	36.8	100	38	100	21.2	100	0.28	100
	Water stress (2 weeks)	35.6	96.7	21.4	56.32	6.4	30.1	0.11	39.6
Justin	Control	34.4	100	29.2	100	9.4	100	0.15	100
	Water stress (2 weeks)	17.4	50.5	18	61.64	6.6	70.2	0.19	126.1
Romina	Control	28.8	100	18.8	100	7.6	100	0.16	100
	Water stress (2 weeks)	15.6	54.1	18.2	96.81	7.2	94.7	0.21	133.4
Splendor	Control	29.4	100	31.2	100	22.4	100	0.37	100
	Water stress (2 weeks)	21.4	72.7	23.4	75.00	10.4	46.4	0.23	62.8

It is well known that water stress has a profound effect on sunflower yield (Muriel and Downes, 1974; Talha and Osman, 1975). In our experience, the yield was affected by water stress with the low status treatment yielding 10-13% less than the control (Fig. 3). The highest productive hybrids under drought conditions were Favorit and Justin but Romina presented a high stability in yield level, both when plants were stressed for one week and for two weeks, too.

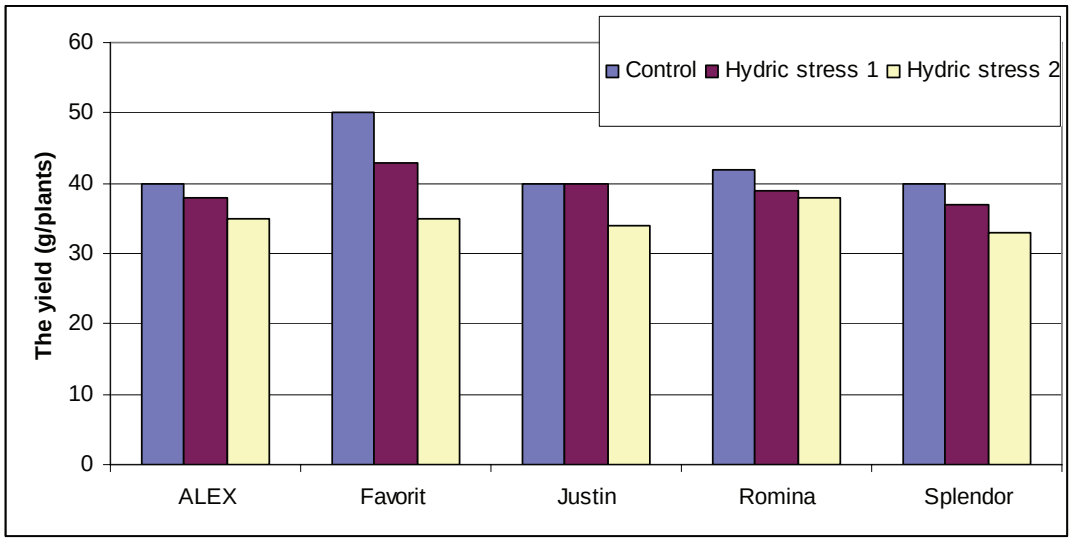

Fig. 3. The effect of water stress on yield of the tested sunflower hybrids

This suggests that although in Justin hybrid most of the nutritive reserve was conducted towards root development, its yield is not influenced.

CONCLUSIONS

The reduction in leaf area, shoot size and biomass accumulation of sunflower seedlings under water stress conditions determined the increase in root/shoot ratio. This suggests that for young plants the main sink was survival. In a late stage of vegetation, the root/shoot ratio decreased under drought stress in some hybrids but increased in other hybrids, this suggesting that for mature plants the main sink was the yield.

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El vuelco en el cultivo de girasol: características anatómicas y mecánicas del sistema radical

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ABSTRACT

The objectives of this study were to identify the anatomical, morphological and mechanical properties of the root system of sunflower plants related to their tolerance to root lodging, and to evaluate the effects of increases in crop population density on these properties. An experiment was carried out using crops of two genotypes of susceptibility to lodging (CF29: tolerant and Zenit: sensitive) sown to achieve densities of 5.6, 10 and 16 plants.m^{-2}. At the R6 (end of anthesis) developmental stage plants were artificially lodged and the following response variables assessed: total root biomass in the root plate (hemisphere of roots and soil formed when lodging occurs) and separated by layers (0–5 and > 5 cm depth), root number (classified in three categories [0–1 mm; 1.1–2 mm; >2 mm diameters]), axial tension required to provoke root failure, and the number and cell wall thickness of vascular bundles in root cross-sections. The CF29 genotype had 1.35 fold greater root biomass than Zenit, mainly located in the first layer of soil (0–5 depth). This higher biomass of CF29 reflected a higher root number than Zenit for all three categories of root diameter. Roots of CF29 exhibited higher axial tension failure thresholds than those of Zenit, and these thresholds increased more sharply with root diameter than for Zenit. In addition, CF29 roots had vascular bundles with thicker cell walls (30% greater) with respect to Zenit. In summary, the better anchorage of CF29 with respect to Zenit arises from several mutually reinforcing characteristics at both root-plate and individual root axis morphology and histology levels.

Key words: cell wall thickness – crop population density – root axial tensile strength – root biomass – root lodging – root number

RESUMEN

Este trabajo tuvo como objetivos identificar las propiedades morfo–anatómicas y mecánicas del sistema radical de plantas de girasol que le confieren tolerancia al vuelco, y evaluar los efectos del aumento en la densidad poblacional del cultivo sobre dichas propiedades. Se realizó un experimento utilizando dos genotipos de comportamiento contrastante al vuelco (CF29: tolerante y Zenit: susceptible) sembrados a densidades crecientes (5.6, 10 y 16 plantas.m^{-2}). En la etapa R6 (fin de antesis), las plantas fueron volcadas artificialmente y se midieron las siguientes variables: biomasa radical total y por estrato (0–5 y > 5 cm prof.) en el plato de raíces (hemiesfera de suelo y raíces formado cuando la planta vuelca), número de raíces (en tres categorías diamétricas [0–1 mm; 1.1–2 mm; >2 mm]), tensión axial requerida para la ruptura radical, número de los haces vasculares en la estela radical y espesor de las paredes celulares. Los resultados mostraron que CF29 tuvo 1.35 veces más biomasa radical en el plato que Zenit, concentrada en los primeros centímetros de suelo. La mayor biomasa radical de CF29 estuvo asociada con un mayor número de raíces con respecto a Zenit en las tres clases diamétricas exploradas. Además, las raíces de CF29 fueron más resistentes a la tensión axial que las de Zenit, diferencias que se incrementaron con diámetros radicales crecientes. La mayor resistencia de las raíces de CF29 estuvo relacionada a haces vasculares con paredes secundarias más gruesas (30% mayor respecto a Zenit). Resumiendo, el mejor sistema de anclaje de CF29 frente a Zenit tuvo su origen en varias características, expresada a los niveles de sistema radical total y de la morfo-histología de ejes radicales individuales, que se reforzaban mutuamente.

INTRODUCCIÓN

El fenómeno de vuelco de las plantas es un factor abiótico importante que limita la producción en el cultivo de girasol, y en éste, como en otros cultivos (*Triticum aestivum*, *Hordeum vulgare* y *Avena sativa*), provoca no solo reducciones significativas del rendimiento en grano, sino también de su calidad (Kelbert et al., 2004). Para Argentina, se ha estimado que un 10% del área se vuelca cada año, causando pérdidas estimadas en 40 millones de U$D por año (Bragachini et al., 2001). La probabilidad de ocurrencia de vuelco depende tanto de cuestiones ambientales (p.ej., velocidad del viento, resistencia al

cizallamiento del suelo mojado), como intrínsecos de la planta (p.ej., rigidez del tallo, propiedades del sistema radical en las inmediaciones de la base del tallo) (Pinthus, 1973; Berry et al., 2000; Cleugh et al., 1998). El vuelco se asocia principalmente con la lluvia (Baker et al., 1998) que debilita el sistema de anclaje de la planta, combinada con la fuerza que ejerce el viento y que actúa sobre la parte aérea de de la planta, dando como resultado un momento de palanca en la base del tallo que excede el momento de quiebre de las raíces (Berry et al., 2004).

Recientemente se ha demostrado que el cultivo de girasol tiene el potencial de incrementar su rendimiento ante aumentos considerables en la densidad poblacional del cultivo por arriba de las densidades comerciales usuales en Argentina (López Pereira et al., 2004). Sin embargo la implementación de esta práctica se ve limitada por la mayor probabilidad de ocurrencia de vuelco. Por otro lado, es sabido que existe una amplia variabilidad en la tolerancia a este fenómeno entre distintos genotipos, habiéndose evaluado al momento dos genotipos de comportamiento contrastante: CF29 (tolerante) y Zenit (susceptible) (Sposaro et al., 2008). Analizar los efectos que tiene el aumento de la densidad poblacional sobre las características del sistema radical de las plantas en genotipos de tolerancia contrastante al vuelco, resulta un paso importante para aumentar el conocimiento de las bases de este proceso. Los objetivos de este trabajo fueron: (i) identificar las propiedades morfo–anatómicas y mecánicas del sistema radical asociadas con la tolerancia al vuelco en dos genotipos de comportamiento contrastante y (ii) evaluar los efectos de la densidad poblacional del cultivo sobre dichas propiedades.

MATERIALES Y MÉTODOS

*Genotipos y diseño experimental:*Se utilizaron dos genotipos de comportamiento contrastante frente al vuelco: uno tolerante (en adelante CF29) y uno susceptible (en adelante Zenit). Los genotipos fueron sembrados a densidades poblacionales crecientes de 5.6, 10 y 16 plantas.m^{-2} en el campo experimental de la Facultad de Agronomía–UBA sobre un suelo argiudol típico. El experimento consistió en un arreglo factorial con "genotipo" (2 niveles: CF29 y Zenit) y "densidad poblacional del cultivo" (3 niveles: 5.6, 10 y 16 pl.m^{-2}) como factores principales en un diseño en bloques aleatorizados (DBCA) con tres repeticiones. Las plantas crecieron durante todo su ciclo bien provistas de agua a través de un sistema de riego por goteo. Se aplicó fertilización en dos momentos del ciclo con 60 kg N.ha^{-1}. Se realizaron aplicaciones con insecticidas y fungicidas para mantener el cultivo libre de enfermedades y plagas.

Variables de respuesta y análisis estadístico: Las plantas fueron volcadas de forma artificial en fin de antesis (R6, Schneiter y Miller, 1981) utilizando la metodología descripta en Sposaro et al. (2008). Brevemente, la técnica involucra aplicar fuerzas crecientes a los tallos de plantas que crecen en subparcelas cuyo suelo ha sido previamente llevado a capacidad de campo hasta producir el vuelco de las mismas. La hemiesfera (o plato) de raíces (masa de suelo y raíces formada cuando se vuelca una planta) así obtenida fue dividida en dos estratos de acuerdo a su profundidad (0–5 cm y >5 cm), luego de lavadas las raíces, éstas se clasificaron en tres clases diamétricas (0–1 mm; 1.1–2 mm; >2 mm). Se obtuvo la biomasa de las raíces de cada estrato a través de su secado en estufa hasta peso constante (72 hs a 80°C) y se contó el número de raíces en cada una de las categorías previamente definidas. Para determinar la tensión axial requerida para la ruptura de los ejes radicales individuales (vivos) se adaptó la metodología descripta por Striker et al. (2006). Las raíces se sometieron a tensiones axiales crecientes causadas por el desplazamiento del pistón de un minicilindro neumático conectado a un circuito de aire presurizado con un regulador de flujo. Dicho sistema estaba conectado a un transductor de presión (ADZ Nagano S–010bar) y a un datalogger para registrar la tensión axial que provocó la ruptura de las raíces. La fuerza de ruptura se midió en raíces de diferentes diámetros en los rangos de las tres categorías definidas con el propósito de estudiar la posible existencia de un efecto del tamaño de las raíces sobre esta variable. Se realizaron cortes transversales de muestras de ejes radicales, tiñendo los mismos con una tinción doble (Safranina y Fast Green) para distinguir las paredes secundarias. El número de haces vasculares y el grosor de sus paredes se determinó mediante microscopía óptica y digitalización de imágenes, utilizando el software ImageTool 3.0 para Windows (University of San Antonio, Texas).

Los datos de biomasa por estrato y número de raíces por categoría se analizaron a través de ANOVAs de dos vías, con "genotipo" y "densidad poblacional de plantas" como factores principales. La relación entre la tensión axial de ruptura de las raíces y el diámetro radical se evaluó por medio de análisis de correlación de Pearson (Steel y Torrie, 1988). La significancia de las diferencias entre genotipos para dicha relación se evaluó con un test de pendientes para las ecuaciones ajustadas. Los datos correspondientes al número de haces vasculares y al grosor de sus paredes se analizaron a través de test de Student. La homogeneidad de varianzas y normalidad de los datos se verificó para todo el conjunto de

datos. Los análisis se realizaron con el software estadístico InfoStat versión 2007 (Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina). Los resultados son presentados cómo valores promedios ± el error estándar.

RESULTADOS Y DISCUSIÓN

Estudios previos han demostrado que se requiere una fuerza significativamente mayor para volcar en forma artificial las plantas del genotipo tolerante CF29 que para volcar las del genotipo susceptible Zenit (67.3 vs. 27.4 N m, respectivamente, ver Sposaro et al., 2008). En el presente estudio se halló que el diámetro del plato de raíces fue significativamente mayor en CF29 que en Zenit (23.7 y 22.5 cm respectivamente). El mayor diámetro del plato de raíces de CF29 se relacionó con una mayor biomasa radical total que en Zenit, para las tres densidades evaluadas (genotipo= $p<0.0001$, genotipo × densidad poblacional: $p=0.53$, Fig. 1). Esto coincide con la idea de que en plantas de girasol una mayor biomasa radical se asocia a una mayor eficiencia en el anclaje de la planta (Ennos, 1993). Para CF29, la biomasa radical total en el plato fue similar para las tres densidades poblacionales ($p=0.20$) mientras que para Zenit se detectó un efecto negativo de la densidad poblacional sobre dicha variable ($p<0.01$, Fig. 1). Por lo tanto, Zenit no solo presentó una biomasa de raíces en el plato equivalente al 42% de la registrada para CF29, sino que el aumento de la densidad poblacional debilitó aún más su sistema de anclaje.

En ambos genotipos la mayor biomasa de raíces se concentró en el primer estrato (0–5 cm de profundidad, Fig. 1) sin detectarse diferencias en la proporción del total de raíces presentes en ese estrato entre las densidades poblacionales: 78% CF29 y 70% Zenit ($p=0.88$, genotipo × densidad poblacional: $p=0.99$). Estudios previos en girasol (Ennos, 1989) han demostrado que la biomasa de raíces en los primeros centímetros de suelo, es la más importante para determinar un óptimo anclaje de la planta. Por otro lado, en el estrato más profundo (> 5 cm) el aumento de la densidad poblacional de plantas determinó una disminución en la biomasa radical en ambos genotipos (densidad poblacional = $p<0.0001$, genotipo × densidad poblacional: $p=0.18$, Fig. 1). De esta manera, ante el aumento de la densidad poblacional ambos genotipos parecieran haber priorizado el mantenimiento de la biomasa radical en el estrato superior del suelo en detrimento de la producción de raíces en el estrato inferior, lo que redundaría en un mejor anclaje de las plantas (Ennos, 1989).

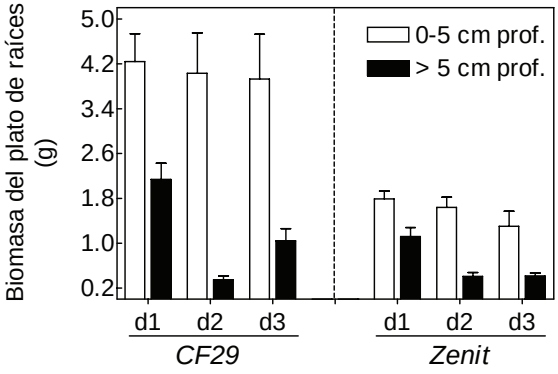

Fig. 1. Biomasa de raíces en el plato (g.planta^{-1}) para los dos genotipos en los dos estratos del plato para tres densidades poblacionales del cultivo (d1: 5.6; d2: 10; y d3: 16 pl.m^{-2}).

Fig. 1. Root plate biomass (g.plant^{-1}) for both genotypes in the two layers of the root plate for three crop population densities (d1: 5.6; d2: 10; y d3: 16 pl.m^{-2}).

El número total de raíces en el plato fue mayor en el genotipo CF29 que en Zenit ($p<0.05$), siendo este atributo uno de los que podría explicar la mayor tolerancia al vuelco que tiene CF29 respecto a Zenit. Al agrupar las raíces por su diámetro, se observó en ambos genotipos que las raíces más finas (0–1mm) fueron significativamente más abundantes que el resto de las categorías diamétricas ($p<0.0001$, Fig. 2, notar cambio de escalas entre el primer panel y los restantes). Otros trabajos han demostrado la importancia de las raíces finas en el conferimiento de la resistencia al vuelco de las plantas (Ennos, 1989; Reubens et al., 2007). El aumento de la densidad poblacional del cultivo produjo un incremento significativo en el número de raíces finas en el plato (0–1mm) sólo para Zenit ($p=0.009$, Fig. 2). Esta podría ser una respuesta del genotipo frente al incremento de la densidad poblacional, dado que una

mayor cantidad de raíces finas proveería una mayor fijación de la planta al suelo (Wu et al., 1988; Wu, 1995) en situaciones adversas donde el riesgo de vuelco se incrementa (Sposaro et al., 2008).

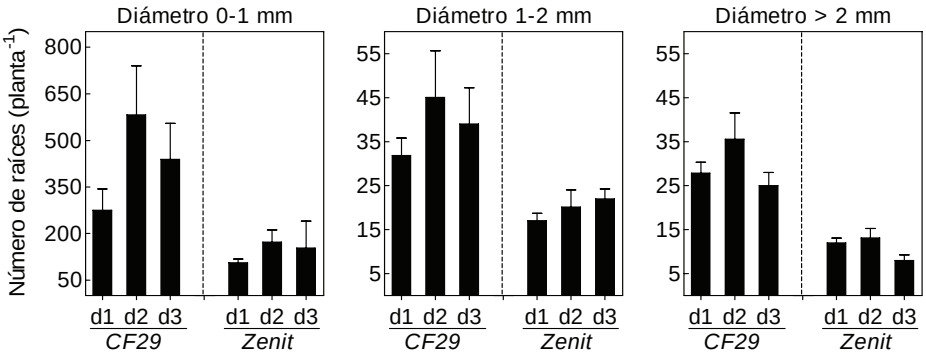

Fig. 2. Número de raíces por plato agrupadas por categorías diamétricas, para los genotipos CF29 y Zenit en tres densidades poblacionales de cultivo (d1: 5.6; d2: 10 y d3: 16 pl.m^{-2}). Notar el cambio de escala entre el primer panel y los restantes.

Fig. 2. Root number per root plate grouped by size category, for genotypes CF29 and Zenit grown at three crop population densities (d1: 5.6; d2: 10; y d3: 16 pl.m^{-2}). Note the change on scale between the left and remaining panels.

La fuerza de tensión axial requerida para provocar la ruptura de las raíces (como sucede durante el vuelco de la planta), fue mayor en CF29 que en Zenit (p=0.003, Fig. 3). Esta propiedad mecánica de las raíces no fue afectada por el aumento de la densidad poblacional de plantas (genotipo × densidad poblacional: p=0.16), por lo tanto, las mediciones hechas para las distintas densidades poblacionales fueron agrupadas, y el análisis se enfocó en las diferencias entre genotipos. En ambos genotipos la fuerza de tensión axial para provocar la ruptura de las raíces se correlacionó positivamente con el diámetro de las mismas (Fig. 3). CF29 presentó una pendiente mayor que Zenit para esta relación (test de pendientes: p< 0.0001, Fig. 3), indicando una mayor resistencia mecánica de sus raíces. El efecto del tamaño de las raíces ("*size effect*") sobre sus propiedades mecánicas ha sido recientemente estudiado en especies leñosas por Genet et al. (2005) y Bischetti et al. (2005). Estos autores demostraron la existencia de una correlación positiva entre el diámetro y la fuerza de ruptura de las raíces. Nuestros datos son consistentes con esos resultados y nuestro trabajo es el primero, para una especie herbácea anual de interés económico, en informar acerca de los efectos del tamaño de las raíces sobre sus propiedades mecánicas.

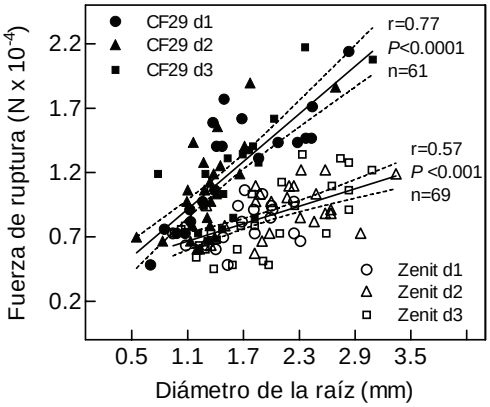

Fig. 3. Relación entre fuerza de ruptura (N) de las raíces y el diámetro para los genotipos CF29 y Zenit sembrados en tres densidades poblacionales de cultivo (d1: 5.6; d2: 10 y d3: 16 pl.m^{-2}). Las ecuaciones para cada genotipo son y= 0.61x + 0.24 para CF29, y= 0.23x + 0.41 para Zenit. Las líneas punteadas indican el intervalo de confianza del 95% para las ecuaciones ajustadas.

Fig. 3. Root failure threshold (N)/root diameter relationship for genotypes CF29 and Zenit, grown at three crop population densities (d1: 5.6; d2: 10; y d3: 16 pl.m^{-2}). Equations for each genotype are y= 0.61x + 0.24 for CF29, y= 0.23x + 0.41 for Zenit. Dashed lines indicate the 95% confidence intervals for the fitted equations.

El análisis anatómico mostró que los genotipos CF29 y Zenit no difirieron en la proporción de estela en la sección transversal de raíz (datos no mostrados), ni en el número de haces vasculares presentes en la sección (Fig. 4). Sin embargo, en el genotipo CF29, cuyas raíces tuvieron una mayor resistencia a la tensión axial, el grosor de la pared de los haces vasculares fue un 30% mayor al encontrado en el genotipo Zenit (Fig. 4c). Trabajos previos realizados sobre cultivos de trigo, cebada y arroz, han demostrado que tanto el número de haces vasculares como el grosor de sus paredes son parámetros altamente correlacionados con la tolerancia a las fuerzas de tensión axial, como las generadas durante el fenómeno de vuelco (Pinthus, 1973; Chatuverdi et al., 1995 citado por Oladokun y Ennos, 2006). Se sabe que el contenido de celulosa y lignina están positivamente correlacionados con la fuerza de tensión axial de las raíces (Hathaway y Penny, 1975; Kokubo et al., 1989; Genet et al., 2005). Por lo tanto, raíces del mismo diámetro y con similar número de haces vasculares, pero con mayor proporción de lignina y celulosa en sus paredes secundarias, brindan una mayor resistencia mecánica a la raíz. En este sentido, la mayor proporción de pared secundaria constitutiva de CF29 sería responsable, al menos en parte, de la mayor resistencia de sus raíces ante fuerzas de tensión axial.

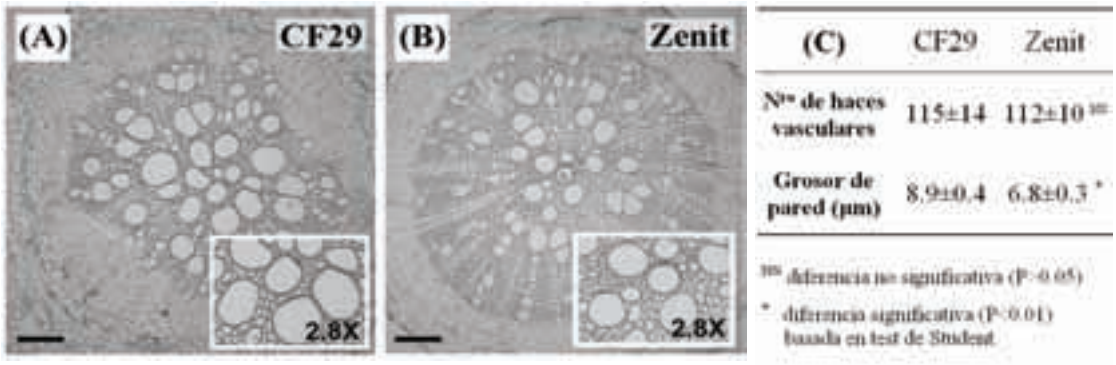

Figura 4. Cortes transversales de raíces de diámetro similar de los genotipos CF29 (A) y Zenit (B). La barra de escala es equivalente a 150 µm. En el cuadro (C) se muestra el número de haces vasculares y el grosor de la pared de dichos haces para cada genotipo. En la parte inferior derecha de (A) y (B) se muestran en detalle los haces vasculares para cada genotipo.

Figure 4. Transverse sections of roots of similar diameter of genotypes CF29 (A) and Zenit (B). Scale bar represents 150 µm. In the table (C) the number and cell wall thickness of vascular bundles are shown for each genotype. Inset photos (A) and (B) shown magnified (2.8X) sections of the stele centered on vascular bundles.

En síntesis, se detectaron diferencias importantes tanto a nivel de sistema radical de la planta como a nivel de raíz individual entre los genotipos que explicarían su tolerancia diferencial al vuelco. A nivel de planta el genotipo tolerante CF29 tuvo una mayor biomasa total en el plato de raíces que el susceptible Zenit. Dicha biomasa estuvo especialmente concentrada en los primeros centímetros del suelo, estrato donde se define gran parte del anclaje de las plantas. A su vez, la mayor biomasa radical registrada para CF29 estuvo asociada con un mayor número de raíces que en Zenit para todos los rangos diamétricos explorados. A nivel de raíz individual, CF29 tuvo raíces más resistentes a las fuerzas de tensión axial que las del genotipo Zenit, detectándose un mayor efecto positivo del diámetro de las raíces sobre dicha variable a favor de CF29. A su vez, la mayor resistencia de las raíces de CF29 estuvo relacionada con la presencia de haces vasculares con paredes secundarias considerablemente más gruesas que en Zenit. De esta manera, estas características diferenciales a nivel anatómico, morfológico y de biomasa radical entre CF29 y Zenit definirían en conjunto un sistema de anclaje más eficiente en el primer genotipo que reducen las probabilidades de ocurrencia de vuelco de sus plantas.

AGRADECIMIENTOS

Se agradece a Gustavo Striker por su constructivo aporte en el análisis de los resultados y edición del manuscrito. Este trabajo se llevó a cabo con fondos del subsidio ANPCyT-ASAGIR PICTO 13159.

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Influence of drought stress on growth, protein expression and osmolyte accumulation in sunflower *Helianthus annuus* L. c.v. Peredovik

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ABSTRACT

Drought stress causes considerable yield losses in agriculture and receives high attention in current sunflower breeding programs. For this study, an *in vitro* system based on MS media (Murashige and Skoog, 1962) supplemented with polyethylene glycol 6000 (Korell, 1997) was established to examine sunflower (*Helianthus annuus* L.) seedlings under artificial drought stress conditions. The objective is to identify parameters applicable as markers for drought stress, which would allow breeders to screen their material efficiently for drought tolerance. For this purpose, different morphological and physiological parameters were analysed by comparing control plants with plants grown under drought stress (-0,6 MPa). The evaluation of growth reveals a significant growth deficit of drought stressed plants compared to control plants concerning hypocotyl length, development of cotyledons and primary leaves, whereas for root fresh weight in relation to shoot fresh weight no significant difference could be detected. Qualitative and quantitative changes in osmolyte accumulation were examined by HPLC and gas chromatography. Osmolyte analyses revealed an accumulation of glucose (25-30fold), inositol (20-30fold), proline (10-20fold), fructose (3-6fold) and sucrose (4-5fold) in extracts from leaves of drought-stressed plants. Changes in protein expression of drought-stressed versus control plants were detected in colloidal Coomassie-stained 2D-PAGE gels. In future studies, differentially expressed proteins will be identified by peptide mass fingerprinting and used to develop molecular markers for breeding programs.

Key words: drought stress – PEG – osmolyte – 2D-PAGE.

INTRODUCTION

Drought is a potential major constraint to crop production all over the world, and causes considerable yield losses in agriculture. Global warming, deforestation, and urbanisation will all increase the severity and frequency of drought in the future, leading to a possible decrease in global food production. Therefore, there is a great need for the development of stable food crops that produce high and stable yields also when drought occurs. Water stress in its broadest sense encompasses both drought and salt stress. Water stress results in stomatal closure and reduced transpiration rates, a decrease in water potential of plant tissue, decrease in photosynthesis and growth inhibition. The accumulation of abscisic acid (ABA) serves as a signal, initiating acclimation reactions such as accumulation of compatible compounds like proline, mannitol, and sorbitol, or the formation of scavenging compounds like ascorbate, gluthathione, α-tocopherol etc. The acclimation process employs processes of differential gene expression leading to new proteins and mRNAs (Yordanov et al., 2003). Obviously, water stress acclimation is a multi-gene acclimation process, in which many different physiological processes and many drought stress-inducible genes are involved. Functionally, these gene products can be distinguished into: osmolyte synthases (Chen and Murata, 2002), protection factors for macromolecules (chaperons, LEA/dehydrin-type genes), proteases, membrane proteins (aquaporins, transporters), detoxification enzymes (GST, SOD), and genes of regulatory proteins like transcription factors, protein kinases, protein phosphatases (Wang et al. 2003; Zhu, 2002). Although the alterations in all of these processes related with drought stress have been widely investigated in many model species and a few crop plant species, reports on sunflower are limited. Physiological and molecular responses have been described by Kane and Rieseberg (2007), Kavas et al. (2006), Bailly et al. (2004), Liu and Baird (2003; 2004), Cellier et al. (1998; 2000) and Poormohammad et al. (2007). The aim of our *in vitro* studies of drought tolerance of sunflower seedlings was to analyse physiological key processes after applying drought stress conditions by supplementation of MS media with polyethylene glycol (PEG) 6000. This evaluation should result in an appropriate test system for breeders to select for drought tolerant breeding material while saving time, space and costs.

MATERIALS AND METHODS

Sunflower c.v. Peredovik seedlings were grown in a liquid MS medium (Murashige and Skoog, 1962) using 0.75 l Weck glasses. The plant material was incubated in climate chamber at 21°C, 16/8 hours light/dark cycle at 150 µM photons m^{-2} s^{-1}. After three days of cultivation half of the seedlings were transferred to drought stress medium, achieved by supplementation of MS medium with PEG 6000 to an osmotic potential of -0.6 MPa (MS6), while the control plants were transferred onto fresh MS0 medium without PEG addition. Seven days later plant growth was characterized by measurement of the hypocotyl length, characterizing the development of cotelydons and primary leaves (using a relative scale from 0 to 6 representing area of leaves up to >4 cm^2) and determination of fresh weight of root and shoots. Primary leaves were frozen in liquid nitrogen and stored at -20°C till extraction of proteins and osmolytes.

Total homogenates of leaves were obtained by grinding plant material in Eppendorf tubes on an ice bath with HEPES buffer (10 mM, pH 7.6) containing protease inhibitors (10 mM PMSF, protease inhibitor cocktail, Sigma P9599 according product information). The soluble protein fraction was obtained after centrifugation of the crude extract at 38,000 g and 4°C. After determination of the protein concentration (Bradford, 1976), these protein extracts (400 µg each) were used for 2D-PAGE according to Fulda et al. (2000, 2006) followed by differential analysis of colloidal Coomassie-stained 2D gels from drought-stressed plants versus control plants using Delta2D software (Decodon).

Qualitative and quantitative determination of osmolytes was done by HPLC and gas chromatography (GC). In the case of HPLC analyses, soluble protein extracts containing 400 µg of protein were taken as raw material. The high molecular contaminants were precipitated with 80% ethanol (containing an internal standard: 100 µg sorbitol) overnight at 4°C followed by centrifugation (28,000 g). Supernatants were dried in a vacuum centrifuge. Dry residues were washed once with 80% ethanol and dissolved in A. bidest. HPLC analyses were performed according to Schoor et al. (1995) using the combination of reversed-phase and ion-moderated partition chromatography. To verify the results of HPLC analyses, additional samples were analyzed by gas chromatography using a Focus GC (Thermo Scientific) equipped with a TR-5MS column (30 m x 0.25 mm x 0.25 µm) and an AS 3000 autosampler. For GC-analyses, a separate ethanolic extraction of ground leaves was performed with 80% ethanol at 68°C for two hours at first, followed by a second extraction at 68°C overnight. The combined extracts were purified by centrifugation at 38,000g, 4°C and dried in a vacuum centrifuge. Trimethylsilyl-derivates of sugars were obtained by incubation with 65 µl pyridine/methoxylamine (20 mg/ml, 90 min at 30°C) and 35 µl N, O-Bis(trimethylsilyl)-trifluoroacetamide (Sigma, 60 min at 60°C). A set of the following standards was chosen for qualitative analysis of the osmolytes: trehalose, maltose, glucose, sucrose, inositol, fructose, glycerol, mannitol, sorbitol, glycine betaine, proline. As an internal standard for quantification sorbitol was used (see above).

RESULTS AND DISCUSSION

In five independent experiments we observed a clear growth inhibition of plants cultivated in drought stress MS6 medium in comparison to MS0 plants (Fig. 1). In each experiment, about 200 plants were evaluated for growth. The rating reveals a significant growth deficit of drought-stressed plants compared to control plants concerning hypocotyl length (Fig. 2a) as well as the development of cotyledons (Fig. 2b) and primary leaves (Fig. 2c).

Fig. 1. Growth of 10-days-old *Helianthus annuus* L. c.v. Peredovik plants cultivated in control medium MS0 and drought stress medium MS6.

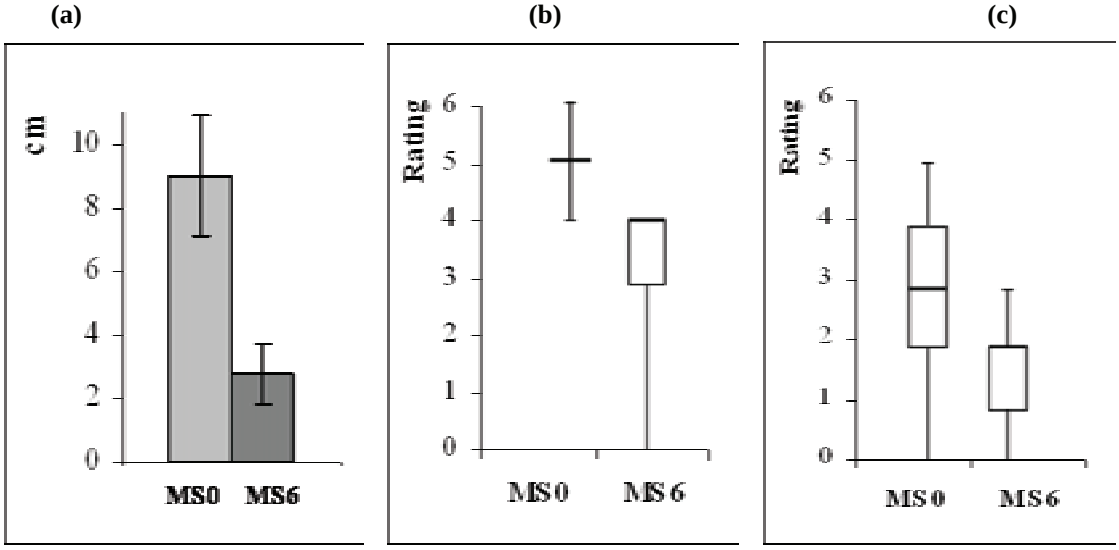

Fig. 2. Development of **(a)** hypocotyl length (cm), **(b**) cotyledons and **(c)** primary leaves of *Helianthus annuus* L. c.v. Peredovik grown in MS media with (MS6) and without (MS0) drought stress. Means and standard deviation (a) or medians (b, c) are given (n for MS0=87, n for MS6=98).

These observations agree with data published by Yordanov et al. (2003). Obviously, drought stress affects growth at the whole plant level leading to a decrease in photosynthesis and associated carbon and nitrogen metabolism. The growth inhibition could be attributed to shrinkage of cells and to the fact that the turgor pressure against cell walls relaxes. Because turgor reduction is the earliest significant biophysical effect of water stress, turgor-dependent activities such as leaf expansion and root elongation are the most sensitive to water deficits. Cell expansion is a turgor-driven process and is extremely sensitive to water deficit so that a decrease in turgor causes a decrease in the growth rate (Taiz and Zeiger, 2007).

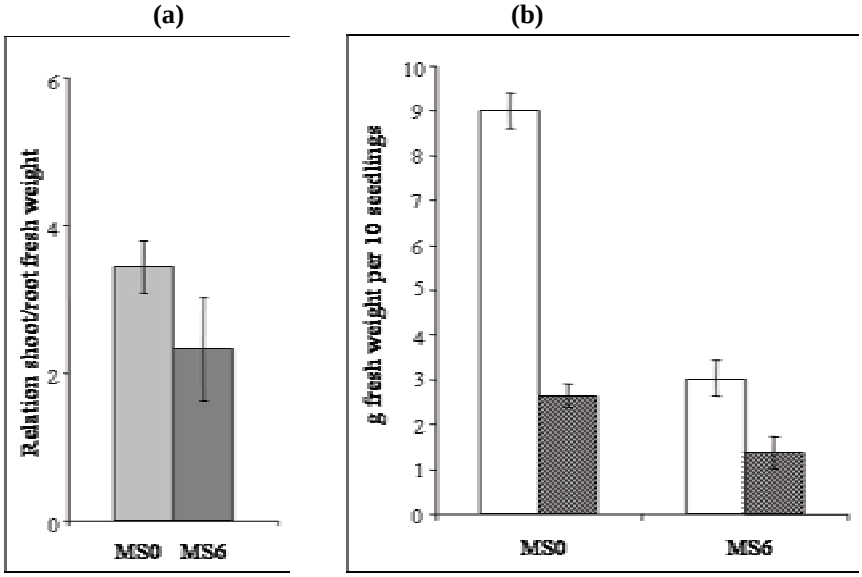

Fig. 3. Comparison of shoot and root development of 10-days-old seedlings of *Helianthus annuus* L c.v. Peredovik grown in MS media with (MS6) and without (MS0) drought stress. Means and standard deviations are given (n for MS0=9, n for MS6=10). **(a)** Fresh weight ratios shoot/root, **(b)** Fresh weight per 10 shoots (white columns) and fresh weight per 10 root systems (grey columns).

For root fresh weight in relation to shoot fresh weight, no significant difference could be detected (Fig. 3b). Karrenberg et al. (2006) investigated responses to salinity in the homoploid species *Helianthus paradoxus* and its progenitors *H. annuus* and *H. petiolaris*. They reported that growth reduction in the

progenitors *H. annuus* and *H. petiolaris* affected roots more than shoots as indicated by a decrease in root mass fraction. In the homoploid hybrid species *H. paradoxus* root biomass allocation did not change in response to salt stress so the relationship between root to shoot growth seems to differ from species to species. On the other hand, development of an optimal root/shoot ratio in relation to water availability is very important for the crop yield. Under natural conditions, plants are able to improve uptake of water by developing an extensive root system, which enables plants to grow into deep soil region with sufficient or improved water supply. Therefore, changes in relative root and shoot growth, leading to an increased root/shoot ratio were often observed with drought stressed plants (Verslues et al., 2006; Sharp et al., 2004). Additional tissue water storage capacity and thickness of the cuticula and water permeability are also of potential importance. Of these, changes in root growth to maximize water uptake are of the greatest importance for crop plants. In our PEG 6000-based hydroponics we observed, for shoot as well as root development, reduced growth in stressed plants (fresh weight, Fig. 3a) that leads to an unaffected root/shoot ratio. This may be due to the fact that PEG 6000-mediated drought stress conditions represent a severe stress.

Accumulation of osmolytes represents one of the central acclimation reactions in drought-stressed plants. Osmolyte analyses, which were done by HPLC and GC, obviously indicate an average accumulation of substances such as glucose (25-30fold), inositol (20-30fold), proline (10-20fold), fructose (3-6fold) and sucrose (4-5fold) in drought-stressed sunflower plants (Fig. 4.).

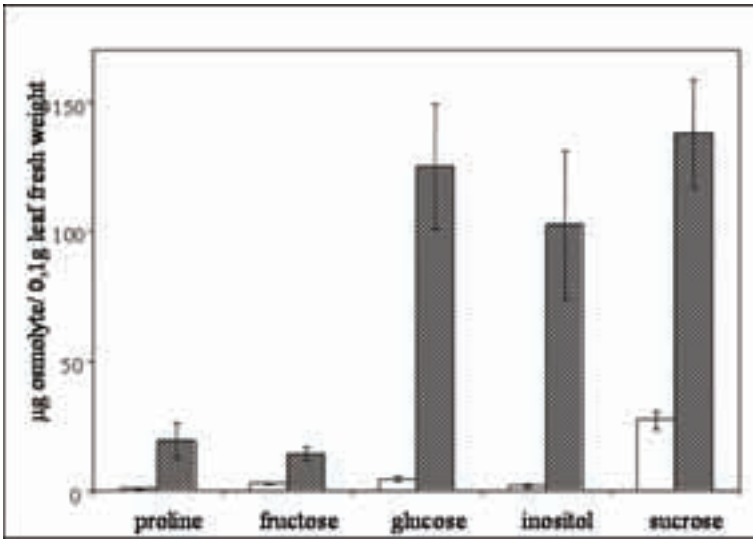

Fig. 4. Osmolyte accumulation in leaf extracts of *Helianthus annuus* L c. v. Peredovik determined by GC. Plants were grown on MS0 medium (white columns) or on MS6 medium (grey columns), respectively. Means of four analyses and their standard deviations are shown.

As expected, decreased water availability requires the accumulation of solutes by cells to decrease cell water potential, which enables plants to absorb water. This osmotic adjustment is a net increase in solute concentration per cell that is independent of the volume changes that result from loss of water. This can be accounted for by an increase in the concentration of a variety of common solutes, including sugars, amino acids, organic acids, polyols and inorganic ions (Taiz and Zeiger, 2007). In the case of our PEG-mediated drought stress system, the hexose glucose, the polyol inositol and sucrose seem to represent the main contributors to osmotic adjustment in primary leaves of *H. annuus* c.v. Peredovik followed by proline and fructose. Sharp et al. (2004), who investigated osmotic adjustment in roots of maize, found that in the maize primary root tip, hexoses are the dominant osmolytes in the basal region of the growth zone, while, in the apical zone proline concentration increases dramatically in water-stressed roots. One of the most frequently found solutes in water-stressed plants is the amino acid proline. Additionally, proline may act as a regulatory or signalling molecule to activate multiple responses that are part of the acclimation process (Maggio et al., 2002). Also, proline is a reliable indicator of the environmental stress imposed on plants (Claussen, 2005). Chechin et al. (2006) found in greenhouse-grown *H. annuus* c.v. Catissol-01 plants a 7-fold increase in proline content in young stressed leaves in comparison to non-stressed plants, but in the case of mature stressed leaves the proline content was increased four fold. In our study, proline was not the dominating compatible compound. These differences may be related to the

use of different sunflower varieties or culture conditions. Myo-inositol and its derivates are commonly studied with respect to cell signalling and membrane biogenesis, but they also participate in response to salinity in plants. Non-methylated inositols are found in all plants but are especially common in legumes. Pinitol and ononitol (methylated derivates) have been reported as a salt-induced response in *M. crystallinum* (Thomas and Bohnert, 1993). The already high concentration of cyclitols in unstressed soybean (*Glycine max*) is further increased in drought-stressed plants, underlying the important role of unmethylated and methylated inositols as osmoprotectants (Schneider et al., 2007; Gagneul et al., 2007).

Adaptation to drought stress requires alterations in the cell machinery that result directly from modifying gene expression. Functional gene expression profiles can be best achieved by proteome analysis. Furthermore, proteins undergo significant levels of post-translational modification of their primary sequences and are readily subjected to targeted proteolysis. Thus, a quantitative analysis of gene expression at the protein level is essential for dissecting responses to drought stress. We used the most common tool for revealing the expression of intact proteins, the two-dimensional gel electrophoresis (2D-PAGE). After staining proteins with colloidal Coomassie stain nearly 250 protein spots could be visualized in sunflower (Fig. 5). Protein pattern of control and drought-stressed sunflower plants were compared. So far, two regions marked on the gels were identified, where remarkable changes in protein expression became obvious. By using same sample replicates (4-5 times), several protein spots could be found, which showed an accumulation in drought-stressed plant material. However, a lot more spots seem to be present in smaller amounts in the drought-stressed plants compared to the control plants. In future experiments, the proteins of these stress-induced protein spots as well as decreased spots will be identified by excising gel plugs from the gel for MALDI-TOF analysis.

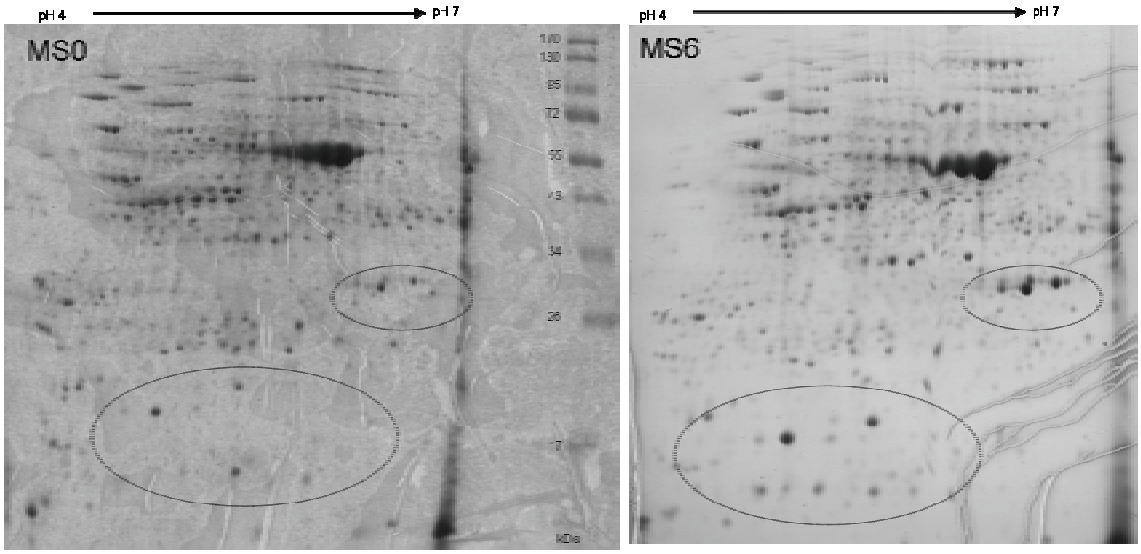

Fig. 5. 2D-PAGE gels of control and drought-stressed protein extracts from *Helianthus annuus* L. c.v. Peredovik. Soluble proteins were separated on 18 cm IPG pH 4-7 strip according to their isoelectric point in the first dimension and then on a SDS-PAG according to their molecular weight in the second dimension. The 2D-PAGE gels were stained with colloidal Coomassie blue. Protein samples were diluted to a load of 400 µg in rehydration buffer.

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Development and validation of a model of lodging for sunflower

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ABSTRACT

Root and stem lodging cause significant yield losses in sunflower (*Helianthus annuus* L.) production in Argentina. Lodging is defined as the permanent displacement of the stem from its vertical position without any possibility of recovery. Few studies have investigated the mechanistic processes of root or stem lodging and not one has attempted to interrelate the complex interactions between climate and soil variables, husbandry practices and plant characteristics for sunflower lodging. A lodging model was developed for wheat and barley and can be used as a basis for a sunflower lodging model. The objectives of this work are to develop a root/stem lodging model for sunflower and to validate it. The root lodging model calculates the root failure wind speed using as its main variable the root plate diameter. The thickness of the epidermis+cortex measured in the lower third of the stem was the main variable used as input by the stem lodging model for the calculation of the stem failure wind speed. This model was tested against individual field experiments, in which natural root or stem lodging had occurred at different crop development stages under different husbandry practices, and it could recreate the differences observed in the field between hybrids and crop population densities in relation to lodging susceptibility. A parametric analysis showed the root plate diameter and epidermis+cortex as the main variables of the model and indicated that sunflower could be more susceptible to root than stem lodging.

Key words: epidermis+cortex – failure wind speed – model testing – root lodging – root plate diameter – stem lodging.

INTRODUCTION

Root and stem lodging cause significant yield losses in sunflower (*Helianthus annuus* L.) production in Argentina. About 10% of sunflower crop lodges annually, representing an estimated loss of US$40 million (Bragachini et al., 2001) due to the impossibility of harvesting the lodged plants. Lodging is defined as the permanent displacement of the stem from its vertical position without any possibility of recovery.

Root lodging is usually associated with rain (Baker et al., 1998) that weakens plant soil-root system (Pinthus, 1973) combined with a wind-induced force acting on the upper sections of the plant (root failure wind speed) that result in a bending moment at its base that exceeds the root failure moment (Berry et al., 2004). Few studies have investigated the mechanistic processes of root or stem lodging in sunflower. Ennos et al. (1993) observed that the most important anchorage component in sunflower was the resistance to the pulling of the roots on the windward side of the plant. Sposaro et al. (2008) found that anchorage strength was determined by the size of the root plate diameter. Stem lodging occurs when wind exerts a force which breaks the stem at its base (stem failure wind speed) that exceeds the stem failure moment. No studies have attempted to interrelate the complex interactions between climate and soil variables, husbandry practices and plant characteristics for sunflower lodging.

Models of lodging have been developed for wheat (Baker et al., 1998; Berry et al., 2003a) and more recently for barley (Berry et al., 2006). By considering the cereal plants as acting as a damped harmonic oscillator subject to a stepped input (Baker, 1995), these models calculate the wind-induced base-bending moment (leverage) of a shoot from plant characteristics. The calculated base bending moment is compared with the failure moments (strength at the point of failure) of the stem base and of the anchorage system to estimate the risk of stem and root lodging, respectively. Although some of the principles of the wheat and barley model could be the same for sunflower, other traits and differences between these crops must be considered. There are important issues that have to be taken into account for a sunflower model. It must firstly be investigated as to whether the sunflower shoot behaves as a damped harmonic oscillator. The area of the plant that is loaded by the wind is very different from cereals because the capitulum is

disc shaped and the leaves present a much greater area. The importance of the root plate diameter in lodging susceptibility has recently been studied (Sposaro et al., 2008). It is also uncertain whether the stem has the strength properties of a cylinder or whether the central pith provides significant strength. It is also possible that there are changes in stem internal anatomy during grain filling due to remobilization.

The objectives of this work are to develop a root/stem lodging model for sunflower and to validate it. This model was tested against individual field experiments in which natural root or stem lodging had occurred at different crop development stages under various crop population densities.

MATERIALS AND METHODS

In order to develop the lodging model a method to estimate the root and stem failure moments was developed during various years of experimentation.

Measurements of root failure moment (B_R)

Root failure moment values (B_R) were obtained by Sposaro et al. (2008). These measurements were carried out for two commercial hybrids (CF29 and Zenit), four crop population densities (3, 5.6, 10 and 16 plant m^{-2}) and three developmental stages (R2, R5.9 and R8 on Schneiter and Miller [1981] scale) in two different soil types (Typic Argiudoll and Typic Hapludoll) during three years.

Measurements of stem failure moment (B_S)

The values of stem failure moments (B_S) needed for the lodging model were obtained during two years of experiments: 2004/05 (E1) and 2005/06 (E2). In both experiments, two hybrids (Experimental Stay green (SG), Advanta Semillas, Argentina and Zenit, Sursem, Argentina), with different stem lodging susceptibility were planted using two plant population densities (5.6 plants m^{-2} [E1] and to 5.6, 10 and 16 plants m^{-2} [E2]) A randomized complete block design with three replications was used.

Measurements were performed at three stages of crop development: when the grain reached: a) 50% of its final dry grain weight (R7); b) 90% of its final dry grain weight (R8); and c) harvest maturity. These stages were selected because it is recognized that during grain filling and harvest maturity are the most stem lodging susceptible stages in sunflower (Abelardo de la Vega, personal communication).

An instrument especially constructed, the same used by Sposaro et al. (2008), was used to measure the force (F, N) required to induce stem lodging by pushing individual stems gradually from their vertical position until stem breakage occurred. The height of the bar that pushed the plant was adjustable, and set at 60% of the plant height (h). The stem failure moment (B_S, N m, i.e., the moment (Nm) needed to induce stem lodging) was obtained as the product of the force F (N) by 0.6h (plant height, m). Once the stem broke, the thickness of the epidermis plus the cortex (Ep+Co, m) was measured at the place where the stem failure occurred.

Plant area expected to be hit by wind gusts (A)

In order to understand how to estimate the area of the plant that is hit by wind gusts, we studied the shape of the leaves and capitulum through development. We measured the diameter and thickness of the capitulum and the length and width of the leaves in the upper third of the stem. The area formed by the leaves and the capitulum in the upper third of the plant was used for the estimation of the area that hits wind gusts.

Plant natural frequency (n)

Natural frequency (n) measurements were carried out for each hybrid at different stages of development. Each individual plant was pulled 10º from its vertical position and allowed to oscillate freely. The time taken for each plant to stop oscillating and the number of oscillations were recorded, and then transformed into number of oscillations per second (Hz) known as natural frequency (Berry et al., 2003b).

Model development

The sunflower model was developed based on exiting models for wheat (Baker et al., 1998; Berry et al., 2003a; Sterling et al., 2003) and more recently for barley (Berry et al., 2006). By considering the plants to act as a damped harmonic oscillator subject to a stepped input (Baker, 1995) these models calculate the wind-induced base-bending moment (leverage) of a shoot from B_R, B_S and plant characteristics. The calculated base bending moment is compared with the failure moments (strength at the point of failure) of the stem base and of the anchorage system to estimate the risk of stem and root lodging, respectively.

Point of application of the wind

In keeping with the work of Finnigan (2000), it was assumed that the top one third of the plant experienced significant wind loading. Hence the overall point of application was changed to 5h/6, where *h* is the height of the plant; this differs from the wheat model which assumes the point of application is at the centre of gravity.

Model validation

During season 2006/07 four meteorological stations (Vantage Pro2^{TM}, Davis Instruments Corp. 3465 Diablo Ave. Hayward, California 94545 USA) were installed at four different locations: Faculty of Agronomy, University of Buenos Aires (FAUBA), Advanta Semillas Research Centre, Venado Tuerto (VT), Daireaux, Buenos Aires; General Pico, La Pampa. At FAUBA, the meteorological station was installed during 2005/06, too. Three sunflower hybrids with different root or stem lodging susceptibility by four crop population densities were implanted at the four sites. The stations registered precipitation, wind speed and direction. At each location the percentage of root or stem lodging was recorded and was represented as a lodging index. The lodging index values are between 0 (no lodging) and 1 (the entire plot lodged).

RESULTS AND DISCUSSION

Methods for calculating root and stem failure wind speed

The root failure wind speed (minimum wind speed that could cause root lodging) V_{gR} (m s^{-1}) can be calculated using Eq. 1:

$$V_{gR} = \sqrt{\frac{2B_R}{\left(\rho A C_d h\left(1+\left(g/\left(2\pi n\right)^2 h\right)\right)\left(1+e^{-\pi\delta\left(\sin\left(\pi/4\right)/\left(\pi/4\right)\right)}\right)\right.}} \quad \text{Eq. 1}$$

Where $B_R = 0.2382x$, being x the root plate diameter cubed multiplied by the soil shear strength (sd^3, Nm, i.e. anchorage strength) (Fig. 1). This robust association between B_R and anchorage strength (sd^3) that held across hybrids, soil types, stages of development and crop population densities (Sposaro et al., 2008) allows a comparison to be made with the same relationship used in the previous lodging model for other crops, specifically wheat and barley. In wheat, the slope of the B_R / sd^3 relationship was 0.39 (Crook and Ennos, 1993) or 0.43 (Baker et al., 1998), while that for barley was 0.58 (Berry et al., 2006). These values contrast with the slope of 0.24 we found in sunflower (Fig. 4), suggesting that sunflower has an inherently lower B_R than the winter cereals for a given root plate diameter (Sposaro et al., 2008).

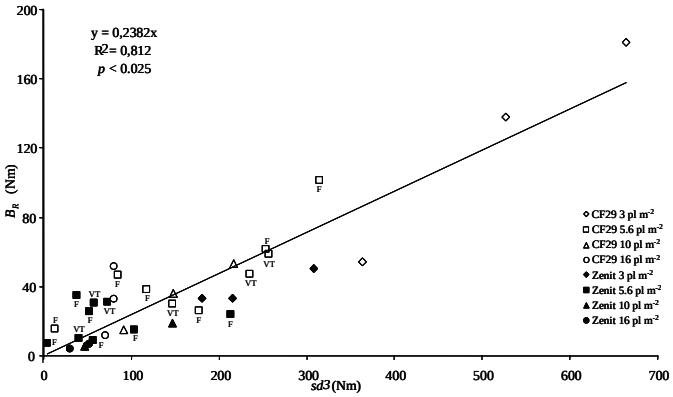

Fig. 1. Relation between B_R (Root failure moment) (Nm) and sd^3 (Plant anchorage) (Nm). Each point is the value for each d^3 (root plate diameter cubed) multiplied by s (soil shear strength) corresponding to four plant densities (3-5.6-10 and 16 plants m^{-2}), two genotypes (Zenit and CF29) and two soil types FAUBA (F), Typic Argiudoll and Venado Tuerto (VT) Typic Hapludoll against each respective value of B_R (from Sposaro et al., 2008).

In the same way the stem failure wind speed (minimum wind speed that could cause stem buckling) V_{gS} (m s^{-1}) can be calculated using Eq. 2:

$$V_{gS} = \sqrt{\frac{2 B_S}{\left(\rho A C_d h\right)\left(1+\left(g/\left(2\pi n\right)^2 h\right)\right)\left(1+e^{-\pi\delta\left(\sin\left(\pi/4\right)/\left(\pi/4\right)\right)}\right)}} \quad \text{Eq. 2}$$

Where B_S = 5980.2*x*, being *x* the Ep + Co (m) measured in the lower third of the stem (Fig. 2).

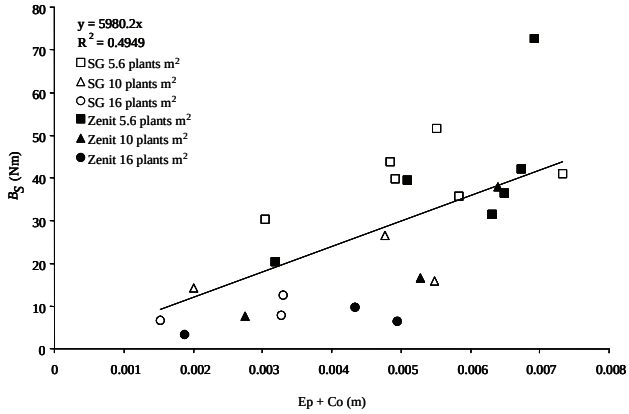

Fig. 2. Relation between B_S (Stem failure moment) (Nm) and Ep+Co (m) from lower third of the stem.

This relationship shows that hybrids with a better maintenance of the stem internal structures during grain filling (i.e. Stay green hybrids) could be more resistant to stem failure at these phenological stages. These results are important in determining the importance of the behavior of the stem as a cylinder providing significant resistance to buckling. Our results are consistent with those of Berry et al. (2003) and Berry et al. (2006) for wheat and barley, respectively.

In Eq. 1 and 2, *n* is the measured plant natural frequency, *A* the area expected to be hit by wind gusts, *h* the plant height, ρ air density, C_d sunflower drag coefficient (0.5), δ the damping ratio (0.08) and *g* the acceleration due to gravity (9.81 m s^{-2}).

Model validation

The model has been tested against various lodging events observed in sunflower experimental plots. Four storms that caused root lodging were recorded: 30/01/05, 17/12/06 (this lodging occurred at a neighbor's experimental plot [Hybrid 1] at FAUBA and was recorded too), 03/03/2007 at FAUBA and 01/12/06 at VT. Stem lodging was registered: 02/03/2007 at FAUBA. The plant parameters (mean of 10 plants per plot) were used with soil information to predict the failure wind speed for each experimental plot lodged. Table 1 shows the recorded and predicted root or stem failure wind speed and the lodging index for each hybrid, crop population density at the location where lodging occurred. If the recorded failure wind speeds were greater than those predicted, root or stem lodging could be expected for that plot. It is remarkable that the predictions of the model recreated the differences in lodging susceptibility between hybrids and crop population densities (Sposaro et al., 2008). For Zenit and CF29 the predicted root failure wind speed diminished when crop population density increased while for Stay green hybrid (SG) stayed stable, which was expected due to the maintenance of the integrity of the Ep+Co of the stem.

The results of the failure wind speed for root or stem lodging are consistent with the lodging index recorded in each combination of hybrid and crop population density. In the cases when the recorded failure wind speed was less or similar than that predicted by the model minimum or no lodging was recorded, and when the differences between recorded and predicted failure wind speed were greater, the recorded lodging index increased.

Parametric analysis

A parametric analysis was made in order to describe what the response of predicted failure wind speed was with changes in each separated variable of the model and to detect which one was the most important in determining lodging susceptibility. Each parameter varied throughout its entire range (between percentile 0 and 100) of values for all hybrids, stages of development and crop population densities.

Table 1. Lodging index, recorded and predicted (n = 10; ± 1 standard error) root or stem lodging wind speed and type of lodging (R, root; S, stem), for the lodged plots at different location, dates and crop population densities.

Hybrid	Crop population density (plant m^{-2})	Date	Location	Type of lodging	Lodging index	Recorded failure wind speed ($m\ s^{-1}$)	Predicted failure wind speed ($m\ s^{-1}$)
SG	5.6	30/01/2005	FAUBA	R	0.5	14.7	6.6 ± 0.7
Zenit	5.6	30/01/2005	FAUBA	R	0.8	14.7	5.7 ± 0.56
CF29	5.6	30/01/2005	FAUBA	R	0	14.7	14.4 ± 0.06
Zenit	3	01/12/2006	VT	R	0.3	13	7.4 ± 0.77
Zenit	5.6	01/12/2006	VT	R	0.37	13	6.1 ± 0.65
Zenit	10	01/12/2006	VT	R	0.6	13	5.6 ± 0.48
Zenit	16	01/12/2006	VT	R	0.85	13	4.3 ± 0.22
CF29	3	01/12/2006	VT	R	0	13	25.2 ± 1.22
CF29	5.6	01/12/2006	VT	R	0	13	16.7 ± 0.85
CF29	10	01/12/2006	VT	R	0.05	13	12.1 ± 0.27
CF29	16	01/12/2006	VT	R	0.05	13	11.5 ± 0.69
Hybrid 1	5.6	17/12/2006	FAUBA	R	1	14.8	6.1 ± 0.52
Zenit	3	03/03/2007	FAUBA	R	0	8.3	8.8 ± 0.78
Zenit	5.6	03/03/2007	FAUBA	R	0.3	8.3	7 ± 0.46
Zenit	10	03/03/2007	FAUBA	R	0.5	8.3	2.6 ± 0.25
Zenit	16	03/03/2007	FAUBA	R	0.95	8.3	2.5 ± 0.24
CF29	3	03/03/2007	FAUBA	R	0	8.3	12.7 ± 0.9
CF29	5.6	03/03/2007	FAUBA	R	0	8.3	10.9 ± 0.57
CF29	10	03/03/2007	FAUBA	R	0.05	8.3	8.6 ± 0.21
CF29	16	03/03/2007	FAUBA	R	0.1	8.3	7.5 ± 1.2
Zenit	5.6	02/03/2007	FAUBA	S	0	8.9	9.9 ± 0.39
Zenit	10	02/03/2007	FAUBA	S	0.95	8.9	5.3 ± 0.61
Zenit	16	02/03/2007	FAUBA	S	0.5	8.9	5.9 ± 0.65
SG	5.6	02/03/2007	FAUBA	S	0	8.9	8.7 ± 0.3
SG	10	02/03/2007	FAUBA	S	0	8.9	8.9 ± 0.48
SG	16	02/03/2007	FAUBA	S	0	8.9	8.9 ± 0.66

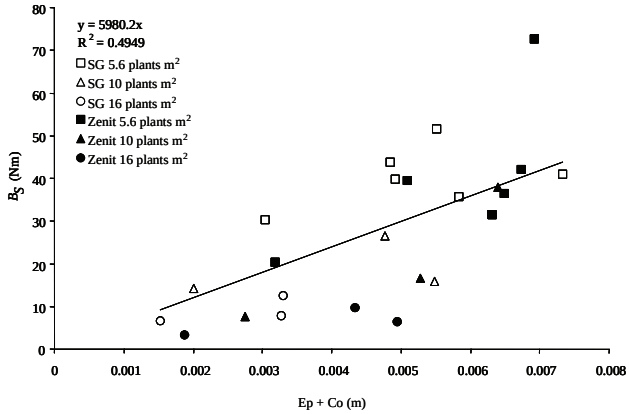

Fig. 3. Parametric analysis for root and stem lodging model. a) Predicted root failure wind speed ($m\ s^{-1}$) and b) stem failure wind speed ($m\ s^{-1}$) for variations between 0 (minimum value) and 1 (maximum value) in model variables: *h*, plant height (m); *A*, area expected to be hit by wind gusts (m^2); *n*, natural frequency (Hz); a) root plate diameter and b) *Ep+Co*, thickness of the epidermis plus the cortex of the stem.

Predicted root failure wind speed (Fig. 3a) was affected mostly by changes in root plate diameter (i.e. root failure wind speed increased in a greater proportion when root plate diameter was higher and decreased when it was lower). Stem failure wind speed (Fig. 3b) was affected mostly by variations in thickness of *Ep+Co* in the same way as the root plate diameter. The area expected to be hit by wind gusts (*A*) was an important variable too for the determination of the failure wind speed, but only when its values were the lowest. Although variations in height (*h*) and natural frequency (*n*) modified the results for failure wind speed, these were of a lesser importance than the other parameters. This is an important finding because most farmers think that plant height is the most important trait that determines lodging susceptibility. These results are similar to those of Berry et al. (2003) for wheat; the root plate diameter and the thickness of the stem wall (i.e. the same as Ep+Co in sunflower) being the most important parameters affecting the predicted failure wind speed. Fig. 3 a) and b) show that the value of the average stem failure wind speed is higher (8.5 m s^{-1}) than the root failure wind speed (7.8 m s^{-1}), indicating that sunflower could be more susceptible to root than stem lodging.

To summarize, the results of this study show, for the first time in sunflower, the developing of a model that can accurately predict the root/stem failure wind speed for crops growing under various husbandry practices (hybrids, crop population densities, soil types). The variables that are inputs of the model can be used in breeding programs to select hybrids that could be more resistant to stem or root lodging.

ACKNOWLEDGEMENTS

We wish to express our particular thanks to Dr. Abelardo de la Vega, Aldo Martínez, Sergio Solián and Hugo Baravalle from Advanta Semillas SAIC for their kind assistance in the performance of the experiments. This research was supported by grants from UBACyT (G048) and FONCyT/ASAGIR (PICTO 13159). MMS was in receipt of an UBA scholarship and AJH is a member of CONICET, the National Research Council of Argentina.

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Exploring genotypic strategies for sunflower drought resistance by means of a dynamic crop simulation model

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ABSTRACT

Although sunflower is often reported as a drought-tolerant crop, it suffers from intense and frequent periods of water deficit in Europe because it is mostly grown on shallow soils, under low rainfall and in rainfed areas. Given the limitations of experimental trials to explore a large number of drought scenarios, a dynamic crop simulation model was developed to determine different phenological (duration of post-anthesis period), morphological (leaf area) and physiological (rate of stomatal closure) putative traits of drought resistance in sunflower. A virtual experimental network was built by combining 4 locations (N-S gradient), 3 soil depths, over 36 weather seasons. In each of the 432 trials, 12 synthetic varieties were evaluated, differing by earliness at maturity (2 levels), leaf area (3 levels), leaf expansion and stomatal regulation sensitivity to progressive soil drying (2 levels: early or late response). This simulation study suggests that the varieties with early stomatal closure could result in the best yields in drought-prone environments, this trait being more determining than leaf area or earliness. In the most productive locations, late varieties, with large leaf area and late stomatal closure should result in the best yield. It is concluded that an additional variety screening including the response to water deficit could improve the choice of optimal sunflower cultivars in France.

Key words: drought resistance – leaf area – phenology – simulation model – stomatal regulation – varietal choice.

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is often reported as a drought-tolerant crop (Unger, 1990; Connor and Hall, 1997). However, in southern Europe it suffers from intense and frequent periods of water deficit because it is mostly cultivated in low rainfall areas, without irrigation, and on shallow soils.

Ludlow (1989) reviewed three main genotypic adaptations to water-limited environments: (a) drought escape, whereby the crop completes its life (or oversteps a critical growth stage) before the onset of severe drought, (b) drought avoidance, where the crop maximizes its water uptake and minimizes its water loss, and (c) drought tolerance, where the crop continues to grow and function at reduced water contents. To these plant strategies, crop management offers additional opportunities (Debaeke and Aboudrare, 2004): (d) drought alleviation or moderation, by the means of irrigation, (e) optimal crop water use pattern, by reducing soil evaporation and increasing the contribution of transpiration during grain filling period (through crop density and N fertilization).

In commercial fields, drought resistance should be achieved by combining optimal cultivar choice and crop management. But limited information is available to characterize the response of sunflower cultivars to water stress, as drought resistance is a complex trait which cannot be evaluated accurately at field level. Field trials where water deficit occurs are generally banned from the official evaluation network because of poor statistical value.

Given the limitation of experimental trials to explore a large number of drought scenarios, dynamic crop modelling may be an alternative to arduous experimentation and is recognized as an adequate tool to identify genotype x environment x cultural practice combinations to achieve the most stable yield over a wide range of soil water availabilities (Agüera et al., 1997; Sinclair and Muchow, 2001; Chapman et al., 2002; Soriano et al., 2004).

Although several models are available for sunflower crop (e.g. Chapman et al., 1993; Villalobos et al., 1996; Pereyra-Irujo and Aguirrezabal et al., 2007), a new simulation framework was developed to represent more explicitly the varietal differences and to support cultivar choice decision in relation with water availability (Casadebaig, 2008). The main original point comes from using genotypic parameters that are measured directly from field or greenhouse trials.

The objective of this communication is to examine, by means of this dynamic simulation model, whether different varietal types defined by earliness, architecture, and response to soil desiccation should

be recommended in France over the sunflower production area when natural water availability (precipitation, soil depth) is changing.

MATERIALS AND METHODS

Model and varietal parameters

The simulation model equations are described in detail in Casadebaig (2008): the daily step model simulates dynamically achene yield and oil concentration as a function of classic weather data (temperature, precipitation, ET_{ref}), soil data (available soil water content, N mineralization), crop management (sowing date, plant density, N fertilization, irrigation) and varietal characteristics (phenology, leaf area dynamics, leaf expansion and transpiration rate response to soil water deficit, biomass allocation).

Phenological parameters are: growing degree days (Tbase: 4.8°C) to reach different characteristic growth stages: emergence (A2), star bud (E1), early anthesis (F1), early grain filling (M0), physiological maturity (M3).

Leaf area (LA) index evolution is simulated on an individual leaf scale basis (Lizaso et al., 2003) and modulated from the measurement of 3 architectural parameters at anthesis: total leaf number, position and length x width of the largest leaf.

The extinction coefficient (k) is determined either directly or through a statistical adjustment using the previous LA parameters.

Genetic harvest index and oil concentration are determined in dense and unstressed sunflower stands.

Modules for development, biomass accumulation and allocation to the achenes were built using robust representations from the literature.

An original screening method was developed in greenhouse to parameterize leaf expansion and stomatal closure response to soil water content. Thresholds were calculated for a range of genotypes from different sources of selection (Casadebaig et al., 2008).

Phenotypic database

A database was built to gather the results of numerous experiments conducted by INRA and Cetiom from 2001 to 2007 on sunflower phenotyping (Debaeke et al., 2004). More than 20 cultivars representing the genetic progress from 1960 onwards (Vear et al., 2003) were fully described.

A virtual multi-environment trial network

From the phenotypic database, 12 virtual cultivars (Table 1) were created by combining 2 variants of earliness (E, early: 1750 °C.day from A2 to M3; L, late: 2160°C.day), 3 levels of plant potential leaf area (S, small: 4000 cm²; M, medium: 8000 cm² ; L, large: 12000 cm²) and 2 extreme levels of plant regulation in response to soil drying (E: early control of leaf expansion and stomatal closure, at a relatively high soil water content ; L , late control, at a rather low soil water content) (Fig. 1). In this study, the term regulation was used to reflect the effects of both response traits (leaf expansion and transpiration control). It was assumed that all the characters of drought resistance were independent.

Table 1. Combination of the 3 phenological, morphological and physiological traits to build 12 virtual varieties

Variety	Code	Earliness	Leaf Area	Regulation
1	ESE	Early	Small	Early
2	ESL	Early	Small	Late
3	EME	Early	Medium	Early
4	EML	Early	Medium	Late
5	ELE	Early	Large	Early
6	ELL	Early	Large	Late
7	LSE	Late	Small	Early
8	LSL	Late	Small	Late
9	LME	Late	Medium	Early
10	LML	Late	Medium	Late
11	LLE	Late	Large	Early
12	LLL	Late	Large	Late

Four regions were selected to sample the main French sunflower cropping area: Midi-Pyrénées (South-West), Provence (South-East), Poitou-Charentes (Center-West) and Parisian Basin (Center-North). Each region was described by one climate station and 3 soil types. The following climate stations from INRA were chosen: Auzeville (Department 31), Avignon (84), Lusignan (86) and Versailles (78). Each climate series was composed of 36 x 365 daily recordings (1971 – 2006). Solar radiation and climatic water deficit were the highest in Avignon and the lowest in Versailles as expected.

The 3 soil types differing by soil depth and available soil water content (ASWC) were extracted from a soil data base from INRA (Brisson et al., 2006): S1 (ASWC: < 60 mm), S2 (80-120 mm), S3 (130-150 mm).

Sunflower crop management was the same in the 12 environments: sowing date on 20 April, 60 kg N/ha applied 15 days after emergence, no supplemental irrigation.

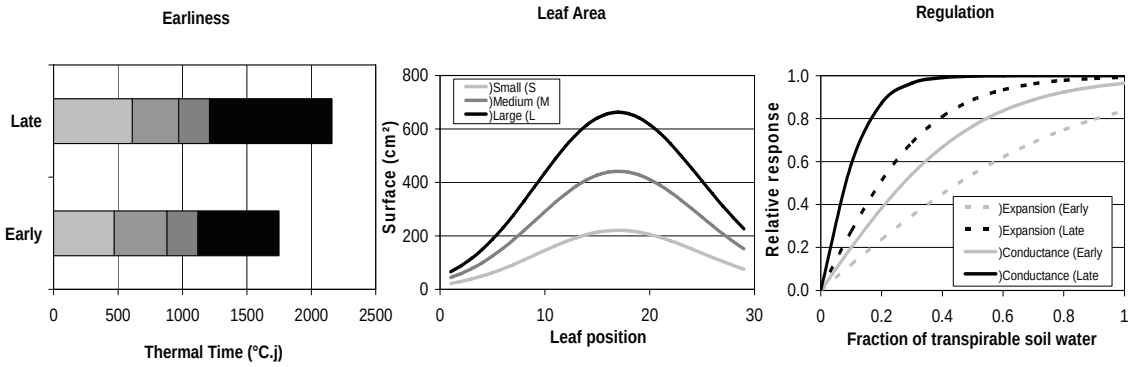

Fig. 1. Extreme values of phenological, morphological and physiological traits (maturity earliness, total leaf area, thresholds for stomatal closure and leaf expansion decline) among a range of 20 cultivars.

RESULTS

The application of the model resulted in different yield performances of the 12 varieties with season and pedoclimatic environment.

The northern situations (LUS, VER) resulted in higher yield levels, whatever the soil depth (Fig. 2). Grain yield was more stable in VER location (especially on S3) and more variable in Auzeville (AUZ). Average grain yield over 36 years ranged from 14 to 28 q/ha depending on soil type and climate. In France, average grain yield in national surveys ranges from 18 to 23 q/ha (at 0 % grain moisture).

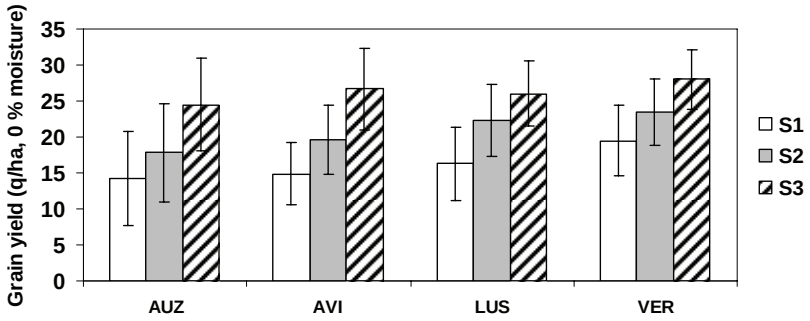

Fig. 2. Effects of location and soil type on grain yield (mean values and S.D over 36 years)
S1 = shallow ; S2 = medium ; S3 = deep soil

The mean effects of variety, earliness, leaf area and regulation on grain yield were displayed on Fig. 3. Late maturation, high leaf area index and early stomatal closure all increased grain yield: the latter trait was the most influential one (+ 3.8 q/ha vs 0.9-1 q/ha for the two other traits). The combination of the 3 traits resulted in GY variations from 18.8 (var. 2) to 24.4 q/ha (var. 11).

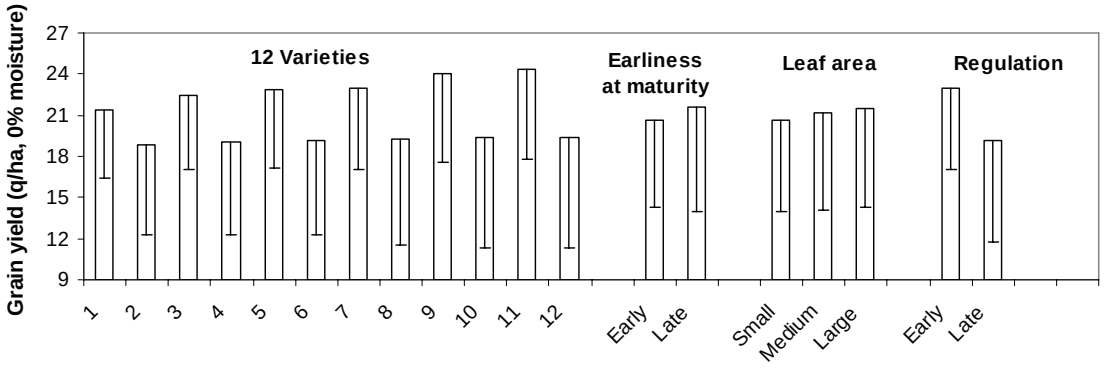

Fig. 3. Grain yield for the 12 virtual varieties and the 3 morpho-physiological traits (mean values and SD over 36 years)

The 3 traits (maturity earliness, leaf area, regulation) had a significant effect on yield ($P < 0.001$) but the 'trait x environment' interactions are not of the same level: highly significant for regulation ($P < 0.001$), significant at $P < 0.1$ for earliness, but not significant for leaf area.

To quantify the importance of a genotypic trait in a given location, an analysis of variance (ANOVA) was attempted as follows: Yield ~ Yr + E + LA + R + Yr*E + Yr*LA + Yr*R, in each environment (Table 2).

Table 2. Variances and statistical significance (*P<0.05; **P< 0.01; ***P<0.001) of the single effects (genotypic traits) and the 'year x trait' interactions in each of the 12 environments.

Location	Soil	Year (Yr)	Earliness (E)	Leaf Area (LA)	Regulation (R)	Yr x E	Yr x LA	Yr x R
AUZ	S1	374 ***	2 ns	12 ***	4043 ***	8.0 ***	0.4 ns	19.8 ***
AUZ	S2	376 ***	7 *	2 ns	5574 ***	5.3 ***	0.7 ns	20.4 ***
AUZ	S3	405 ***	163 ***	12 ***	1757 ***	8.9 ***	2.3 ***	37.7 ***
AVI	S1	183 ***	109 ***	63 ***	1089 ***	2.4 ***	0.3 ***	8.3 ***
AVI	S2	196 ***	63 ***	4 ***	2633 ***	2.4 ***	0.5 ***	4.9 ***
AVI	S3	319 ***	77 ***	2 *	1885 ***	9.7 ***	2.8 ***	8.3 ***
LUS	S1	201 ***	63 ***	12 ***	3283 ***	7.6 ***	0.7 ns	11.6 ***
LUS	S2	211 ***	87 ***	2 ns	1850 ***	7.3 ***	1.0 ns	22.3 ***
LUS	S3	185 ***	249 ***	22 ***	753 ***	5.0 ***	1.1 *	19.2 ***
VER	S1	217 ***	90 ***	82 ***	996 ***	6.2 ***	1.7 **	24.0 ***
VER	S2	204 ***	112 ***	60 ***	364 ***	7.1 ***	4.3 ***	19.1 ***
VER	S3	156 ***	567 ***	407 ***	33 ***	3.9 ***	1.5 ***	1.9 ***

If the advantage conferred by the R character was major whatever the environments (except for VER_S3 and VER_S2, where water stress was minimum), the impact of the other characters was dependent on the environment. Earliness (E) played a role in the medium and deep soils, where late varieties can take advantage of a longer growing duration. Leaf area, although its effect was significant in most of the environments, played only a significant role in the most extreme environments (AVI_S1, VER).

All the characters exhibited significant interactions with year, although Yr x R interactions were the most important. The existence of such interactions between the traits and the climate suggests that a varietal choice based only on the mean performance of a variety in a given location might be irrelevant in some years.

The R character was obviously the most determining one to explain yield variations in this simulation exploration: depending on the regions, regulation though leaf expansion and stomatal closure may be responsible for mean gaps of 7 q/ha in South-West (between varieties differing only by this character) but of 1.7 q/ha in the Parisian Basin region. These gaps were related to the intensity of soil water deficit, stomatal closure being a response to soil desiccation. Earliness had a lower influence: from 0.4 q/ha (South-West) to 1.8 q/ha (Parisian Basin). Concerning leaf area, the mean gap between two modalities ranged from 0.2 q/ha (South-West, Center-West) to 1.7 q/ha (Parisian Basin). Increasing LA had a negative impact on yield in the most drought-prone environments (AUZ, AVI) but only for varieties with late stomatal closure. The model suggested that « early regulation » has more impact than a variation in potential LA in these environments. In dry environments, reducing LA might not be a good strategy as potential yield would be reduced too much and soil evaporation could increase as well.

From the ANOVA, the best varietal choice (combination of 3 traits) among 12 candidates was determined for each of the 12 environments on the basis of 36 virtual trials (climate series) (Table 3).

At a regional level, the variety "11" or LLE (late maturing, large leaf area, early stomatal closure) would be systematically the best choice. The ideotype LLE was relevant 5 years out 10 in South-East and Parisian Basin and 7 years out 10 in the South-West and Center-West regions. Three years out of 10, the ideotype LLL, with late stomatal closure, would be a better choice in the Parisian Basin and the ideotype LSE, with a smaller LA, would be a better choice in South-East.

The soil type had no marked effect on the best choice within a region. But the frequency of the best yielding cultivar changed from one environment to another (from 46 % to 89 %). In general, early regulation should be recommended in shallow soils, because delaying soil water depletion in this way is a good strategy to sustain a large leaf area (and light interception). On the contrary, in the Parisian Basin region, in deep soils (VER_S3), where water deficit and global radiation are the lowest, the model selected a late maturing ideotype, with a late stomatal closure when exposed to water deficit (more photosynthesis in spite of more water transpired in the first part of the cycle) and a large LA value.

Table 3. Potential yield, mean GY value of the best ranked variety, and best ranked varieties in each simulated environment[1]

Location	Soil	Best year (q/ha)	Best variety (q/ha)	1st rank for variety n (%)	1st choice	2nd choice
AUZ	S1	27.0	18.0	71	**11** - 9 - 5	7 - 3 - 1
AUZ	S2	30.0	22.3	66	**11** - 9 - 7	5 - 3 - 1
AUZ	S3	34.1	28.1	63	**11** - 9	7 - 5 - 3
AVI	S1	25.4	17.7	89	**11**	9 - 5 - 3 - 7
AVI	S2	29.9	22.7	51	**11** - 9 - 7	5 - 3 - 1
AVI	S3	36.7	29.6	43	9 -11 - **7**	5 - 3 - 1
LUS	S1	26.4	20.4	83	**11** - 9	7 -5 -3 - 1
LUS	S2	30.9	25.7	66	**11** - 9 - 7	5 - 3 - 1
LUS	S3	33.1	29.0	71	**11** - 9	7 - 5 - 3
VER	S1	29.6	22.8	74	**11** - 9	5 - 3 - 7
VER	S2	32.6	26.0	46	**11** - 9	5 -7 - 3
VER	S3	35.0	30.5	63	11 - **12**	10 - 9

[1]The underlined variety number corresponds to the best ranked one in term of frequency over 36 years.

DISCUSSION

According to the model and to its multi-environment application, early regulation would be a relevant physiological trait to select in sunflower. According to Casadebaig et al. (2008), early stomatal closure is not frequent among commercial cultivars. This behaviour is closer to what is observed on isohydric species such as sorghum and maize. Conversely, Sinclair and Muchow (2001) did not simulate a significant increase in grain yield in sorghum, by manipulating this trait, as this crop was already well adapted to production under water stress.

In sunflower, changing cultivar earliness (from early to late type) did not result in huge differences in grain yield over the French cropping area, contrary to what is reported in Mediterranean environments (Debaeke and Aboudrare, 2004). In this paper, only differences in the anthesis-maturity were explored. The date of anthesis (about 10 days from early to late type) could have been modulated as well but probably with minor consequences on drought escape at anthesis. However, choosing an early maturing variety appeared as a good decision in the most stressful environments (AUZ_S1). Sowing date would probably have more effect on drought escape, especially sowing in autumn instead of spring as practised in the most southern regions of Europe (Soriano et al., 2004).

The optimal level of leaf area index results from a trade-off between transpiration, soil evaporation and light interception. In general, the lowest values of LA were not optimal in France even in the most stressed environments; plant density should rather be increased in this case.

Other traits have been reported as influencing grain yield in drought-prone environments: water extraction pattern and early vigour (Sadras and Hall, 1989; Agüera et al., 1997; Sinclair and Muchow, 2001). These traits could be explored by the model provided that experimental evidence of genotypic variation could be supplied.

The simulation of virtual genotypes, which is of interest for testing new combinations of traits, was based on characters expressing a sensitivity to water stress. From a practical angle, the farmer's decisions are based on cultivar potential productivity and disease tolerance (which were not considered in the simulations). Potential productivity corresponds to the LLL type in environments where water is not a limiting factor. With the exception of earliness, the characters involved in water stress resistance are not

evaluated in the official trials and for that reason they cannot be exploited by the advisers. As cultivar choice results from a complex evaluation of a range of characters (some are measured, others result from expertise), the varietal supremacy of LLE type would be probably less visible in trials. The interaction with the weather may change the optimal choice. For that reason, 2 or 3 years of field evaluation as currently practised are not sufficient to explore the advantages and limits of a new variety. The simulation of varietal strategies may help the adviser to promote a cultivar with a stable yield over a wide range of pedoclimatic conditions.

ACKNOWLEDGEMENTS

Our partners CETIOM, ONIDOL, and OLEOSEM are gratefully acknowledged for their financial support and other facilities.

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Dynamics of dry matter accumulation in sunflower

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ABSTRACT

This paper deals with the effect of sowing density on dynamics of dry matter accumulation in sunflower. The experiment was established on the chernozem soil, in a six-crop rotation system, in the two-factorial split-plot design. The main plots were three sunflower hybrids: NS-Dukat, NS-H-111 and NS-H-103. The subplots were six densities: 30000, 40000, 50000, 60000, 70000 and 80000 plants per hectare. The dynamics of dry matter accumulation and the distribution of dry matter among the various plant organs were dependent on competition between the plants. The largest amount of dry matter was accumulated by the flowering stage, while the peak of accumulation occurred 30 days after the flowering. The period of the most intensive dry matter accumulation was from budding till flowering.

Key words: dry matter – stand density – sunflower.

INTRODUCTION

Since sunflower hybrids differ in plant height, number, size and position of leaves, lodging and disease resistance, vegetative space, nutrient and water uptake as well as photosynthetic activity, it is necessary to establish optimal plant density for each hybrid. Different sunflower hybrids react differently to the environmental conditions. This leads to a different realization of yield potential.

Control of dry matter distribution between different plant organs is the basis for crop yield (Wardlaw, 1990). Special conditions for nutrient uptake, its distribution between different organs, and photosynthesis efficiency are created under the influence of different vegetative space and plant number. This is one of the causes of differences in sunflower yield in the same agro ecological conditions (Hall et al., 1990). Villalobos et al. (1992) found that, in order to create a model of crop growth, it is necessary to have a knowledge of assimilate distribution between plant parts and how the environment affects dry matter distribution and yield.

It was found that semi-dwarf sunflower hybrids had a higher dry matter content in head and a lower one in the stem, when compared with the hybrids with the standard height (Maid and Schneiter, 1988). No differences were observed in total dry matter accumulation in the hybrids of different heights, although taller hybrids formed a larger amount of dry matter.

There is increased competition between plants at high stand densities. As the competition between plants should be decreased in order to promote efficient use of water, nutrients and sunlight, it is necessary to determine optimal plant density in order to maximize the expression of the yield potential.

The aim of this experiment was to determine optimal plant densities for sunflower hybrids with a high yield potential which differ significantly in their growing habits.

MATERIALS AND METHODS

Field experiments were conducted at the Rimski Sancevi experiment field of the Institute of Field and Vegetable Crops, Novi Sad, Serbia, for three years. The experiment was established on a chernozem soil, in a six-crop rotation, following a two-factorial split-plot design. The main plot contained three cultivated sunflower hybrids: NS-Dukat, NS-H-111 and NS-H-103.

NS-Dukat is an early hybrid that matures in 90 to 95 days. The average stem height is 145 to 155 cm, the genetic potential for seed yield 4 t ha^{-1}, and the seed oil content from 47 to 49%. The hybrid is genetically resistant to downy mildew, broomrape and the sunflower moth. It is recommended for late sowing (15 May to 15 June) in fields that could not be sown before for some reason.

NS-H-111 is a medium early hybrid that matures in 105 to 115 days. The stem is firm, 165 to 185 cm tall on average. The genetic potential for seed yield is 5 t ha^{-1}, the seed oil content from 48 to 50%. The hybrid is genetically resistant to downy mildew, rust, broomrape and the sunflower moth, and tolerant to *Phomopsis*. The hybrid is adaptable to a wide range of agroecological conditions.

NS-H-103 is an experimental hybrid that matures in 120 to 130 days. The stem is firm, 90 to 100 cm tall on average. The genetic potential for seed yield is 4 t ha^{-1} and the seed oil content ranges up to 50%. The hybrid is genetically resistant to downy mildew, rust, broomrape and the sunflower moth.

The experiment subplots were six stand densities: 30,000, 40,000, 50,000, 60,000, 70,000 and 80,000 plants per hectare. Manual planting was done in early April, by placing 3-4 seeds per hill. At the stage of 1-2 pairs of leaves, the stand was thinned to one plant per hill, to obtain the desired number of emerged plants. Timely cultural practices were performed, applying the conventional technology. The experiment was conducted in four replications. The elementary plots consisted of six 10-meter rows.

Dynamics of dry matter accumulation during vegetative period of sunflower plants was observed at the main stages of the plant development:

1. 6 pairs of leaves
2. budding
3. flowering
4. seed forming
5. 30 days after flowering

Samples were taken from 12 plants (3 plants from each repetition) from the following variants: 30000, 80000 plants/ha in all three hybrids; 60000 plants/ha in NS-Dukat, 50000 plants/ha in NS-H-111 and 70000 plants/ha in NS-H-103. Average sample for each variant was used for the determination of dynamics of dry matter accumulation. Each sample was dried at 105° C, and dry matter percentage in different plant parts was determined.

RESULTS AND DISCUSSION

Dry matter accumulation dynamics varied depending on the development stage, plant density and the hybrid.

Dry matter accumulation at the stage of 6 pairs of leaves was low, and the plant density did not have any effect on plant dry matter production (Fig. 1). A higher proportion of total dry matter content in leaves compared to the stem was observed at this stage, especially in the hybrid NS-H-103 (Fig. 2). This is in accordance with the results of Horie (1977), who found predominance of assimilative distribution in the leaves at this stage.

Sunflower plants developed slower and accumulated less dry matter till the budding stage. Merrien (1986) explained this phenomenon by the fact that until the budding, most of the assimilates produced in the leaves are transported towards the root, which develops intensively during that stage. Bud appearance causes the inversion of the main direction of assimilate transportation. At that moment, the capitulum becomes the main assimilates consumer. In hybrids NS-Dukat and NS-H-111, the capitulum had a higher dry matter content than the leaves. According to Villalobos et al. (1994) and Trapani et al. (1994), this is the direct consequence of the increased competition between plants caused by increase in stand density. In contrast to NS-Dukat and NS-H-111, hybrid NS-H-103 dry matter content was higher in the leaves than in the capitulum.

The relationship between dry matter accumulation per plant and per acreage in different stand densities changed significantly at the budding stage. The highest dry matter content per plant was obtained at the lowest stand density, while the lowest dry matter content was recorded at the highest stand density. This was not the case with the dry matter accumulation per acreage, where the opposite trend was observed. Relations between dry matter production per plant and per hectare at the different stand densities are affected by competition between plants. Competition between plants starts at the stage of the intensive growth. The competition starts at the 13-14 pair of leaves stage, first in the stands with a higher number of plants, and, at the later stages of the development, in the stands with the lower plant densities. Percentage of dry matter formed at the budding stage increased with the increase in the plant density in all three tested hybrids.

The highest quantity of dry matter was accumulated from emergence till the flowering stage. Similar results were obtained by Merrien (1986). Dry matter accumulation was most intensive between budding and flowering, which is in accordance with the results of De Giorgio et al. (1990) and Sfredo et al. (1985).

Competition between plants for light, water and nutrients was more evident at the flowering stage. Till flowering, relations between plants were such as to enable the development of each plant even at higher stand densities, i.e. the plants developed faster and formed a larger quantity of dry matter at the stages of development in which the competition between plants was lower.

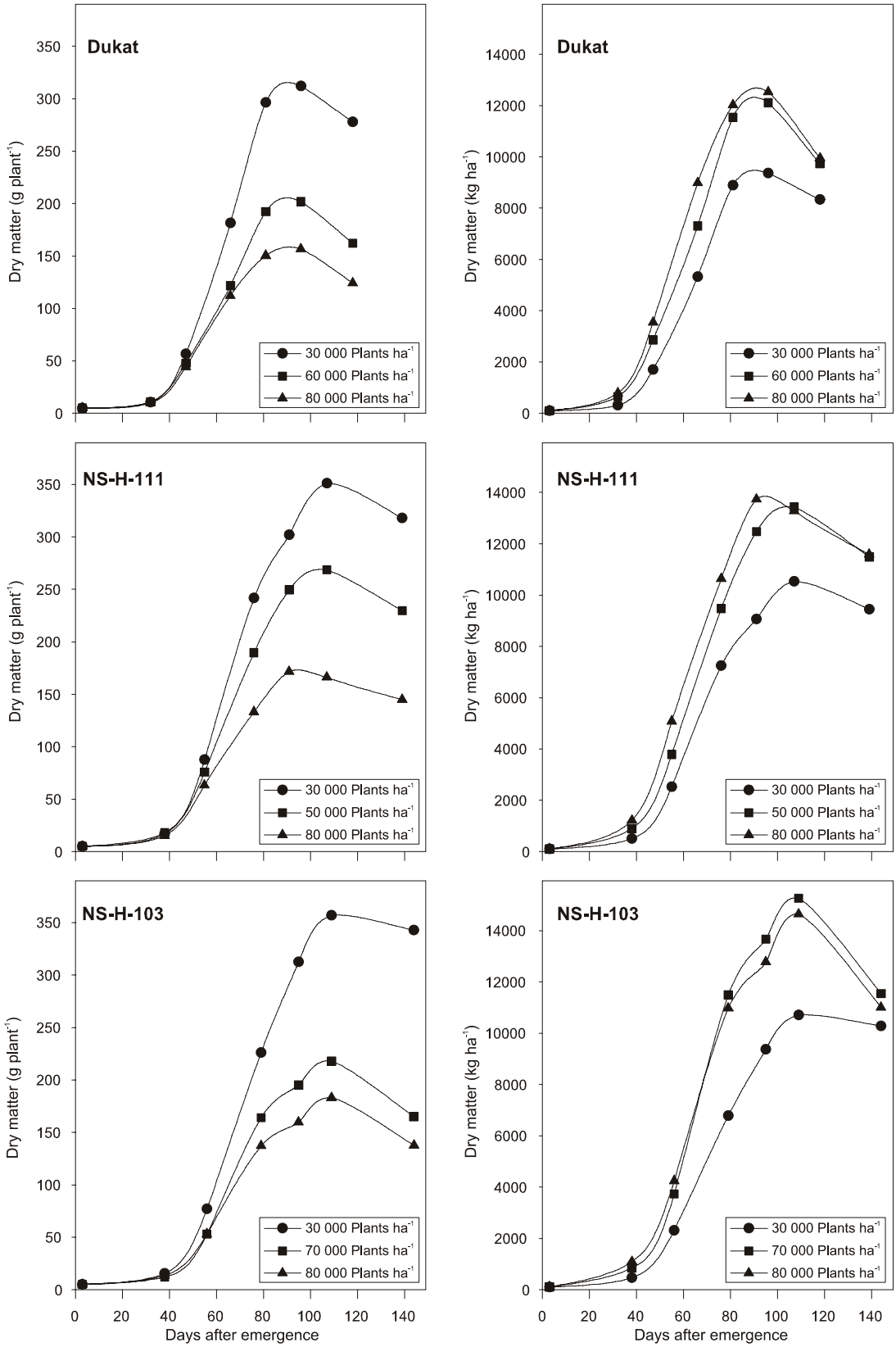

Fig. 1. Dynamics of dry matter accumulation during vegetative period of sunflower plants at different plant densities.

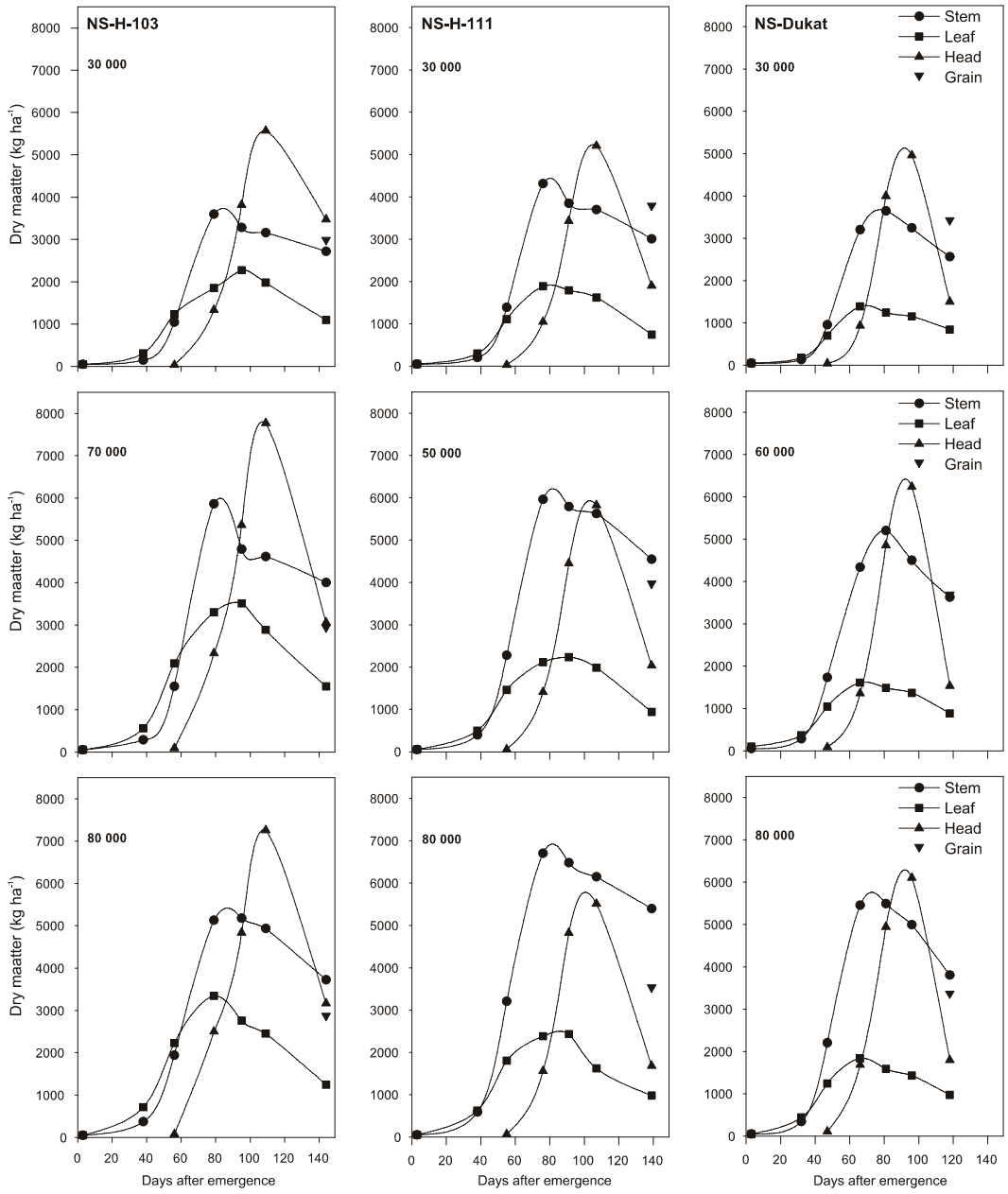

Fig. 2. Dry matter distribution between plant organs of sunflower plants at different plant densities.

Similar results were obtained by Blanchet et al. (1988). At this stage, in all three tested hybrids, the highest dry matter content was found in the stems, followed by the dry matter content in the leaves. In hybrids NS-Dukat and NS-H-111 dry matter content increased in the stem and decreased in the leaves with the increase in plant density. This is in agreement with the results of Horie (1977), Villalobos et al. (1994) and Trápani et al. (1994). In NS-H-103, the opposite trend of dry matter accumulation was observed. According to De Giorgio et al. (1990) and Sfredo et al. (1995), when there is no competition between plants caused by the size of vegetative space, the highest dry matter content can be found in the leaves, followed by the dry matter content in the stems.

Intensive dry matter accumulation continued from flowering till 30 days after flowering. This was especially the case of the hybrid NS-Dukat, in which, due to its growing habit, dry matter accumulation was not that much affected by competition. Proportion of stem in total dry matter content was still significant, but lower compared to the flowering stage. Significant decrease in dry matter, compared to

the flowering stage, was observed in leaves as well. Proportion of head in total dry matter content increased significantly at the expenses of assimilates from stem and leaves, as well as current assimilation. Similar results were obtained by De Giorgio et al. (1990). Proportion of stem in total dry matter content increased with the increase in stand density, while the proportion of leaves and capitulum decreased.

Maximal dry matter content was observed at the stage of 30 days after flowering. This is in accordance with the results of Vrebalov et al. (1983). Dry matter distribution between plant organs had the same tendency as in the previous developmental stage. Similar to the results of Sfredo et al. (1985), the proportion of dry matter of stem and leaves in total dry matter content decreased, and the proportion of dry matter in the capitulum increased.

The highest dry matter content in the stem was observed in NS-H-111 at all stages of the development. The other two tested hybrids had similar dry matter content in the stem, although it was non-significantly higher in NS-Dukat. Hybrid NS-H-103 had the highest dry matter content in leaves, NS-H-111 being the second and NS-H-Dukat the third.

In our work, we have found that the dynamics of dry matter accumulation and dry matter distribution between different sunflower plant organs depended on the competition between plants, which started from the budding stage. Dry matter content per plant decreased and dry matter per acreage increased with the increase in stand density. At the initial stages of the dry matter accumulation, leaves had priority in dry matter distribution. At the later stages of the development, that priority was derived to the stem till flowering, and later on to the capitulum. In all tested hybrids, the maximum dry matter accumulation was at the stage of 30 days after flowering. The most intensive period of dry matter accumulation was from budding till flowering stage.

ACKNOWLEDGEMENTS

This work was supported by Ministry of Science, Republic of Serbia.

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IAA/GA_3 quantitative ratio of some sunflower genotypes representing CMS-Rf system

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ABSTRACT

Quantitative ratio of IAA/GA_3 (indole-3-acetic acid/gibberellic acid) was studied during the growth and development of different sunflower genotypes that represent a CMS-Rf system. It has been shown that IAA/GA_3 ratio is variable and depends on ontogenesis stages, organs and genotype. Thus, IAA/GA_3 ratio had maximal values for male sterile line and minimal ratio for restorer line RW637Rf. The highest IAA/GA_3 ratio was registered in cotyledon leaves and subsequently decreased during ontogenesis whereby the hormonal ratio in reproductive stages was higher in inflorescences than in leaves. Gibberellic acid exogenously applied increased the hormonal ratio in the male-fertile line. The specificity of IAA/GA_3 balance in male sterility-fertility phenotype expression and in GA-induced pollen sterility is discussed.

Key words: CMS-Rf system – gibberellic acid – IAA/GA_3 ratio – indole-3-acetic acid – male fertility – male sterility.

INTRODUCTION

Hormonal regulation of plant growth and development including interaction between different classes of hormones remains an important research topic in biology. Plant growth regulators, endogenous or exogenously applied, are involved in male reproductive development, regulating sex differentiation (Ciailahean, 1988) and male (genetic and cytoplasmic) sterility promoting (Luis and Durand, 1978; Kaul, 1988; Rastogi and Sawhney, 1990; Nakajima et al., 1991) at various species. Our previous work has shown that CMS sunflower lines contain lower amounts of gibberellins than fertile genotypes, including homozygote line with Rf genes (Duca et al., 2003). This evidence suggests an auxin and gibberellin interaction in microsporogenesis processes by their quantitative ratio.

It is known that auxin and cytokinin interaction plays a decisive role in cell division and elongation (Inoue et al., 1991), in induction of root and stem development (Jacobsen et al., 1995). Also, the gibberellin and abscisic acid interaction was shown to regulate the beginning of seed germination through gene expression regulation (Collett et al., 2000; Zentella et al., 2002). It is also known that GA induces synthesis and secretion of a number of hydrolytic enzymes in germinating seed endosperm (Muthukrishanan et al., 1984; Jacobsen et al., 1995), and GA activity can be suppressed by abscissic acid (White et al., 2000).

To reveal the functional role of IAA/GA_3 balance in male sterility-fertility phenotype expression, the hormonal quantitative ratio was studied during the growth and development of different sunflower genotypes, representing a CMS-Rf system.

MATERIALS AND METHODS

Plant material

Sunflower plants were cultivated in the experimental field of Moldova State University according to conventional technologies during four years. Two isonuclear lines MB514 and MB514CMS with mitochondrial *orfH522*, RW637Rf with nuclear homozygote restoration nuclear gene *Rf* and hybrid F_1 obtained by cross between these lines (MB 514 CMS x RW637Rf) with restored male fertility (*Rf_*) were chosen for analyses. For comparative studies, SW501CMS was additionally used. Phenocopies method was applied (Duca, 1998).

The treatment with exogenous gibberellic acid (GA_3) solution by plant spray was carried out at the development period of the inflorescence buds. At this stage, prior to the opening of the inflorescence, male meiosis occurs in disc flower anthers (Anaschenco, 1971). Non-GA_3 treated plants (control) were sprayed with distilled water. For assaying non-GA_3 treated plants (control) and GA_3 treated plants, 24 h post-treatment were used.

Chromatographic analysis

The plant material was collected at various vegetative stages that were correlated with development and microsporogenesis (Duca, 1998). Fresh plant material (about 10g) was homogenized and fixed in cold (-20°C) 80% acetone (1:30 ratio) and extracted over-night at 3-5ºC during 24h. After a series of organic extractions and purifications, the extracts were dried in vacuum at 40°C. The residue was dissolved in 0.1 ml N,O-bis(trimethylsilyl)-acetamid with the addition of 0.05 ml of trimethylclorosilan (1%) and then subjected to chromatography.

Quantitative analysis of phytohormones was performed using gas-liquid chromatographic method and indole-3-acetic acid and gibberellic acid (Sigma) as internal standards, as described previously by Cavell et al., 1967 with modifications (Duca et al., 1997).

The chromatograph FRACTOVAP 4200 equipped with a detector of flame ionization, line programs for temperature MOD 410, integrator MEGA SERIES SP 4270, rustproof column (2m x 4mm) with 5% SE-30 DMCS Cromoton W, 60/80 mesh (0.15-0.2 mm) was used for analysis with gas carrier N_2 - 25 ml/min. Air flow was maintained at 300 ml/min, while hydrogen flow was 25 ml/min. The injector temperature was + 210^0C, and the detector temperature was +210^0C. The phytohormones were determined in the following temperature regime: after injection, the temperature was maintained at 60^0C for 4 min, then the temperature rate increase was 12^0C/min until the temperature of 220^0C was achieved. This temperature was maintained until the end of the analysis. The phytohormones content was expressed in ng per gram of fresh weight (ng/g fwt).

Data are presented as means ± SE (standard errors) of three separate experiments (n = 6 for each experiment) and Student's *t* test ($P< 0.05$and $P<0.09$) was used to determine the statistical significance of differences between genotypes.

RESULTS AND DISCUSSION

In our previous work, it was shown that the IAA and GA_3 content in vegetative and reproductive tissues was variable and depends on ontogenesis stages, organs and genotype (Duca and Port, 2002; Duca et al., 2003). But the ascertained changes of IAA and GA_3 concentrations are insufficient for revealing their functional role in male fertility-sterility phenotype expression. For this purpose, IAA/GA_3 ratio was analyzed in ontogenesis of sunflower plants using CMS-Rf system.

The highest IAA/GA_3 ratio was found in the cotyledon leaves with maximal values higher than 9 (Table 1). But, the RW 637 Rf line, in contrast to the all studied lines, had the minimal hormonal ratio – 7.9 resulting from a higher gibberellins content only, because no obvious genotypic differences in auxin content were observed.

Table 1. IAA/GA_3 ratio in leaves of different sunflower genotypes

Genotype	Stages of plant growth and development				
	Cotyledons	First leaves	Bud developing	Active growth	Blossoming
F_1	9.5	4.5	4.4	4.3	2.5
MB 514	9.7	3.6	3.9	3.5	2.5
MB 514 CMS	9.7	5.9	6.1	4.6	2.9
RW 637 Rf	7.9	3.3	2.9	2.8	3.8
SW 501 CMS	-	6.6	7.4	5.5	3.6

The IAA/GA_3 ratio was twofold decreased during the first true leaves stage and its values subsequently decreased in ontogenesis down to the lowest values (2.5-3.6) ascertained at blossoming stage. Heterozygote hybrid F_1 with restored male fertility and self-pollinate homozygote male fertile lines showed almost similar values of IAA/GA_3 ratio, while male sterile plants showed the highest hormonal ratio (Table 1).

The IAA/GA_3 ratio in inflorescences tissues, as in leaves, showed higher values at male sterile plants, while male fertile genotypes had nearly the same values of IAA/GA_3 ratio (Table 2).

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Table 2. IAA/GA_3 ratio in inflorescences of different sunflower genotypes

Genotype	Reproduction stages		
	Bud developing	Active growth of inflorescence	Blossoming
F_1	4.4	4.9	4.9
MB 514	4.1	4.4	5.0
MB 514 CMS	10.2	11.0	7.7
RW 637 Rf	3.3	3.9	5.1
SW 501 CMS	10.3	10.1	6.9

Significant results were observed for the RW 637 Rf line, which showed the lowest values of this ratio both in the leaves and in the inflorescences, assayed at the stages of bud development and active growth of inflorescence, when flower development and microsporogenesis occurred.

Similar features of the variable ratio were also determined for disc flowers (Table 3). Thus, during the flower development, the values of the studied index decreased in male sterile lines. But in comparison with male fertile genotypes, IAA/GA_3 ratio in sterile flowers was higher in archesporogenesis and sporogenesis phases. Meanwhile, in the following reproduction stage (carpogenesis) almost the same values of IAA/GA_3 ratio were found for all studied genotypes.

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Table 3. IAA/GA_3 ratio in disc flowers of different sunflower genotypes

Genotype	Microsporogenesis stages		
	Arhesporogenesis	Sporogenesis	Carpogenesis
F_1	4.7	5.0	5.1
MB514	4.1	4.8	5.0
MB514 CMS	8.9	6.6	4.6
MB514 + GA_3	6.9	6.0	5.0
RW637 Rf	5.4	5.1	4.5
SW501 CMS	11.7	7.4	4.4

A special interest related to physiological and genetic aspects of this study represents the variation of the IAA/GA_3 ratio at isogenic lines under exogenous gibberellins treatment (Table 4).

Table 4. IAA/GA_3 ratio in plants treated with gibberellins

Lines		Post-treatment period, hours						
		0	24	48	72		96	
					leaves	inflorescence	leaves	inflorescence
MB 514	control	4.5	4.8	4.5	5.1	4.6	4.9	4.6
	+ GA_3		5.4	5.1	5.9	4.9	5.7	4.7
MB514	control	6.1	5.6	5.3	6.0	4.6	6.6	5.6
CMS	+ GA_3		5.6	5.6	5.7	4.6	5.1	5.2

The GA3 exogenously applied at inflorescence bud developing stage increased the values of IAA/GA_3 ratio in leaves and inflorescences tissues of male fertile lines almost to the level of the values found for MB 514 CMS line. This increasing effect was significant after 72 and 96 hours post-treatment. The revealed variations in hormonal balance were not noticed for CMS plants (Table 4). In spite of these genotypes being considered hormone susceptible, the effect of GA-treatment on endogen IAA/GA_3 ratio was different. Similar effect of ``genotype correction`` to the normal hormonal status under exogenous phytohormone influence was also reported for several hormone metabolism mutants (Fadeeva et al., 1980).

The following analysis of IAA/GA_3 ratio at entire plant level (Fig. 1) provided the information on genetic and physiologic interactions in self-regulation of CMS-Rf system at sunflower (Fig. 1).

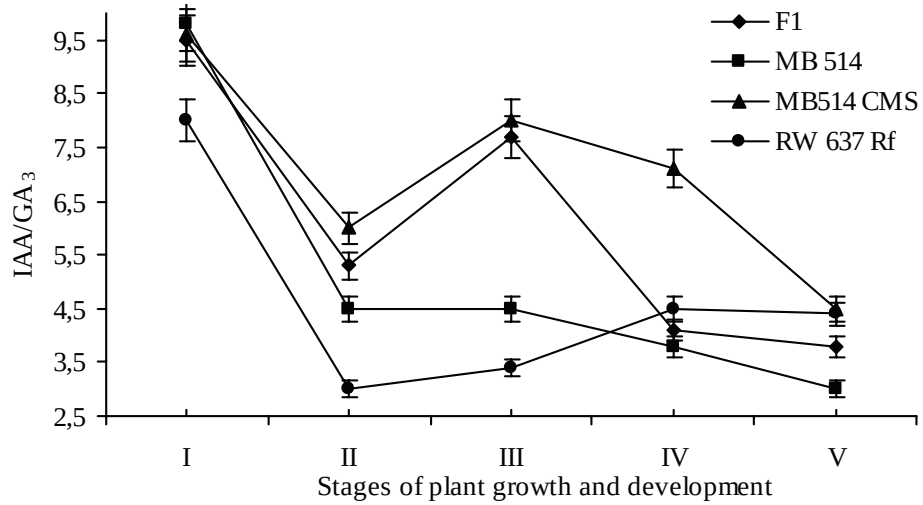

Fig. 1. Hormonal ratio of different sunflower genotypes at following stages of growth and development: I-cotyledons; II-first leaves; III- bud developing; IV- active growth; V – blossoming.

Thus, the most significant differences were revealed at the developing inflorescence bud stage. From the physiological point of view, this stage represents the stage of floral bud evocation and induction, because it was shown that microsporogenesis and microgametogenesis in sunflower occurs prior to the opening of inflorescence, when the diameter of the inflorescence bud reaches 2.5 – 4.5 cm (Smart et al., 1994). A high auxines/gibberellines ratio was ascertained at the stages of bud development (7.9) and active growth of the inflorescence (6.9) for the MB514CMS line, characterized by Srfrf genotype. A significantly lower ratio was observed for RW637 Rf (FRfRf) and MB514 (Frfrf), suggesting that the values of hormonal ratio of the CMS line are much higher than the optimal balance, which, according to our results, is approximately 4 for male fertile genotypes. The hybrid F_1 (SRf) contained sterile cytoplasm with nuclear Rf genes, which restore male fertility in homo- and heterozygote combination, resulting in the normalization of physiologic and biochemical processes in plants (Dmitreva et al., 1971). The fertility restorer gene presence in a genotype of these plants probably resulted in the IAA/GA_3 ratio decreasing at active growth and blossoming stages of reproduction development. Low values of this hormonal ratio are characteristic of fertile genotypes and high values are typical for sterile ones. The hormone ratio alterations observed at the critical stages of reproduction development, especially at microsporogenesis phases, reveal the phytohormonal mechanism of CMS-Rf genetic system control, because in F_1 the ratio of analyzed phytohormones is already restored at the next stage of the growth and development.

The hormonal balance and interactions between various plant hormones, as well as the cell capacity to receive the hormonal signal, play an important role in physiological spatial and temporal regulation of ontogenesis (Egorov et al., 1990; Braedford and Trewavas, 1994; Ross and Neill, 2001).

Our results have revealed the structural changes as a result of different auxins and gibberellins content and their ratio. Therefore, male sterile genotypes are characterized by a high IAA/GA_3 ratio. Also, the GA_3 treatment of fertile plants, resulting in phenotype male sterility, induced the increase in the IAA/GA_3 ratio, caused by the augmentation of endogen auxins and gibberellin amounts with a different intensity, which finally led to a ratio approximately similar to that in male sterile genotypes (Table 4).

It would seem that the hereditary cytoplasmic and GA-induced male sterility can be explained by the change in the phytohormone ratio and not in their concentration. It can be assumed that the phenotypic expression of the morphogenetic program, especially microsporogenesis realization, depends on the IAA/GA_3 ratio. The hormonal balance plays an essential role during the key stage of microsporogenesis (bud development and active growth of inflorescence).

These conclusions are sustained by the reported data. Thus, it was established that IAA/GA_3 regulates the primary differentiation of conductive fascicles, and, if this ratio is high, short phloem fascicles are developed (Roni et al., 1990). Also, it is well known that cytoplasmic and induced male sterility appear at the level of sporophyte tissues, because mononuclear microspores of the tetrads develop

normally up to the stage of binuclear pollen (Simonenko, 1982). This process is characterized by the break of interaction between the anther nests and parenchyma tissues of receptacle and an insufficient supply of nutritive substances (Frenchel, 1982), that finally cause tapetum tissue degeneration and the disruption of pollen formation (Roni et al., 1990).

ACKNOWLEDGEMENTS

We are grateful to Ph.D., Rotaru T. (SRC ``Magroselect``, Soroca, Republic of Moldova) for providing us with sunflower seeds and fruitful discussion during the investigation. The paper was written with support from Project Nr.4032 at Science and Technology Center in Ukraine.

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Genetic-phytohormonal interactions in male fertility and male sterility phenotype expression in sunflower (*Helianthus annuus* L.)

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ABSTRACT

Amounts of indole-3-acetic acid (IAA) and gibberellic acid (GA_3) have been investigated in vegetative and reproductive tissues of diverse sunflower genotypes using gas-liquid chromatography. Quantification of endogenous GA_3 content from homozygote MB514 line, characterized by cytoplasmic male sterility (CMS), revealed a lower level in comparison to the fertility restorer RW637Rf line, which contained a higher hormone quantity. The largest amount of IAA was found in the heterozygote F_1 hybrid obtained by crossing these lines, regardless of the tissues and ontogenesis phases analyzed. Similar features were found in leaves, apex, inflorescence, and disc flowers in most of the variants investigated.

Key words: auxins – CMS-Rf system – gibberellins – *Helianthus annuus* L. – male fertility – male sterility.

INTRODUCTION

Genetic CMS-Rf system (*cytoplasmic male sterility – fertility restoration of pollen*) is a well known and versatile phenomenon that has been the subject of many studies due to its importance in commercial hybrid breeding and heterosis (Vrânceanu and Stoenescu, 1971; Voscoboinik, 1977). Besides, this genetic system represents a useful model for revealing nucleus-cytoplasm interaction mechanisms in male sterility-fertility expression. It has been shown that CMS in sunflower is associated with mitochondrial gene *orfH522* (Laver et al., 1991; Horn et al., 1994) that can be suppressed in F_1 hybrids based on CMS by the action of nuclear-encoded fertility restorer Rf genes both in homo – and heterozygous condition (Vrânceanu and Stoenescu, 1971; Anascenco and Duca, 1985). Also, male sterility can be induced by gibberelic acid (GA_3) treatment of plants and the same class of phytohormones restores male fertility to sterile plants (Anascenco, 1971). These phenomena sustain the hypothesis that plant hormones regulate the nucleus and other cell structure activity by the induction or suppression cytoplasmic systems of genes expression (Collett at al., 2000). It is assumed that the phenotype expression of hereditary male fertility/sterility trait is regulated by the phytohormones.

In the present research, the quantification of IAA and GA_3 endogen levels has been studied in several sunflower genotypes during their ontogenesis to reveal the interaction between genetic (nuclear and mitochondrial) factors and phytohormones in CMS–Rf phenotype expression and how these relationships influence the physiological and biochemical basis of microsporogenesis. Also, we studied two functional states of male gametophyte (male sterility/fertility) in the same nuclear context, using the phenocopies method, to obtain information on nuclear influence on the mitochondrial genome expression related to the CMS- Rf genetic system.

MATERIALS AND METHODS

Plant materials

Two sunflower isonuclear lines distinguished only by cytoplasm genes (MB514 and MB514CMS with mitochondrial *orfH522*), the line RW637Rf with nuclear homozygote restoration nuclear gene *Rf*, and the F_1 hybrid obtained by cross between these lines (MB 514 CMS x RW637Rf) with restored male fertility (*Rf_*) were used in this study. The plants were cultivated in the experimental field of Moldova State University according to conventional technologies during four years. Sunflower seeds were kindly provided by SRC ``Magroselect`` (Soroca, Republic of Moldova).

The apex decapitation at two leaves stage is a good way to obtain phenocopies, known as being convenient models for functional activity studies of a gene. As a result of apical domination excluding, two lateral sprigs have grown, one of them was treated with exogenous gibberellic acid. Thus, two inflorescences, fertile and with induced male sterility, were obtained from the same sunflower plant.

Treatment with exogenous gibberellic acid

GA_3 (Sigma) solution was prepared by dissolving GA_3 in the minimal amount of ethanol 96%, and bringing it up to remaining volume in distilled water to make a final concentration of 0.005%. The treatment with GA_3 solution by plant spray was carried out at the developing inflorescence buds period. At this stage, prior to the opening of the inflorescence, male meiosis occurs in disc flower anthers (Anascenco, 1971). Non-GA_3 treated plants (control) were sprayed with distilled water. For assaying non-GA_3 treated plants (control) and GA_3 treated plants, 24 h post-treatment were used.

Phytohormones extraction. The plant materials were collected at various vegetative stages that were correlated with development and microsporogenesis. Phytohormones assays were performed on cotyledons, apex with true 2-3 leaves, inflorescences without bracts, and inflorescence flowers without parenchyma tissues of peduncles. Slicing was performed from radial to head to analyze the anthers at different developmental stages on the single inflorescence. Fresh plant material (about 10g) was harvested in the morning. The samples were homogenized and fixed in cold (-20^{o}C) 80% acetone (1:30 ratio) and extracted over-night at 3-5^{o}C during 24h. After a series of organic extractions and purifications the extracts were dried in vacuum at 40^{o}C. The residue was dissolved in 0.1 ml N,O-bis(trimethylsilyl)-acetamid with addition of 0.05 ml of trimethylclorosilan (1%) and then subjected to chromatography.

Chromatographic analysis. Quantitative analysis of phytohormones was performed using gas-liquid chromatographic method and indole-3-acetic acid and gibberellic acid (Sigma) as internal standards, as described previously by Cavell et al. (1967) with modifications (Duca et al., 1997).

The chromatograph FRACTOVAP 4200 equipped with a detector of flame ionization, line programs for temperature MOD 410, integrator MEGA SERIES SP 4270, rustproof column (2m x 4mm) with 5% SE-30 DMCS Cromoton W, 60/80 mesh (0.15-0.2 mm) was used for analysis with gas carrier N_2 at a flow of 25 ml/min. Air flow was maintained at 300 ml/min, while hydrogen flow was 25 ml/min. The injector temperature was 210^{0}C, the detector temperature was 210^{0}C.

The phytohormones were determined in the following temperature regime: after the injection, the temperature was maintained at 60^{0}C for 4 min, from then on the temperature rate increase was 12^{0}C/min until the temperature of 220^{0}C was achieved. This temperature was maintained until the end of the analysis. The phytohormone content was expressed in ng per gram of fresh weight (ng/g fwt).

Data are presented as means ± SE (standard errors) of three separate experiments (n = 6 for each experiment) and Student's *t* test ($P< 0.05$and $P<0.09$) was used to determine the statistical significance of differences between genotypes.

RESULTS AND DISCUSSION

Plant hormone metabolism and maintaining the levels of hormonal balance in appropriate temporal and spatial patterns influence the intensity, localization, structure and quality of all morphogenetic processes. The pathway of GA biosynthesis and catabolism and their physiological role has been investigated over many years by a variety of approaches, including the application of active GAs, chemical inhibitors of GA biosynthesis, and the analysis of mutants of plants such as maize, pea, and Arabidopsis (Kende and Zeevaart, 1997).

IAA and GA_3 are essential hormones that act synergetically on diverse developmental processes in plants (Ross and O'Neil, 2001); moreover, auxins stimulate the gibberellin biosynthesis (Symoons and Reid, 2002). Based on this information, quantitative analyses of the hormonal balance variation have been performed in some sunflower genotypes including hybrid F_1 and parents lines, during different ontogenesis stages. Our results have shown the quantitative variation of IAA and GA_3 levels depending on the plant tissues, development stages (Duca and Port, 2002) and environmental factors (Duca et al., 2003).

The most interesting data obtained were related to hormone amounts in different sunflower genotypes making up the CMS-Rf genetic system. Thus, hybrid F_1 was shown to contain the highest IAA amount versus RW637Rf, male fertility restorer line, which had the lowest hormone level. These features were found for apex, leaves and inflorescences (Table 1). Hormone levels in roots showed no significant quantitative variations between studied genotypes.

It is known that IAA induces DNA replication. The highest IAA level of F_1 associated with increased mitotic activity (Capatana, 2004) and with other morphological and physiological indices (Duca and Port, 2002) suggests a correlation between IAA amount and heterosis. It is also possible that the low IAA amount at homozygote RW637Rf line is the cause of the reduced height of these plants.

Table 1. IAA amount of different sunflower genotypes, ng/g fwt

Genotype	Plants number: *fertile* / *sterile*	Phenotype Genotype	Ontogenetic phases			
			The first pair of true leaves		Inflorescence bud developing	
			Roots	Apex	Leaves	Inflorescence
F_1	*76* / *0*	fertile S Rf	32.79 ± 0.12	70.98 ± 0.31	62.99 ± 0.64	81.64±0.29
MB 514 CMS	*2* / *58*	sterile S rfrf	30.47 ± 0.24	57.03 ± 0.05	60.15 ± 1.17	77.40±0.83
RW 637 Rf	*78* / *0*	fertile F RfRf	31.71 ± 013	50.41 ± 0.25	54.99 ± 4.70	61.05±1.86
LSD	*0.95*		*0.093*	*0.432*	*0.265*	*0.367*
	0.99		*0.140*	*0.654*	*0.401*	*0.556*

S – male sterile cytoplasm. containing mitochondrial *orfH522*; F **–** male fertile cytoplasm.

It is important to accentuate that the gibberellin level in all studied tissues and genotypes of sunflower was four-six fold less than IAA, as has been shown for maize (Polevoi, 1992). The highest GA_3 concentration was found in the male fertile genotypes, F_1 hybrid and the RW637Rf line that was distinguished by the increased biosynthesis during ontogenesis (Table 2). The intensity of phytohormones accumulation, expressed by harmonic mean, was also significantly higher for RW637Rf line than for F_1 and MB 514 CMS line (Duca, 1998).

Table 2. Gibberellin content at different sunflower genotypes, ng/g fwt.

Genotype	Plants number: *fertile* / *sterile*	Phenotype Genotype	Ontogenetic phases			
			The first pair of true leaves		Inflorescence bud developing	
			Roots	Apex	Leaves	Inflorescence
F_1	*76* / *0*	fertile S Rf	2.04 ± 0.05	16.94 ± 0.03	18.07 ± 0.21	14,3 ± 0,24
MB 514 CMS	*2* / *58*	sterile S rfrf	0.36 ± 0.03	11.82 ± 0.87	7.21 ± 0.63	9,50 ± 0,42
RW 637 Rf	*78* / *0*	fertile F RfRf	0.97 ± 0.04	17.40 ± 0.20	17.30 ± 1.03	17,40 ± 0,52
LSD	*0.95*		*1.868*	*0.493*	*1.050*	*0.709*
	0.99		*2.828*	*0.747*	*1.590*	*1.074*

S – male sterile cytoplasm. containing mitochondrial *orfH522*; F – male fertile cytoplasm.

The gibberellins level showed maximum values in roots and leaves of heterozygote plants and in apex and inflorescences of homozygote plants, but these differences were not statistically significant, because they are not reliable either for 0.95 nor for 0.99 probability levels.

Isogenic lines and phenocopies of sunflower are a good experimental genetic system for investigation of phytohormones interactions and their role in gene expression. Thus, the IAA level during the ontogenesis of three sunflower lines: MB514, MB514 CMS and MB514 treated with exogenous GA_3 showed lower values in the homozygote line with male sterility than its male fertile analogue, characterized by normal bisexual flowers with fertile pollen (Table 3). As a result of an exogenous hormonal treatment, the microsporogenesis was blocked, this phenomenon being associated with significant increases in IAA amount during the inflorescence buds' developing and active growth stages.

Also, it was found that the nucleic acid level (especially of RNA) and protein biosynthesis was increased (Duca, 1998; Duca and Savca, 1998). But by blossoming phase the auxin content and the above mentioned parameters decreased as their levels became lower than those found at CMS lines (Table 3). At this reproduction stage, CMS plants and those treated with gibberellins displayed abnormally developed anthers and lack of pollen.

Table 3. Auxin content of three isogenic sunflower lines, ng/g fwt.

Genotype	Plants number	Phenotype Genotype	Ontogenetic phases		
			Bud development	Active growth	Blossoming
			Apex Inflorescence	Apex Inflorescence	Apex Inflorescence
MB 514	*60*	fertile	60.57 ± 1.14	61.50 ± 0.92	49,53 ± 2,49
		F rfrf	75.80 ± 1.23	76.90 ± 2.26	85,40 ± 0,28
MB 514 CMS	*58*	sterile	58.53 ± 2.08	59.13 ± 1.16	48,00 ± 2,08
		S rfrf	73.20 ± 2.22	74.10 ± 1.02	85,00 ± 1,41
MB 514 +CA_3	*10*	sterile	60.77 ± 0.94	85.57 ± 1.28	45,50 ± 0,59
		F rfrf	75.57 ± 0.77	88.47 ± 3.21	83,80 ± 0,71
LSD	*0,95*		*0,120*	*0.526*	*0.138*
			0,064	*0.241*	*0.037*
	0,99		*0,182*	*0.797*	*0.210*
			0,097	*0.365*	*0.057*

S – male sterile cytoplasm, containing mitochondrial *orfH522*; F **–** male fertile cytoplasm

Our results have shown that the maximum GA_3 content was in apex and inflorescence tissues of MB 514 line, and also in plants exogenously treated with GA_3 in contrast to the lowest hormonal content ascertained at the cytoplasmic male sterile analogous MB514 CMS (Table 4).

Table 4. Gibberellins content in three sunflower isogenic lines, ng/g fwt

Genotype	Plants number	Phenotype Genotype	Ontogenetic phases		
			Bud development	Active growth	Blossoming
			Apex Inflorescence	Apex Inflorescence	Apex Inflorescence
MB 514	*60*	fertile	17.90 ± 0.14	18.13 ± 0.47	24.50 ± 0.14
		F rfrf	18.50 ± 0.33	16.26 ± 0.32	18.37 ± 1.18
MB 514 CMS	*58*	sterile	9.50 ± 0.42	12.7 ± 0.19	16.70 ± 0.45
		S rfrf	7.20 ± 0.57	6.70 ± 0.47	12.33 ± 0.43
MB 514 +CA_3	*10*	sterile	17.90 ± 0.09	20.43 ± 0.58	18.40 ± 0.19
		F rfrf	18.50 ± 0.33	16.80 ± 0.19	13.40 ± 0.52
LSD	*0.95*		*0.786*	*0.570*	*0.506*
			1.082	*1.051*	*0.573*
	0.99		*1.190*	*0.863*	*0.766*
			1.693	*1.591*	*0.868*

S – male sterile cytoplasm, containing mitochondrial *orfH522*; F **–** male fertile cytoplasm

During blossoming stage, the gibberellins quantity of GA_3 treated MB514 line decreased by approximately 30% in comparison to CMS analogue and by 20% compared to untreated MB 514. MB 514 CMS plants had low concentrations of this hormone compared to male fertile plants during all the ontogenetic phases studied. Gibberellins content boost at MB514 fertile line happened during ontogenesis stages, reaching higher levels at blossoming stage (24.5 ng/g fwt). Exogenous gibberellins application changed its internal concentration. Maximum values of endogen IAA and GA_3 content were determined at inflorescence development stage, 24 hours post treatment, and also during active growth phase.

Data on isogenic lines study provided us with more complete information related to auxin-gibberellin regulation of generative differentiation processes in sunflower. A comparative analysis of endogen auxins and gibberellins levels at different microsporogenesis stages (Fig. 1 and 2) revealed that phytohormone concentration decreased in disc flowers from the centre of the inflorescence to the periphery during the microsporogenesis in all studied genotypes.

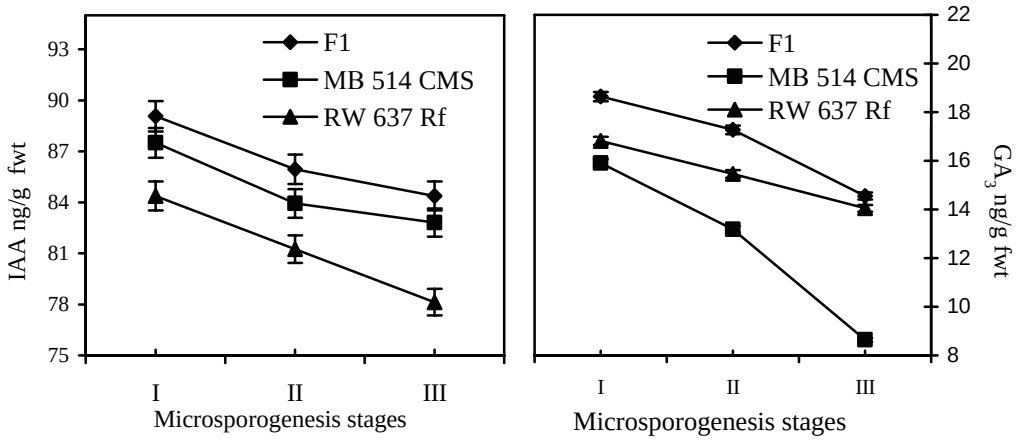

Fig. 1. Phytohormone levels in flowers at various microsporogenesis stages:
I – arhesporogenesis; II – sporogenesis; III - carpogenesis**.**

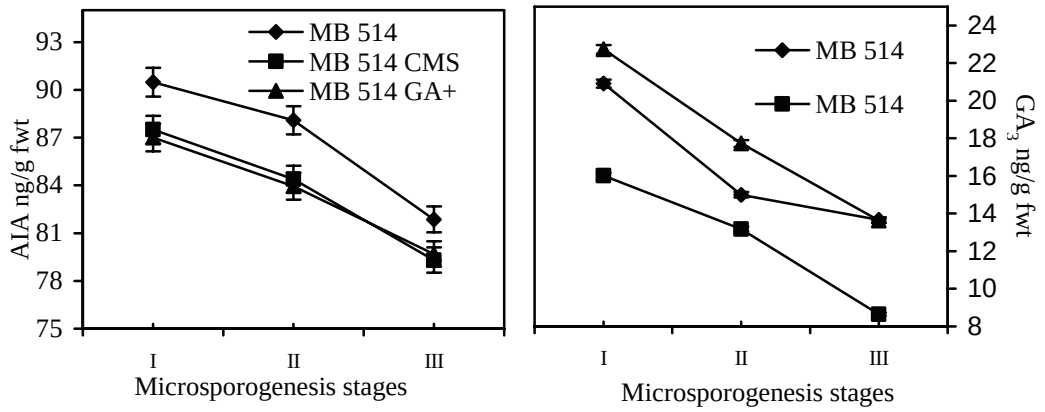

Fig. 2. Differences in phytohormone levels in flowers of three isogenic sunflower lines at following stages of microsporogenesis: I– arhesporogenesis; II – sporogenesis; III - carpogenesis

Our data support the findings related to the higher contents of both hormones in hybrid plants and the lowest IAA level in the male fertility restorer lines, but the lowest GA_3 level in the CMS plants.

Exogenous GA_3 treatment induced a decrease of the IAA concentration in disk flowers. The results obtained have shown no significant differences between plants with the male sterility induced and CMS plants. But the endogen gibberellin content in the treated genotype was higher than those detected in another two isogenic lines.

Besides the complex functional role of the studied parameters, the investigation of the IAA and GA_3 content in different sunflower genotypes during the ontogenesis has revealed several features regarding growth regulators levels and genetic CMS-Rf system. Thus, the outcomes showed a high level of GA_3 at RW 637 Rf line in comparison to other genotypes. MB 514 CMS line contained the lowest level of gibberellins, which increased during all the analyzed phases, even in disc flowers, where for RW 637 Rf line and F_1 a diminution in this hormone quantity was found. In fertile line MB 514 (as in other male fertile genotypes) there was a high auxin and gibberellin content during all studied phases in comparison to its male sterile analogue.

Genotypic peculiarities related to the auxin content were less considerable and less specific than those revealed by gibberellins content, which apparently verified their insignificant functional role in the phenotypic expression of CMS-Rf system. However, it could be supposed that a high gibberellin content is associated with restored male fertility, and a low auxin content with pollen sterility.

Evidence of the requirements of GAs in male reproductive development of flowering plants has resulted from genetic and physiologic studies of GA biosynthesis mutants. Typically, in addition to the dwarf stature, the GA-deficient mutants exhibit various defects of reproduction development (Kende and Zeevaart, 1997). Out of the majority of the plant growth regulators used as gametocydes (Frank et al., 1978), only gibberellins induce male sterility (Anascenco, 1971). These data together suggest that microsporogenesis development occurs normally at a sufficient level of GA. A low level of this hormone in the MB514 CMS line and a high level at RW 637 Rf line (and at all male fertile line) could support the hypothesis proposed. Also, these conclusions are sustained by the reported data that have shown that tomato sl_2 gene mutants (nuclear male sterility) contain a higher IAA and abscissic acid quantity but a lower gibberellin content (Santokh and Sowhneu, 1993).

Thus, it can be concluded that quantitative differences in auxins and gibberellins in various sunflower genotypes have revealed that self-regulation of the CMS-Rf system in sunflower is mediated by endogenous phytohormone concentration, depending on the genotype, ontogenesis phase and organ studied.

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Direct and indirect effects of morphophysiological traits on seed yield of sunflower (*Helianthus annuus* L.)

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ABSTRACT

In this paper, we have studied the interdependence of seed yield per plant and the following morphophysiological traits: total leaf number per plant, total leaf area per plant, plant height, total seed number per head, head diameter, weight of 1,000 seeds and oil content. Path coefficient analysis was used to separate direct and indirect effects of studied traits on seed yield, and to identify traits that could be used as selection criteria in sunflower breeding. The research was conducted during two vegetation seasons on 21 experimental sunflower hybrids, produced within the breeding program at the Institute of Field and Vegetable Crops. Among the large number of examined traits, significant and highly significant correlations were found. A strong positive correlation between the weight of 1,000 seeds and seed yield (0.791^{**}) was determined. On the contrary, a strong negative correlation between oil content and seed yield was found (-0.649^{**}). The biggest highly significant positive effect on seed yield was determined for the following traits: the weight of 1,000 seeds (0.789^{**}), total seed number per head (0.473^{**}) and total leaf number per plant (0.199^{**}). Total leaf area per plant has demonstrated a significant direct positive effect on seed yield (0.139^{*}). The weight of 1,000 seeds and total seed number per head were the most important traits for seed yield. Based on the coefficient of determination in F_1 generation (R^2=0.92), it can be concluded that the influence of all traits involved in the study on total variability of seed yield per plant, was 92%.

Key words: correlations – hybrids – path analysis – quantitative traits.

INTRODUCTION

The basic direction in sunflower breeding at the Institute of Field and Vegetable Crops, Novi Sad is the creation of hybrids with high genetic potential for seed yield (above 5t/ha) and the seed oil content (>50%) providing oil yield per hectare over 2.5 t (Miklič et al., 2008).

Breeding for high seed yield, components of seed yield and creating new sunflower ideotypes demands an increase of genetic variability of sunflower by interspecies hybridization (Škorić et al., 2007).

High seed yield and oil content are the two important criteria for introducing new hybrids in the production. These two traits, however, pose problems for breeders because they are both characterized by low heritability and affected by genotype x environment interaction.

In sunflower breeding for productivity, it is important to find morphophysiological traits, which are easy to score and, at the same time demonstrate a causal connection with the seed yield, and, therefore which could be used as selection criteria (Škorić et al., 2002). The mutual connection of seed yield with morphophysiological traits is often studied by the simple correlation coefficient analysis (Škorić, 1974; Marinković, 1992; Hladni, 2006). Since the simple correlation analysis cannot fully explain the relationships between characters, the path coefficient analysis is introduced for more successful breeding work. This type of analysis enables the partition of correlation coefficients to their components, which, in turn, allows the separation of a direct effect of one variable from indirect effects of other variables, thus giving a clear picture of the individual contribution of each variable to the seed yield.

Positive direct effects of total leaf area and plant height (Hladni et al., 2004), total seed per head (Gonzales et al., 2000) and weight of 1,000 seeds (Marinković, 1992; Gonzales et al., 2000) on seed yield were found. However, different results were obtained for the effects of the oil content on the seed yield. Positive direct effects (Chaudhary and Anand, 1993; Razi and Assad, 1999) as well as negative direct effects (Doddamani et al., 1997) of oil content on seed yield per plant were established.

In this paper, we studied mutual relationships between several morphophysiological traits on one side and seed yield on the other, as well as the direct and indirect effects of these components on seed yield of sunflower hybrids of CMS inbred lines originating from interspecies crosses.

MATERIALS AND METHODS

In this research, 21 experimental hybrids, developed by using new divergent (A) CMS inbred lines were used. Female inbred lines (NS-GS-1, NS-GS-2, NS-GS-3, NS-GS-4, NS-GS-5, NS-GS-6, NS-GS-7) developed from interspecies hybridization and restorer inbred lines with good combining characteristics (RHA-R-PL-2/1, RHA-N-49, RUS-RF-OL-168) were created at the Institute of Field and Vegetable Crops, Novi Sad.

The experiment was set up at an experimental field of the Institute of Field and Vegetable Crops at Rimski Šančevi, in a randomized complete block system with three replications, during the period of two vegetation seasons. The soil was characterized by 2.8% humus content, moderate content of phosphorus and potassium and pH 6.92 (Vasin et al., 2002).

The basic sample for analysis of the examined trait consisted of thirty plants (ten plants per replication) sampled from middle rows of each block.

Plants in the flowering stage were transferred to the laboratory and the total leaf number per plant (TLN), as well as the total leaf area per plant (TLA; cm^2/plant) were measured on the leaf area meter (LI-300-LiCOR, USA). At the stage of physiological maturity the plant height (PH) and head diameter (HD) were measured (cm) in the field. After the harvest, the seed yield (SY) produced in free fertilization for every single plant was measured by technical balance in the laboratory. The number of full seeds per head (total seed number-TSN) was determined by counting. On a random sample of completely pure and air dried seed the weight of 1,000 seeds (M1000S) was determined (g). The analysis of oil content (OC) in seed was carried out nondestructively on a nuclear magnetic resonance (NMR) analyzer. The determination of main values and the correlation coefficients (r) was carried out according to Hadživuković (1991). The strength and the direction of the correlation was determined according to the Roemer-Orphalov scale.

Mutual relationships of the examined characteristics and direct and indirect effects on seed yield were analyzed by the path coefficient analysis (Wright, 1921; Dewey and Lu, 1952). Statistical analysis was performed using Mstat C (1991) and SAS System Software (2003) programs.

RESULTS AND DISCUSSION

Knowing the mutual relationships between different yield components as well as the dependence of seed yield on different yield components is an important precondition for a successful application of suitable selection criteria in sunflower breeding. Presence or absence of correlations can contribute to the right choice of examined traits so as to enhance the efficiency of some selection criteria.

Positive highly significant interdependence between SY and M1000S (0.791^{**}), TLA (0.623^{**}), HD (0.446^{**}), TSN (0.369^{**}), is shown in (Table 1). Similar results of highly significant correlations between SY and: M1000S, TSN (Dagustu, 2002; Dušanić et al., 2004), TLA (Merrien et al., 1982; Joksimović et al., 1999; Hladni et al., 2001), and HD (Hladni et al., 2003; Mijić et al., 2006) were obtained by others.

Table 1. Phenotypic coefficient of correlation among analyzed traits

Trait		TLA	PH	HD	TSN	M1000S	OC	SY
		X2	X3	X4	X5	X6	X7	y
TLN	X1	-0.202^{ns}	0.566^{**}	-0.452^{**}	-0.075^{ns}	0.010^{ns}	0.168^{ns}	0.087^{ns}
TLA	X2		-0.161^{ns}	0.602^{**}	0.253^{*}	0.461^{**}	-0.461^{**}	0.623^{**}
PH	X3			-0.544^{**}	0.040^{ns}	0.220^{*}	-0.011^{ns}	0.199^{ns}
HD	X4				0.297^{*}	0.291^{**}	-0.589^{**}	0.446^{**}
TSN	X5					-0.164^{ns}	0.090^{ns}	0.369^{**}
M1000S	X6						-0.786^{**}	0.791^{**}
OC	X7							$-0,649^{**}$

** F test significance at level P<0.01 * F test significance at level P<0.05 ns- not significantly different

X1 total leaf number (TLN)
X2 total leaf area per plant (TLA)
X3 plant height (PH)
X4 head diameter (HD)
X5 Total seed number per head (TSN)
X6 Mass of 1000 seed (M1000S))
X7 Oil content (OC)
Y seed yield per plant (SY)

Highly significant negative interdependence was established between SY and OC (-0.649^{**}), which is in agreement with the research of Doddamani et al. (1997), and in disagreement with the research of Chaudhary and Anand (1993) and Razi et al. (1999).

There was no correlation between TLN and SY, which is in disagreement with the research of Chaudhary and Anand (1993), El-Hosary et al. (1999) and Dagustu (2002), who determined a positive and significant correlation of TLN and SY. Significant positive interdependence was not established

between PH and SY which was detected by others (Marinković, 1992; Hladni i sar., 2003; Mijić et al., 2006).

A positive highly significant interdependence was established between TLN and PH (0.566**); TLA and HD (0.602**); TLA and M1000S (0.461**); HD and M1000S (0.291**). The positive significant connection between TLA and HD was determined by Hladni et al. (2004).

Negative highly significant interdependence was established between M1000S and OC (-0.786**), HD and OC (-0.585**), PH and HD (-0.544**), TLA and OC (-0.461**), TLN and HD (-0.452**). These results are in agreement with the investigations of Punnia and Gill (1994) who determined the negative significant interdependence of M1000S and OC.

The correlation relations were further analyzed by using path coefficient analyses which include the involvement of correlation coefficients in direct and indirect effect on a specific trait (Table 2).

M1000S (0.789**) and TSN (0.473**) have the biggest positive effect on SY, which justifies the existence of a highly significant simple correlation and confirms that these two traits are important components of seed yield. These results are in agreement with the work of Marinković (1992) and Dušanić et al. (2004).

A positive direct influence of TSN on SY was also demonstrated by others (Škorić, 1974; Marinković and Škorić, 1988; Punia and Gill, 1994). A high direct effect of the M1000S on SY was noted both under good water supply conditions as well as under limited water conditions (Razi et al., 1999).

TLN has shown an important direct effect on SY. These results are in agreement with the research of Razi et al. (1999) and Nirmala et al. (2000), but are in disagreement with the results published by Marinković and Škorić (1988).

TLA has shown a positive significant direct effect on seed yield. These results are in agreement with the work by Alba et al. (1979).

Other traits in the investigation have shown a significantly lower direct influence, which means they had an indirect influence through other traits. The direct influence of HD and SY was not significant, which means that the HD had a high and indirect positive influence through M1000S and TSN. Different results were obtained by Green (1980) and Nirmala et al. (2000), who state that HD has a significant direct influence on SY, while according to Škorić (1974), Fick et al. (1974) and Hladni et al. (2004) that influence was negative.

Negative direct influences of OC on SY was not significant which means that OC had a high indirect influence and a negative one through M1000S.

Table 2. Path coefficient analysis of grain yield

Traits	Direct effects	Indirect effects via							r* SY
		TLN	TLA	PH	HD	TSN	M1000S	OC	
TLN	0.1990*		-0.0281	-0.0373	-0.0151	-0.0354	0.0079	-0.0038	0.082
T L A	0.1390*	-0.0402		0.0106	0.0201	0.1196	0.3637	0.0104	0.6232
P H	-0.0659	0.1126	-0.0224		-0.0182	0.0189	0.1736	0.0002	0.1988
H D	0.0334	-0.0899	0.0837	0.0358		0.1404	0.2296	0.0133	0.4463
TS N	0.4726**	-0.0149	0.0352	-0.0026	0.0099		-0.1294	-0.0020	0.3688
M1000S	0.7889**	0.0020	0.0641	-0.0145	0.0097	-0.0775		0.0178	0.7905
OC	-0.0226	0.0334	-0.0641	0.0007	-0.0197	0.0425	-0.6201		-0.6499

r*- Correlation coefficient

Determination coefficient: R^2= 0.918

The differences in acquired results can be explained by different plant material which the authors used in their research.

In sunflower breeding, attention should be paid to the ways in which the increase in morphophysiological components influences the SY.

In this research, with the increase in TLA, HD, M1000S ,SY also increased, but OC decreased. Similary the increase in HD led to the increase in TNL, M1000S and SY, and to the decrease in OC. The increase in TSN and M1000S would cause an increase in SY. In short, the increase in TLN, HD, TSN and M1000S influences the increase in SY.

Path coefficient analysis helped to separate direct and indirect effects of individual traits on SY and identify traits such as M1000S and TSN, which should be used as selection criteria in sunflower breeding.

CONCLUSIONS

A positive highly significant interdependence has been established between seed yield per plant and total leaf area per plant (0.623**), head diameter (0.446**), total seed number per head, (0.369**) and mass of 1000 seed (0.791**). Highly significant negative effect was established between seed yield per plant and oil content (-0.649**).

The path coefficient analysis applied gave a somewhat different picture from what the correlation analysis did. The path coefficient analysis partitioned the direct and indirect effects of the morphophysiological yield components on seed yield of sunflower. It allowed us to detect those components which exhibit the highest effect on yield expression. The data obtained in this investigation, as well as various literature data, indicate that the morphophysiological character: mass of 1000 seed, total seed number per head, total leaf number and total leaf area per plant are the main yield components which should be used as selection criteria in sunflower breeding .

The coefficient of determination (R^2) was 0.92 which indicates that the influence of all traits involved in the study affected 92% of total variability in seed yield per plant.

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Determination of maximum achene size in sunflower

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ABSTRACT

It has been suggested that the maximum volume of grain could determine its potential dry weight, and that grain water content can be used as a surrogate of grain volume, but this issue has not been investigated in sunflower. The aim of this work was to examine the relationships between final achene weight and the dynamics of achene volume and achene, pericarp and embryo water contents. Seven experiments were conducted between 2002-6 in Argentina, testing a total of 10 sunflower genotypes. Achenes were collected every 2-3 days, and the fresh and dry weights of the achene, pericarp and embryo, achene dimensions (length, breadth, width) and volume were determined. The pericarp played a dominant role in achene water content throughout grain filling. Maximum achene and pericarp water contents were reached early in development, and these maxima were good predictors of final achene and embryo dry weights across genotypes. Achene water content and volume showed parallel increases between anthesis and peak achene water content, but achene volume and dimensions continued to increase for a few days after the time of peak achene water content. We have concluded that the relationship between final achene dry weight and peak values for achene water content is not causal, and that the strong correlation between these variables arises because of the correlation between achene water content and volume during the phase where both variables increase together and the fact that, at the time of peak achene water content, the achene has reached close to 90% of its final volume.

Key words: achene dimensions – achene size – pericarp – sunflower – water content.

INTRODUCTION

In sunflower, as in other grain species, dry matter accumulation in the achene is related to achene moisture dynamics, and physiological maturity for sunflower is achieved with a grain water concentration of 38% (Rondanini et al., 2007). It has been suggested that maximum grain water content may be used as surrogate for maximum grain volume and that maximum grain volume may limit maximum final grain dry weight (Borrás et al., 2004). The relationship between maximum achene water content and achene final dry weight has not been explored for sunflower. Because pericarp maximum dry weight in sunflower is achieved well before maximum embryo dry weight during grain filling (Mantese et al., 2006), and if the pericarp represents a physical restriction to the growth of whole achene, maximum pericarp volume could determine the final achene size across different sunflower genotypes. The ease (relative to cereal grains) with which pericarp of the growing sunflower achenes can be separated from its contents increases the possibility of studying this issue.

The aim of this work was to relate final sunflower achene weight to the dynamics of achene volume, achene dimensions, and achene, pericarp and embryo water contents.

MATERIALS AND METHODS

Seven experiments were conducted between 2002-6 at FAUBA (Buenos Aires) and the Advanta Semillas Biotechnology Centre (Balcarce). Ten genotypes were evaluated, having contrasting final achene size (30-105 mg achene^{-1}) and pericarp proportion (17-35 %). They included inbred lines (HA89, IM9), white striped hybrids (Paraiso30, M734, CF19, CF29) and black striped hybrids (Paraiso20, VDH488, Aguara, DK4050). Experimental units were individual plants, and each experiment had three replicates. Starting at anthesis, achenes were collected every 2-3 days, and their volume (in genotypes DK4050 and CF29 only), dimensions (length, breadth, width, in genotypes Paraiso30, HA89, DK4050, and CF29 only), fresh weight and dry weight (all genotypes) were determined. Water content in achene, pericarp and embryo was calculated as the difference between the respective fresh and dry weights.

RESULTS AND DISCUSSION

Pericarp and embryo contributions to whole achene water content dynamics

The dynamics of dry and fresh weight, as well as those of water content and concentration for the whole achene, pericarp and embryo exhibited consistent patterns across genotypes and environments, and are illustrated for the genotype CF19 in Fig. 1. Achene and pericarp fresh weight increased rapidly during early grain-filling, to fall after achieving peak values, while embryo fresh weight increased until physiological maturity (Fig. 1B). The pericarp was the dominant component of whole achene water content right up to physiological maturity (Fig. 1C), and exhibited a higher water concentration than the embryo throughout, even after achene water concentration had fallen to harvest maturity values (i.e., ca. 17%, Fig. 1D). Sunflower achene water dynamics (Fig. 1 C) contrast with those of wheat (which show an extended plateau) and soybean (which achieve maximum water content very close to physiological maturity) grains (Egli, 1998).

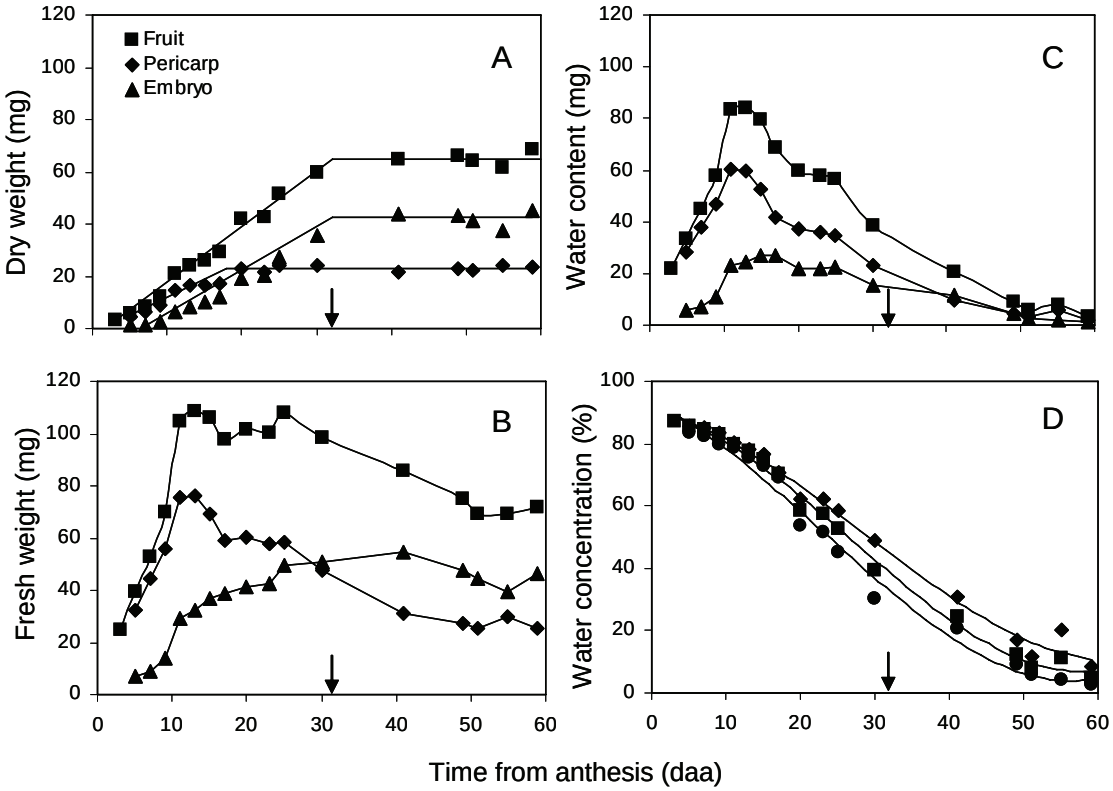

Fig. 1. Post-anthesis dynamics of whole achene, pericarp, and embryo dry weights (A), fresh weights (B), absolute water contents (C), and water concentration (fresh weight basis, D) for achenes from the peripheral position on the capitulum of hybrid CF19 (Exp. 1). Fitted functions are bi-linear (A) and cubic polynomials (D). Arrows on the "x" axis indicate PM. Each datum point is the mean of three replicates.

Averaged across genotypes, maximum pericarp water content was 1.9 times greater than maximum embryo water content, in spite of the fact that average embryo final dry weight was 3 times greater than average pericarp final dry weight (data not shown). Across genotypes, 62-78 %of the water in the achene was contained within the pericarp at the time of whole achene maximum water content (Table 1). This was unexpected, given that the pericarp constituted between 17-35 % of the final achene dry weight and, even at achene maximum water content, embryo dry weight was beginning to approach that of the pericarp.

Recently it has been shown that the achene dry weight/water concentration relationship (in both absolute and relative to maximum dry weight terms) for sunflower can be described using a trilinear relationship, in which the first section (for achene water concentrations in the 90-80% range) has a much steeper slope than the subsequent section (Rondanini et al., 2007). The slopes of the first two sections of the corresponding trilinear relationship for maize show the opposite behaviour (Borrás and Westgate, 2006). In this initial phase of the grain-filling process, most of the water in the achene is found in the pericarp. Thus, pericarp dominance of achene water content dynamics underpins the contrast between the

grain dry weight/water concentration relationships of sunflower and that of other grains like maize (Borrás and Westgate, 2006) and true seeds such as soybean (Egli, 1998).

Table 1. Achene water and dry weight contents at the time of achene maximum water content and proportions (%) of these water and dry matter contents present in the pericarp (P) and embryo (E). Values are means of three replicates.

Genotype	At the time of maximum achene water content					
	Achene water content			Achene dry weight		
	(mg)	Proportion in		(mg)	Proportion in	
		P	E		P	E
P30	140	65	35	30	58	42
M734	133	74	26	31	70	30
CF19	86	72	28	22	67	33
P20	83	75	25	18	71	29
VDH	80	74	26	19	67	33
Aguara	72	73	27	18	67	33
HA89	59	62	38	17	66	34
M734i	60	78	22	14	79	21

Associations between final achene and embryo dry weights and achene and pericarp maximum water contents

Achene final dry weight showed strong associations (r= 0.95) with achene and pericarp maximum water contents (Fig. 2). Given the dominant contribution of the pericarp to achene maximum water content (Table 1), the strong association between final achene dry weight and pericarp maximum water content (Fig. 2), and the fact that maximum pericarp dry and fresh weights are achieved before those of the embryo contained within the pericarp (Fig. 1), we also tested the association between embryo final dry weight and pericarp maximum water content. We found a strong relationship (r= 0.92) between these two variables (Fig. 2). Relationships between achene and pericarp final weights and their respective maximum fresh weights were also strong (data not shown).

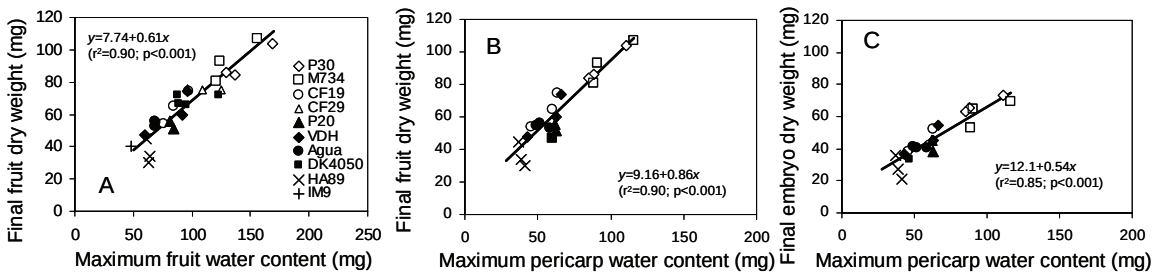

Fig. 2. Relationship between final achene dry weight and achene maximum water content (A), pericarp maximum water content (B), and relationship between final embryo dry weight and maximum pericarp water content (C) for 10 sunflower genotypes.

Dynamics of achene volume and achene dimensions

In the genotypes for which achene water content, volume and dimensions were followed simultaneously, achene water content reached its maximum value early during the development, some time before achievement of maximum achene volume (as illustrated for DK4050 in Fig. 3A). Achene volume peaked at 18 daa (Fig. 3B) and then decreased slightly. Maximum achene length was achieved before the maxima for other dimensions of the achene, whereas maximum achene breadth was determined later during the development, and matched to the timing of maximum achene volume (Fig. 3A and 3B).

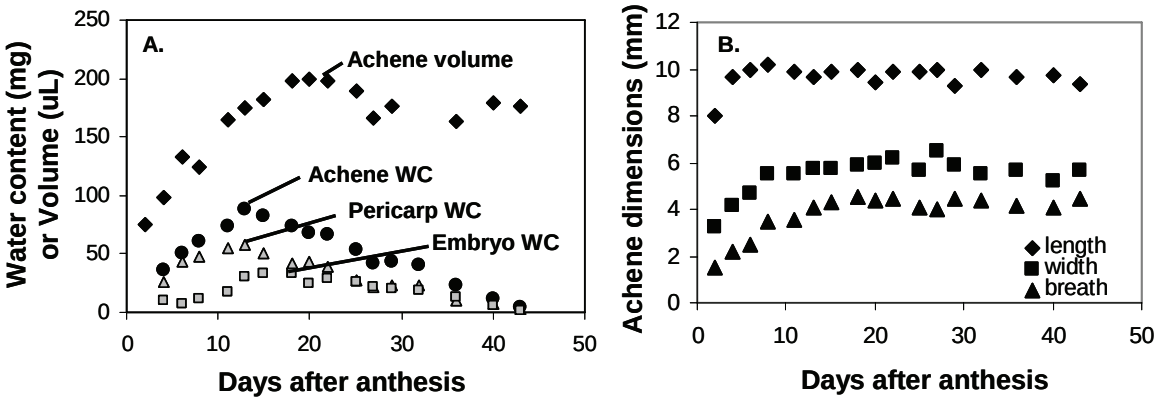

Fig. 3. (A) Dynamics of achene, pericarp and embryo water content (WC) and achene volume; and (B) dynamics of achene dimensions, in sunflower genotype DK 4050.

Plots of the variables shown in Fig. 3 in relative (to the respective maxima) terms against time from anthesis (Fig. 4) showed a strong parallelism for the trajectories of achene relative water content, relative volume and pericarp relative water content. At the time of peak achene and pericarp water contents, achene volume had reached 88-90% of its peak value (Fig. 4A). Achene dimensions showed different trajectories, with achene breadth being the last to achieve its peak value (Fig. 4B), coinciding with peak achene volume.

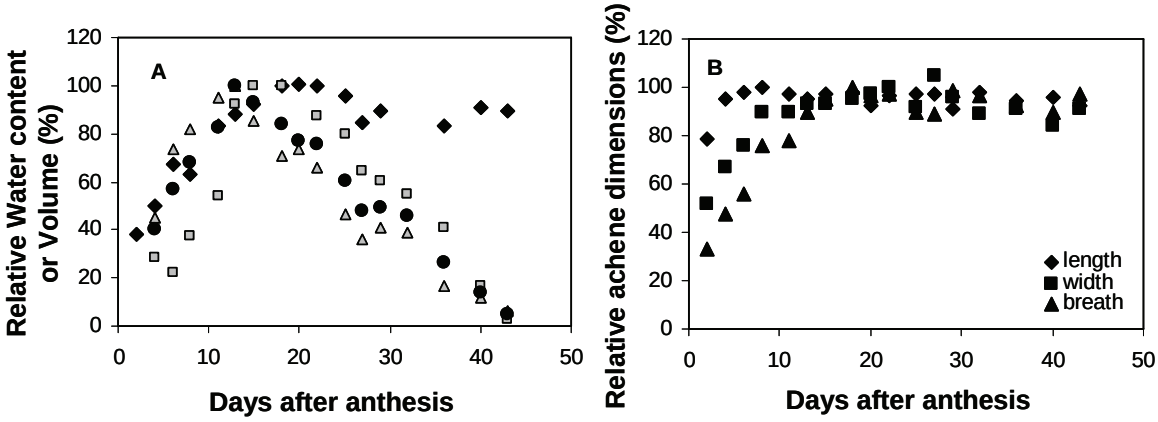

Fig. 4. (A) Relative (to the maximum value for each variable) dynamics of achene, pericarp and embryo water content (symbols as in Fig. 3 A); and (B) achene dimensions. Data for the hybrid DK 4050.

Achene volume and achene water content relationship

During the anthesis (maximum achene water content phase), volume and water content were positively associated (Fig. 5), but the relationship was not 1:1, indicating that achene volume is not all occupied by water.

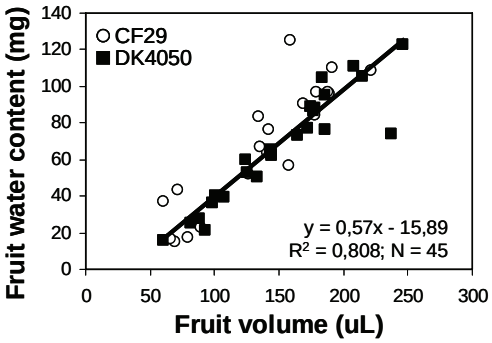

Fig. 5. Achene water content/volume relationship (for the anthesis/maximum WC phase) in two sunflower genotypes.

We conclude that, in sunflower, maximum achene water content is reached early in the development and that pericarp water content dynamics play a dominating role in achene water content dynamics (Fig. 1, Table 1). Differences in grain water content dynamics between sunflower and other grain crops (cereals, soybean) are probably due to a lesser importance of the pericarp in the structure of these latter grains. Because maximum achene water content and volume are determined fairly early during grain filling (e.g., over 60% of peak volume is achieved 10 days after anthesis, Fig. 4), achene capacity to compensate in size for early exposure to adverse environmental conditions later during grain filling may be limited, in contrast to that of other species such as soybean (Egli, 1998). Peak values for both achene and pericarp water content are good predictors of final achene and embryo sizes across genotypes (Fig. 2), However, the association between achene water content and volume (Fig. 5) only holds for the interval during which both variables increase in parallel (Fig. 4), and is clearly not causal, suggesting that not all the fruit volume is filled with water during the first phase of grain-filling. We speculate that the effectiveness of peak achene and pericarp water contents as predictors of final achene dry weight (Fig. 2) arises from the fact that peak values for the former two variables are achieved when achene volume has reached about 90% of its peak value (Fig. 4) and because achene volume is strongly associated with achene water content (Fig. 5). Complete resolution of the determinants of final achene size probably requires a study of the dynamics of pericarp epidermis cell division and expansion and of the contribution which cell destruction on the inner face of the pericarp during grain filling makes to the volume available for embryo growth.

ACKNOWLEDGEMENTS

We thank Rafael Mac Donough for allowing us to harvest achenes from the genotypes included in his experiments; and Alberto León and Mónica Morata for their help at Advanta-Balcarce. This research was financed by grants FONCyT 08-5264 and UBA G-048. DPR was in receipt of a UBA fellowship. A.J.H, D.M. and D.R. are members of CONICET, the national research council of Argentina.

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Abscisic acid content of a nondormant sunflower (*Helianthus annuus* L.) mutant

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ABSTRACT

A sunflower (*Helianthus annuus* L.) mutant was observed in the progeny of a cross between the sunflower cultivar HA 89 and an amphiploid of a *H. divaricatus* L. × P21 cross that exhibited loss of dormancy induction in the developing embryo. Seeds of this mutant frequently germinate on the head about 40 d after pollination (DAP). In contrast to other nondormant sunflower mutants reported previously, the cotyledons of this mutant remain green, whereas other nondormant mutants exhibit loss of pigmentation. The objective of this investigation was to compare the level of abscisic acid, a plant hormone that induces dormancy in developing embryos, in the nondormant green mutant (*ndg*) and HA 89 from which *ndg* was derived. Immunoassays showed that abscisic acid was present in *ndg* and the levels decreased from a maximum at 5 to 20 DAP to basal levels at 25 DAP. The levels of abscisic acid were not significantly different from those in the control plant HA 89. We conclude that the nondormancy trait is due to a mutation that renders *ndg* insensitive to abscisic acid.

Key words: abscisic acid – *Helianthus annuus* – mutant – nondormancy – sunflower.

INTRODUCTION

Seed dormancy is a physiological strategy evolved by plants to ensure survival of the species. A dormant state prevents germination into a temporarily favorable, but unstable, environment that could become adverse shortly after germination and lead to plant death. Of the several types of dormancy, physiological dormancy is the most common mechanism that has evolved and is present in both gymnosperms and angiosperms. It can occur at a deep, intermediate, or non-deep level (Baskin and Baskin, 2004).

Cultivated sunflower seeds (*Helianthus annuus* L.) undergo a Type 2 non-deep physiological dormancy period (Baskin and Baskin, 2004). In Type 2 dormancy, seeds initially have greater germination potential at higher temperatures, but gradually improve their ability to germinate at lower temperatures during the progression from dormancy to nondormancy. Abscisic acid (ABA) is a known inducer of dormancy in sunflower as it is in many plants (Le Page-Degivry and Garello, 1992). Dormancy in sunflower seed can be broken by application of gibberellic acid or ethylene, by cold stratification, or by excision and culture of the embryo on appropriate medium (Fick, 1978; Corbineau et al., 1990; Jridi et al., 2004).

An albino sunflower mutant, *nd-1*, that exhibited loss of seed dormancy was previously reported by Fambrini et al. (1993). The mutant was found in the selfed progeny of an *in vitro*-regenerated plant and displayed visibly reduced pigmentation by carotenoids. Analysis showed that a defective ζ-carotene desaturase caused the loss of pigmentation (Conti et al., 2004). Because carotenoids are precursors of ABA, altered ABA biosynthesis was likely responsible for nondormancy in *nd-1*.

Within our sunflower germplasm enhancement program, we recently identified a nondormant sunflower mutant that occurred during an interspecific gene transfer from a wild *Helianthus* species into cultivated sunflower to find resistance to the newly evolved broomrape (*Orobanche cumana* Wallr.) race F in Spain. Resistance genes were found in an interspecific cross with the pedigree of *H. divaricatus* 830/P21 amphiploid//P21/2/HA 89. In 1999, a single plant among the sib-pollinated progeny with 2n=34 chromosomes of this pedigree was observed to have seed germinated on the head. The amphiploid *H. divaricatus* 830/P21 has 2n=68 chromosomes; therefore, it took several backcrosses and sib- or self-pollinations to reduce the 2n chromosome number to 34, the same as cultivated sunflower, while continuing to monitor the broomrape resistance. Continued self-pollination maintained the nondormancy trait until F_{14}, and one homozygous F_{14} nondormant line was selected in 2003 for this study.

In this mutant, dormancy was not induced in the developing embryo. Instead, developing seeds of the mutant sunflower began to germinate in the head about 40 d after pollination (DAP). This nondormant mutant differed from *nd-1* in that pigmentation appeared normal. Hence, we use the term *ndg* to describe this *nondormant green* sunflower mutant. Because ABA is known to induce dormancy in physiological dormancy, we investigated the levels of ABA in the developing seeds of *ndg* at various stages after

pollination. When the ABA levels in *ndg* were compared to those in HA 89, which is in the pedigree of *ndg*, we found that the ABA content of *ndg* was not significantly different from HA 89.

MATERIALS AND METHODS

Plant material

Both HA 89 and the mutant *ndg* sunflower were grown in the greenhouse (16-h light) and self-pollinated. After pollination, developing seeds of HA 89 and *ndg* were removed from the head of a single plant at 5, 10, 15, 20, 25, 30, 40, 50, and 60 DAP. At each harvest date, 20 to 40 achenes of *ndg* and 25 to 50 achenes of HA 89 were removed from the head and stored at -80° C prior to ABA analysis.

ABA determination

ABA content was determined in both mutant *ndg* and HA 89 achenes. The frozen achenes from each harvest date were thawed and the hulls separated from the kernels when possible. Both the hulls and kernels were weighed and placed in a desiccator (Moisture Gone desiccant, Hiatt Distributors, Ltd., Long Beach, CA, USA)[1] and allowed to dry overnight. For each sampling date the hulls and kernels were weighed separately and then the hulls or kernels from each were pulverized in liquid nitrogen using a mortar and pestle and extracted with 4 mL of 80% (v/v) aqueous acetone using a Polytron homogenizer. After evaporation of the acetone with a stream of nitrogen and extraction of oil with hexane, the homogenate was diluted with 5 mL of 1 M formic acid and partially purified by separation on an Oasis SepPak (Waters, Milford, MA). The ABA was eluted with 5 mL of methanol and the eluate was evaporated to dryness using a stream of nitrogen. The samples were reconstituted in 1.0 mL of Millipore-purified water. Dilutions of 10× or 100× in Millipore-purified water were used for immunoassay. Samples containing ABA were subjected to quantitative analysis for ABA content by an enzyme-linked immunosorbant assay (Suttle and Hultstrand, 1994; Walker-Simmons, 1987). The (±)ABA used for the standard curve was purchased from Sigma (St. Louis, MO, USA) and the (±)ABA concentrations were doubled for calculation of the physiologically active (+)ABA racemate. (+)ABA concentrations in the embryos and the hulls were expressed as nmol·g dry wt^{-1}. ABA analyses from at least three different plants were conducted for the time points between 5 and 25 DAP, and two ABA determinations were typically made for 30 to 60 DAP. Between 5 and 15 DAP, the ABA content of *ndg* was determined on the whole achene because the embryos were too small to be successfully separated from the hull. From 20 DAP and later, the ABA contents in the embryos and hulls were determined separately.

RESULTS

ABA levels in ndg mutant and HA 89 sunflower

The quantitation of ABA levels at the early stages of seed development showed high variability among samples, likely due to the small size of hulls and embryos and varying stages in initiation of development. Both HA 89 and the *ndg* mutant showed similar patterns of ABA levels in the achenes during the period following pollination (Fig. 1). ABA concentrations in each declined during achene development until basal levels were reached by 30 DAP. We detected a slightly higher ABA concentration in HA 89 compared to *ndg* at 15 DAP, but the differences were otherwise not statistically significant. For both HA 89 and mutant *ndg*, the concentrations of ABA in the hulls and embryos were the same (Fig. 2). By 40 DAP, the developed seeds of *ndg* typically began to germinate on the sunflower head.

DISCUSSION

Sunflower normally undergoes a dormancy period during and following seed development. The length of dormancy is dependent on cultivar and on the environment. Under normal conditions at room temperature, gradual dormancy release for cultivated sunflower typically begins about 45 to 50 DAP (Fick, 1978). For wild sunflower species, the dormancy period is often much longer and highly variable in length.

[1] Mention of trade names or commercial products in this paper is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

Several studies on sunflower dormancy have been reported. By inhibiting ABA synthesis with fluridone, Le Page-Degivry et al. (1990) demonstrated that sunflower seed dormancy was dependent on ABA synthesis, but that dormancy induction was not concomitant with ABA accumulation. Their results for developing seeds showed an increase in embryo ABA content that reached a maximum at 13 DAP and then decreased to low levels by 25 DAP. They concluded that ABA induced embryo dormancy during seed maturation.

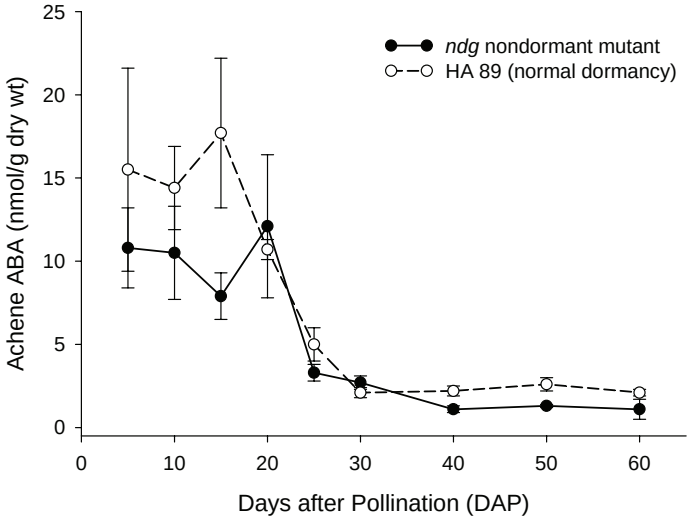

Fig. 1. ABA content (dry weight basis) of achenes of sunflower mutant *ndg* (●) and HA 89 (○) cultivated sunflower at various stages of achene development. Data are means of 3 or more individual plants for 5 to 25 DAP, and of 2 individual plants for 30 to 60 DAP ± SE.

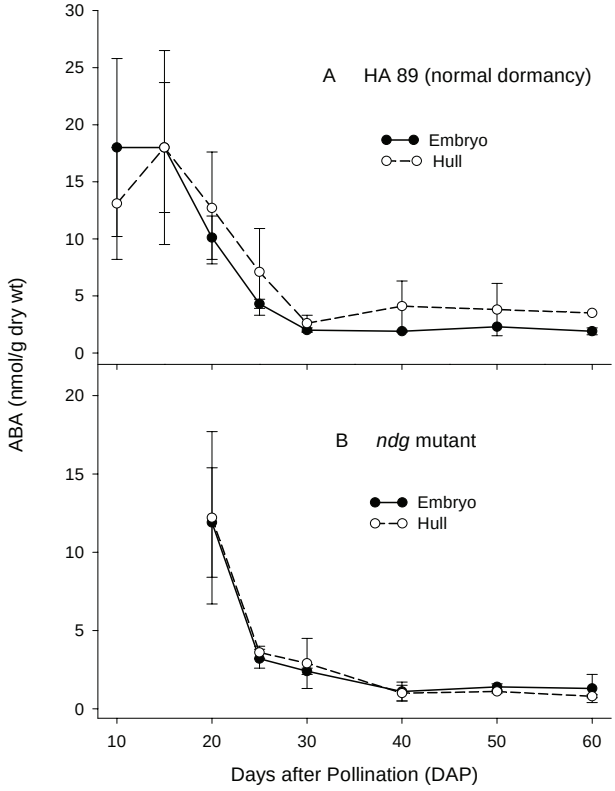

Fig. 2. ABA content (dry weight basis) of embryos (●) and hulls (○) in A) cultivated sunflower HA 89 and B) mutant *ndg* sunflower. Data are means of 3 or more individual plants for 5 to 25 DAP, and of 2 individual plants for 30 to 60 DAP ± SE.

Because expression of total ABA content in an embryo is dependent on the size of the embryo, we avoided using total ABA per embryo. Instead, we expressed ABA content on a dry weight basis of the whole achene so that equivalent comparisons could be made between ABA concentrations in the *ndg* mutant and HA 89. Our results clearly showed the presence of ABA during early achene development in both HA 89 and the *ndg* mutant (Fig. 1). The differences in ABA content between the *ndg* mutant and HA 89 were not statistically significant at most stages of achene development.

We believe that the nondormancy observed in the *ndg* mutant is due to loss of sensitivity to ABA. In our terminology, loss of sensitivity is used in the broad sense to include a defect in any component of the dormancy induction mechanism. A mutation in the ABA receptors that results in reduced affinity to ABA, or a mutation in any of the proteins involved in ABA signal transduction could lead to impaired transcriptional activation of ABA-inducible gene expression. Koornneef et al. (1984) have reported a similar ABA-insensitive mutant (*abi-3*) of *Arabidopsis thaliana* that is green and exhibits nondormancy that also is not reversed by exposure to ABA.

While the synthesis of ABA in the mutant *ndg* embryo appears to be normal, or at least near normal, we cannot rule out the effect of a slightly reduced capacity for ABA synthesis. White et al. (2000) proposed that an adequate ABA:GA ratio is critical for suppression of germination and induction of dormancy, rather than the absolute amounts of the two hormones. In the case of *ndg*, it may be that a slightly reduced content of ABA leads to a ratio shift in favor of GA, and the result is a failure to induce dormancy at a critical time during embryo development. Indeed, Fong et al. (1983), in a study on maize vivipary, proposed that there is a narrow window of embryo development in which ABA is able to induce dormancy. Accelerated catabolism of ABA during after-ripening or a reduced rate of seed desiccation might also result in loss of dormancy.

We did not investigate these alternative possibilities for the observed nondormancy of *ndg*. The aim of this study was to examine whether ABA synthesis was altered in seeds of the nondormant mutant. Our results showed that ABA levels in the mutant *ndg* were the same as in the control line HA 89. Thus, we believe that the *ndg* mutant is defective in the signaling pathway of ABA recognition and subsequent induction of gene expression leading to dormancy.

Introduction of the nondormancy trait into a breeding program could be a useful tool for sunflower breeders. We have determined that nondormancy in the *ndg* mutant is under the control of a dominant gene(s) (unpublished), and if introduced during development of a germplasm line could be a useful tool to advance generations quickly. Use of the nondormancy trait could circumvent the utilization of embryo rescue techniques to avoid the normal dormancy period between germplasm generation advances. Seeds could simply be transferred from the sunflower head directly to large pots without the need for chemical treatment to break dormancy. Finally, the nondormancy trait could be eliminated in the last phase of germplasm line development by selection for segregating lines having normal seed dormancy.

ACKNOWLEDGEMENTS

The technical assistance of Lisa Brown for greenhouse maintenance of the plants is greatly appreciated. We also thank Linda Huckle for assistance with the abscisic acid immunoassay.

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Producción de girasol (*Helianthus annuus* L.) en valles altos de México

Sunflower (*Helianthus annuus* L.) production at México highlands

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RESUMEN

El girasol (*Helianthus annuus* L.), por su uso integral, es un recurso genético potencial para valles altos, donde los cambios en la producción están ligados al manejo del cultivo y las condiciones ambientales. El objetivo de este estudio fue determinar la variación de la fenología, biomasa total, rendimiento de aceite y semilla, contenido de aceite de la semilla y su relación con factores e índices climáticos durante 1992 a 2001. El cv. Victoria de girasol se sembró en Montecillo México (clima BS1 y 2240 m de altitud) bajo condiciones de temporal, a la densidad de 5 plantas m^{-2} en surcos a 80 cm y con orientación este-oeste en las fechas siguientes: (I) 20/5/1992; (II) 01/5/1995; (III) 18/5/1996 ; (IV) 13/5/1997; (V) 01/6/1998; (VI) 26/5/1999; y (VII) 22/5/2001. La fenología mostró cambios debidos a fecha de siembra. El rendimiento de aceite mostró mayor variabilidad (cv=45%), seguido del de semilla (cv=40%), la biomasa total (cv=20%),el contenido de aceite de la semilla (6%) y la radiación fotosintéticamente activa (RFA, cv=8%), la evapotranspiración de cultivo (ETc, cv=6%) y las unidades calor (cv=4%), siendo en promedio de 100 g m^{-2}, 256 g m^{-2}, 1,224 g m^{-2}, 38%, 1,111 MJ m^{-2}, 360 mm, y 1402 UC, respectivamente. Los cambios en la biomasa total pueden estimarse mediante la ETc, y el rendimiento de semilla mediante la ETc, RFA y la humedad relativa mínima.

Palabras clave: biomasa – contenido de aceite - evapotranspiración - rendimiento - unidades calor.

ABSTRACT

The integral use of the sunflower (*Helianthus annuus* L.) is an important potential genetic resource for the highlands, where crop production changes are linked to management practices and environmental conditions. The aim of this study was to determine the phenology, total biomass, seed yield, oil yield and seed oil content of sunflower and their relation with climate factors and indices from 1992 to 2001. The sunflower cv. Victoria was sown in Montecillo Méx (climate BS1 and 2240 m of altitude) in the following dates: (I) 20/5/1992; (II) 01/5/1995; (III) 18/5/1996; (IV) 13/5/1997; (V) 01/6/1998; (VI) 26/5/1999; y (VII) 22/5/2001. The phenology was affected by sowing date. The oil yield variability was the highest (cv=45%), followed by seed yield (cv=40%), total biomass (cv=20%), oil seed (cv=6%), the radiation photosynthetically active (PAR, cv=8%), heat units (cv=4%) and the evapotranspiration (ETc, cv=6%) with mean values of 100 g m^{-2}, 256 g m^{-2}, 1224 g m^{-2}, 38% , 1111 MJ m^{-2}, 1402 UC and 360 mm, respectively. Total biomass changes can be estimated by the ETc, and seed yield by the ETc, PAR and minimum relative humidity.

Key words: biomass – evapotranspiration – heat units – oil seed - yield.

INTRODUCCIÓN

El girasol (*Helianthus annuus* L.) es un recurso genético cuyas semillas son ricas en aceite para consumo humano (como aceites para usos culinarios, elaboración de margarinas y confitería). Tiene también usos industriales (producción de ceras, fosfatina, lecitinas y tocoferoles), ornamentales y para alimento del ganado (Alba y Llanos, 1990) y aves. Además de uso potencial como biocombustible. Se estima que por hectárea se pueden producir 890 L de biodiesel, (Biodiesel Uruguay, 2007; Kavalov y Jensen, 2000). Rodríguez et al. (1998) mencionan el uso de los residuos de girasol aplicados al suelo para el control de la maleza que de manera natural emergen en los cultivos. No obstante el uso integral de la planta y de que las condiciones de clima y suelo no parecen ser limitantes para el girasol en México, su cultivo no es muy extensivo y solo se siembra en algunos estados del país (Baja California, Tamaulipas, Nayarit, Jalisco y Durango) con un rendimiento medio de semilla de 0.8 t ha^{-1} (SAGARPA, 2000), donde el manejo del

cultivo y las condiciones del clima son cruciales para determinar la producción de girasol. Así, el objetivo del presente estudio fue determinar el grado de variación de la fenología, biomasa total, rendimiento de semilla, contenido de aceite y rendimiento de aceite del girasol de temporal en valles altos y su relación con factores e índices del clima durante el período 1992-2001.

MATERIALES Y MÉTODOS

El estudio se realizó en Montecillo Méx., (clima BS1, el menos seco de los áridos y 2,240 m de altitud) donde una reproducción del cv. Victoria se sembró bajo condiciones de temporal, a la densidad de 5 plantas m^{-2} en surcos a 80 cm con orientación este-oeste y fertilización de 100-100-00 de NPK en las fechas siguientes: (I) 20/5/1992; (II) 01/5/1995; (III) 18/5/1996 ; (IV) 13/5/1997; (V) 01/6/1998; (VI) 26/5/1999; y (VII) 22/5/2001 en un suelo Fluvisol mólico (Flm) de textura arcillosa, 2% de materia orgánica, pH 7.5-8.0 y sin problemas de salinidad. En cada año se evaluó la fenología de acuerdo con el criterio de Schneiter y Miller (1981), biomasa total (materia seca, g m^{-2}), rendimiento de semilla (materia seca, g m^{-2}), el índice de cosecha (IC) como una medida de eficiencia de la acumulación de materia seca en la semilla en relación a la total , el contenido de aceite en la semilla (%) mediante resonancia magnética nuclear (Granlund y Zimmerman, 1975), utilizando un analizador modelo MKTIIA (Newport Instruments Bucks G. B.). El rendimiento de aceite se calculó mediante el rendimiento de semilla y el contenido de aceite de la semilla (%), la evapotranspiración del cultivo (ETc) como una medida que estima el requerimiento hídrico para el crecimiento, mediante la evaporación del tanque de tipo A (Ev), utilizando 0.6 como coeficiente de tanque (Kt) y 0.8 como coeficiente de cultivo (Kc) y el planteamiento: Etc= Ev*Kt*Kc (Doorenbos y Pruitt, 1986). Las unidades calor (UC) se calcularon mediante el método residual (Snyder, 1985) tomando como temperatura base 6 ºC. La radiación fotosintéticamente activa (RFA, MJ m^{-2}), la evaporación del tanque tipo A, la temperatura media máxima (Tmáx) y mínima (Tmín), y la humedad relativa máxima (HR máx) y mínima (HR mín) fueron proporcionadas por la estación agrometeorológica del Colegio de Postgraduados. A cada variable en estudio se le determinó la media y el coeficiente de variación (cv %) y un análisis de correlación entre las variables en estudio y regresión múltiple procedimiento paso a paso para determinar el mejor modelo que estime la producción de biomasa y el rendimiento.

RESULTADOS Y DISCUSIÓN

Factores e índices climáticos

En los años de estudio la oscilación de la media estacional de la Tmáx fue de 27.3 y 29.3 ºC; la T mín entre 6.6 y 9.4 ºC; la HR máx entre 93 y 98%, la HRmín entre 32.9 y 44.8%; la ETc fluctuó entre 329 y 393 mm, las UC entre 1353 y 1503 ºC y la RFA entre 1017 y 1259 MJ m^{-2} (Tabla 1).

Tabla 1. Factores e índices del clima durante el desarrollo del girasol (*Helianthus annuus* L.) cv. Victoria durante 1992-2001. Suma y promedio estacional

Experimento	ETc mm ∑	UC ºC ∑	RFA MJm^{-2} ∑	Tmáx ºC	Tmín ºC	HRmáx %	HRmín %
I) 1992	329	1353	1017	28.2	8.4	96.8	41.2
II) 1995	380	1450	1168	28.5	8.5	97.2	40.4
III) 1996	393	1371	1259	27.3	7.5	96.4	34.3
IV) 1997	367	1379	1143	28.5	7.9	95.3	32.9
V) 1998	331	1503	1036	29.3	9.4	93.0	37.5
VI) 1999	358	1374	1076	28.0	6.6	93.5	39.0
VII) 2001	362	1488	1079	28.8	8.0	98.0	44.8
Media	360	1417	1111	28.4	8.0	95.7	38.6
S	24	62	84	0.63	0.87	1.9	4.1
CV (%)	6	4	8	2.2	10.8	2.0	10.6

S=desviación estándar; cv (%)= coeficiente de variación

Fenología, biomasa, índice de cosecha, rendimiento de aceite y sus componentes

La fenología del girasol cv. Victoria mostró cambios entre años de estudio. Así, la emergencia (Ve) ocurrió entre los 7 a 12 días después de la siembra (dds), el inicio de floración (R5) entre los 77 y 84 dds y la madurez fisiológica (R9) entre los 113 y 120 dds (Ve, R5 y R9 son etapas fenológicas; Schneiter y Miller, 1981). En la Tabla 2 se observa que durante el período de estudio, el rendimiento de aceite mostró mayor variabilidad (cv=45%), seguida del rendimiento de semilla (cv=40%) , la biomasa (cv=20%) y el contenido de aceite de la semilla (cv=6%). El índice de cosecha (IC) mostró valores entre 11 y 23% (cv=23%), donde los valores más bajos de IC son indicativos de una posible limitada disponibilidad de agua durante el período reproductivo que generó una menor acumulación de materia seca en la semilla (Escalante, 1999). El promedio de biomasa total, rendimiento de semilla, de aceite y contenido de aceite durante el período de estudio fueron de 1,430 g m-^{2}, 256 g m-^{2,} 100 g m-^{2} y 38%, respectivamente. La más baja variabilidad en el contenido de aceite en la semilla, sugiere que para lograr incrementos en la producción de aceite a nivel de superficie, se requiere buscar incrementos en la acumulación de materia seca en la semilla.

Tabla 2. Biomasa total (g m^{-2}), índice de cosecha (IC), rendimiento de semilla (g m^{-2}),contenido de aceite (%) y rendimiento de aceite (g m-^{2}) en girasol, cv. Victoria, en Montecillo Méx. de 1992 a 2001.

Experimento	Biomasa g m^{-2}	Rendimiento de semilla g m^{-2}	IC (%)	Aceite semilla (%)	Rendimiento de aceite g m^{-2}
(I) 1992	1,050	120	11	36	43
(II) 1995	1,675	336	20	42	141
(III) 1996	1,840	370	20	41	152
(IV) 1997	1,511	352	23	39	137
(V) 1998	1,075	151	14	36	54
(VI) 1999	1,403	271	19	37	100
(VII) 2001	1,456	191	13	37	71
Media	1,430	256	17	38	100
S[1]	290	102	4	2	44
cv (%)	20	40	23	6	45

[1]S=desviación estándar; cv= coeficiente de variación.

Relaciones entre la biomasa, el rendimiento y la ETc, UC y RFA

Los resultados del análisis de correlación presentados en la Tabla 3, indican que la biomasa, el rendimiento de aceite y de semilla presentaron una correlación alta con la ETc y la RFA. Asimismo, el contenido de aceite en la semilla está altamente asociado con los cambios en la producción de biomasa y el rendimiento de semilla, lo que sugiere que la mayor producción de materia seca y de aceite en girasol cv. Victoria estuvieron afectados por la disponibilidad de agua y la capacidad del dosel en la intercepción de la radiación solar (Escalante, 1992).

Tabla 3. Coeficiente de correlación (r) entre la biomasa (Bio), índice de cosecha (IC), rendimiento de semilla (Ren) y aceite (RA), contenido de aceite en la semilla (aceite), evapotranspiración (ETc) y la radiación fotosintéticamente activa (RFA) estacional en girasol cv. Victoria. 1992-2001.

	Bio	Ren	IC	aceite	RA	ETc	RFA
Bio	-------	0.90***	0.73*	0.88**	0.92***	0.99***	0.94***
Ren	0.90**	-------	0.94**	0.86*	0.99***	0.91**	0.91***
IC	0.72*	0.94***	------	0.72*	0.92***	0.73*	0.73*
aceite	0.88***	0.87*	0.72*	-----	0.91**	0.89**	0.89**
RA	0.92***	0.99***	0.92***	0.91***	------	0.92***	0.92**
ETc	0.99***	0.91***	0.73*	0.89**	0.89**	-------	0.94**
RFA	0.95***	0.90**	0.73*	0.89**	0.89**	0.94***	------

*,**,*** Prob <0.05, 0.01, 0.001, respectivamente.

Por otra parte, la acumulación de calor estacional y la producción de biomasa y el rendimiento mostraron una relación cuadrática. Las ecuaciones que describen dichas relaciones son: Biomasa= -25254+356.8 UC-0.125 UC^{2}; R^{2}=0.63*; y el Rendimiento=-90898+128.3UC-0.045UC^{2}; R^{2}=0.73**.

Dicha relación también fue encontrada en frijol por Escalante et al. (2001) y sugiere la existencia de uno o más factores que limitan una mayor respuesta del girasol al calor acumulado, donde deben de considerarse en primera instancia ajustes en las prácticas de manejo del cultivo.

Modelo para estimar la biomasa , rendimiento de aceite y sus componentes en función de los índices y factores del clima

De los índices y factores del clima que mejor estiman la biomasa y el rendimiento de aceite y sus componentes tenemos a la ETc, RFA y HRmín. Las ecuaciones que mostraron un coeficiente de determinación (R^2) superior a 0.80 se presentan en la tabla 4.

Tabla 4. Modelo para estimar la biomasa, rendimiento de aceite y sus componentes en función de los índices y factores del clima. Selección con base al procedimiento "paso a paso".

Variable	Ecuación	R^2
Biomasa ($g\ m^{-2}$)	Biomasa=-2997.74+12.29 ETc	0.99***
Rendimiento de semilla ($g\ m^{-2}$)	Ren= - 428.2+6.6ETc-1.039RFA-14.38HRmín	0.97***
Indice de cosecha	IC=20.48+0.40ETc-0.95RFA-1.08HRmín	0.95***
Aceite en la semilla (%)	Aceite=9.68+0.025RFA	0.80**
Rendimiento de aceite ($g\ m^{-2}$)	RA=-441.01+0.4869RFA	0.85**

,* P<0.01, 0.001, respectivamente; Ren= rendimiento de semilla; ETc= evapotranspiración estacional; RFA=radiación fotosintéticamente activa; HRmín= humedad relativa mínima; IC=índice de cosecha; RA=rendimiento de aceite.

Finalmente, en concordancia con Dirks y Bolton (1981), este tipo de estudios pueden contribuir al conocimiento de los principales factores ambientales de riesgo para la producción en cada región agrícola, así como apoyar a la generación de modelos de predicción de rendimiento.

CONCLUSIONES

Durante el período de 1992 a 2001 en la región de influencia de Montecillo Méx., la ocurrencia de las etapas fenológicas es variable. Asímismo, la variabilidad del rendimiento de aceite y de semilla es mayor que la de la biomasa. El contenido de aceite de la semilla muestra mayor estabilidad. Los cambios en la biomasa presentan una relación alta con la evapotranspiración, de manera que éste índice puede considerarse como un estimador apropiado de la producción de materia seca total. El rendimiento del girasol cv Victoria puede estimarse en función de la evapotranspiración, radiación fotosintéticamente activa y la humedad relativa mínima.

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Proceedings of the

17th International Sunflower Conference

Vol. 2

Córdoba, Spain

June 8-12, 2008

Sponsored by The International Sunflower Association, Paris, France

Instituto de Investigación y Formación Agraria y Pesquera
CONSEJERÍA DE AGRICULTURA Y PESCA
CSIC
International Sunflower Association

Título: Proc. 17th International Sunflower Conference, Córdoba, Spain (2008), Vol. 2

© Edita: JUNTA DE ANDALUCÍA - Consejería de Agricultura y Pesca

Publica: Secretaría General Técnica. Servicio de Publicaciones y Divulgación

Colección: Congresos y Jornadas

Serie: Cultivos Herbáceos

Depósito Legal: SE-3054-08

Impresión: German López - Servicios Gráficos

Proceedings of the 17th International Sunflower Conference
Córdoba, Spain. June 8-12, 2008

Editor: Leonardo Velasco

Photography: *Feral sunflowers growing in a sunflower field in Santa Cruz, Córdoba, Spain.* Courtesy of Marie-Hélène Muller.

Foreword

The proceedings of the *17th International Sunflower Conference* contain 142 contributions from scientists of 24 countries. They include plenary lectures in several disciplines and regular communications presented in posters during the conference and discussed in the corresponding workshops. The manuscripts are classified by disciplines. They offer a good picture of the current state of the art of sunflower research and cultivation around the world.

The manuscripts in the *Proceedings* have been reviewed by an editorial committee with the main objective of helping the authors to improve their manuscripts through a critical reading. The authors received the edited manuscripts together with the comments of the reviewers and then went on to draft their final version. All the manuscripts received have been published in the *Proceedings*. The contents of the manuscripts are the responsibility of the authors. They should be considered as being privileged communications that require the express consent of the authors to be reprinted in part or as a whole. We wish to thank both the members of the Editorial Committee for their dedication to the task of editing such a large number of manuscripts, as well as all the authors for their collaboration throughout the whole edition process.

The Organizing Committee would also like to thank Diana Badder and José A. Palacios for their excellent editorial assistance in the preparation of these *Proceedings*. We are indebted to the Spanish Association of Sunflower Breeders (Asociación Española de Mejoradores de Girasol), which collaborated actively in the organization of the conference, and, very especially, to Juan Parejo, who was in charge of the financial side.

Finally, we would like to thank all the participants in the conference, who have contributed to its success by a careful preparation and revision of manuscripts and posters, presentation of their research in the workshops, and stimulating discussions throughout the conference on the scientific and technical aspects of sunflower research and cultivation in the world.

The Organizing Committee
17th International Sunflower Conference
Córdoba, Spain. June 8-12, 2008

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CROP PRODUCTION – PHYSIOLOGY

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WILD SPECIES AND GENETIC RESOURCES

Early responses to high crop population density in sunflower: Controls and effects of the crop self-organization process

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ABSTRACT

Plants in the rows of high crop population density sunflower crops exhibit a self-organizing process that commences shortly after seedling emergence: a given stem inclines towards one inter-row and the neighbouring stem inclines towards the opposite inter-row. To date, the causes and consequences of this process have not been explored in sunflower or in other grain crops. The aim of this work was to describe the dynamics of sunflower self-organization, focusing on the causes that elicit this crop response. Five experiments were conducted at the Facultad de Agronomía, Universidad de Buenos Aires. Factors affecting crop responses that were examined included: crop population density, quality of light incident on upper leaves of the plants, and the mechanical restriction of the self-organization process. Dynamics of the process were followed at four-day intervals or, in a more detailed fashion, using time-lapse photography to document the organization process and the degree of shading between neighbouring plants at 10-minute intervals. Effects of self-organization on grain yield and grain number were determined in some experiments. The self organization process (as exhibited by the proportion, of all plants in a row, of inclined stems) was greater and occurred earlier in the high population density plots. Thus, 70% of plants deviated from the vertical position at 14 pl. m^{-2} (equally distributed among the two inter-row positions) at floral initiation as against 0% at 5 pl. m^{-2} . Low red/far red ratio light (but not low blue light) incident on the upper portion of otherwise unshaded plants induced a high incidence of stem inclination (82%) and greater inclination angles (30º) than in the controls (0%, 0º). Analysis of time-lapse photography records for individual plants showed that the initiation of stem inclination took place shortly after the plant began to be shaded by its neighbours. Grain yield was significantly lower in plots in which the self-organization process was mechanically restricted. It is concluded that a) shading of the target plant by the leaves of neighbouring plants reduced the red/far red ratio of the light incident on the target plant, inducing stem inclination; b) stem inclination diminished the negative effects of the increased crop density on grain number and grain yield per plant.

Key words: crop density – light influence – radiation interception – self organization –stem inclination.

INTRODUCTION

Under non-limiting conditions, sunflower crop yield responds positively to increases in plant population density above those usual (ca. 5 pl.m^{-2}) in commercial production in Argentina (López Pereira et al., 2004a). The effects of increased crop population density involve changes in light quality within the canopy. Plants can perceive changes in light quality (i.e. proportion of red/far red, blue light) and respond by exhibiting modifications in morphological and physiological attributes during the early stages of crop development (Ballaré et al., 1987). For example, at the bud visible stage, sunflower basal internodes became longer and heavier as crop population density increased (2 to 14 plants m^{-2}) (López Pereira et al., 2004b). De la Vega and Hall (pers. comm. 2002) observed that in denser stands (>5 plants m^{-2}) the plants within a row adopt a regular arrangement: one stem inclines towards one inter-row and the neighbouring stem inclines towards the opposite inter-row, and at physiological maturity this pattern is evident along the whole of the row. Preliminary experiments have demonstrated that this alternating inclination of stems along the row could not arise by chance (López Pereira et al., 2004b). This self-organization process within a given row might increase radiation interception and aerial biomass production in early crop development phases, and possibly affect yield.

While the movement of leaves (Lang and Begg, 1979; Casal and Sadras, 1987) and stems (Buda, 2003) has been widely studied in sunflower, there are no reports of self-organization responses to increments in crop population density, either in sunflower or in other grain crops. The aims of this work were: i) to describe the dynamics of the process of stem inclination in early stages of crop development; ii) to determine which of the possible light quality signals known to be involved in plant-to-plant

communication (red/far red light [R/FR] ratio and /or blue light [B]) are involved in this process; and iii) to evaluate the effects of stem inclination on yield and generation of reproduction structures.

MATERIALS AND METHODS

Five experiments were conducted at the experimental field of the Facultad de Agronomía, Universidad de Buenos Aires (34º35 ´ S., 58th 29'W.) to achieve the objectives indicated above. The plots were fertilized (60 kg N ha^{-1}) and irrigated, and diseases, insects and lodging controlled. Crops were over-sown and desired crop population densities were established by removing plants at the two-leaf stage. The distance between rows was 0.70 m. The same hybrid (Paraíso 20) was sown in all experiments. Details of the experiments are provided below.

i) Dynamics of the self-organization process

Two experiments were conducted; *Experiment 1*: 2003-04. The experiment was laid out in a randomised complete block design with three replications. Crop population densities covered a broad range of densities (0.2 to 14.3 plants m^{-2}). Crop rows were oriented N-S and number of inclined stems for 40 contiguous plants in a row and their inter-row orientation (E or W) was evaluated every fourth day from 20 days after crop emergence (dae) until the bud visible stage. *Experiment 2*: 2005-2006. In this experiment time-lapse photography was used to generate a more detailed record of the self-organization process. Plants were sown at a very high crop population density (20 plants m^{-2}), and a system of web-cams and a CPU were devised which allowed plant position to be registered every 10 minutes. Three cameras, each focused on a section of row, were supported on a framework above a 1-m length of row. The images were analyzed using JPG video version 1.05.0.0-freeware. To simplify the analysis, the total image data base was segmented, and apex position (distance from the row axis) and proportion (with respect to total) of leaf area per plant shaded by neighbouring plants were quantified at 9, 11, 13, 15 and 18 hrs of each day, beginning 8 dae, when mutual shading between neighbours and deviation of the plant apex from the vertical started to become evident, and continued up to 23 dae. A daily integral of the fractional shaded area per plant was synthesized from the five daily observations. Shading was computed up to about 280 hours from the start of imaging (at later stages of the process this became too difficult) but apex position was documented for a further 50 hours.

ii) Controls of stem inclination: light signals

In *Experiment 3* (2004), the quality of light incident on the upper leaves of the plants was manipulated using filters that absorbed or reflected solar light in different wavelengths, generating: i) a low R/FR ratio; ii) a normal (sunlight) R/FR ratio (i.e., control), and iii) a low blue irradiance. The filters (0.08 m x 0.08 m) were centred over the apices of ten plants per treatment. Plots were sown at a low crop population density (5 plants m^{-2}) so that light quality impinging on the apex and leaves of the target plant were not affected by the leaves of the nearby plants. At 96 hours after the beginning of the first day in which filters were put in place, the number of treated plants that had stems which deviated from the vertical were registered and the angle of stem inclination (with respect to the vertical) measured.

iii) Effects of stem inclination

During the 2004-05 (*Experiment 4*) and 2005-06 (*Experiment 5*) seasons experiments using high crop population densities (10 and 14 plants m^{-2}) were conducted. A randomized complete block design was used, with two factors (crop density, self-organization [natural vs. none, in which stems were forcibly restrained in the vertical position using guide-wires] and three replicates per treatment. At physiological maturity, yield and total grain number were determined on a sample of 30 plants per treatment.

RESULTS

In Experiment 1, the self-organization process began early (before 27 dae) at 14 $pl.m^{-2}$, while at 5 $pl.m^{-2}$ very few plants had deviated from the vertical at 49 dae (Fig. 1). The process was gradual, with increasing proportions of plants showing inclination, so that almost all stems in the 14 $pl.m^{-2}$ plots were inclined at 49 dae, with almost equal proportions in the E and W inter-rows. Although not shown in Fig. 1, the final pattern was regular, with neighbouring plants inclining toward opposite inter-rows. The small proportion of plants not showing inclination at 49 dae in the 14 $pl.m^{-2}$ plots were usually suppressed (little growth) by their neighbours. Interestingly, the process began in different sectors of the row, in patches, and some degree of “re-orientation” was involved as patches began to overlap. At the beginning of the process, the

proportion of stems inclining towards the E was higher than the proportion of stems inclined towards the W. At the end of the process, the proportion of stems inclined towards each inter-row became similar because the proportion of plants inclined towards the W increased. At the highest crop population density (14 plants m^{-2}), the stem inclination process began 15-20 dae, before the beginning of floral initiation. A large (≈70%) proportion of the stems showed inclination at the time when floral initiation was completed in these plots (Fig. 1).

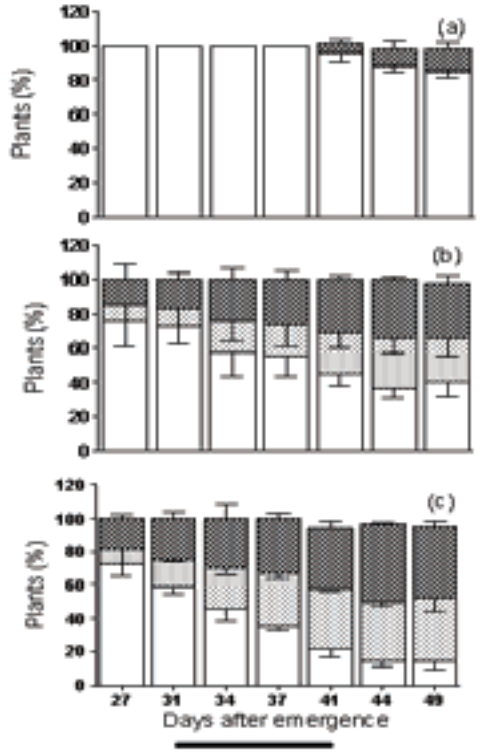

Fig. 1. Dynamics of stem inclination from 20 days after emergence to bud visible in crops sown at three crop population densities: a) 5 plants m^{-2}, b) 10 plants m^{-2}, and c) 14 plants m^{-2}. Each bar represents the proportion of plants not inclined (white), inclined towards the E (heavy stippling) and towards the W (light stippling). The line below panel c) represents the duration of the floral initiation period in these crops. The vertical lines on the bars indicate ± a standard error, n= 3 (40 plants per replication). Experiment 1.

In Experiment 2, stem inclination began shortly after neighbouring plants began to be shaded by their neighbours (Fig. 2), with the shaded plant inclining away from the row axis towards the inter-row space. The plants that first experienced shading were the first to show this response (e.g., Plants 1 and 2 vs. Plant 3, Fig. 2). The effects found in the 14 remaining plants evaluated in this experiment (data not shown) were consistent with those illustrated for the three plants shown in Fig. 2.

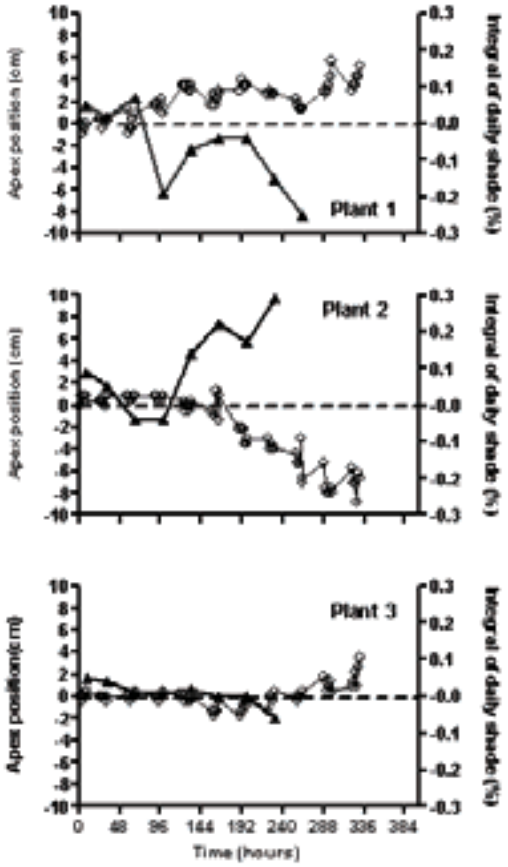

Fig. 2. Dynamics of the apex position (cm from row axis, circles, five observations per day) and daily shade integral (mean daily fraction of total plant leaf area, triangles) for three plants growing in plots sown at 20 plants m^{-2}. The dashed horizontal line indicates the row axis. The negative and positive values represent the two inter-rows, and are used to show the position where the shading took place and position of the plant apex. Daily shade integral estimates were not made after about 280 hours after the start of observations (8 dae) because the degree of plant-to-plant interference became too difficult to resolve in the two-dimensional images.

In Experiment 3, proportion of inclined plants and the angle of stem inclination evoked by low R/FR incident on the upper leaves were greater than in the other two treatments (little response in low blue light treatment and insignificant for the control (neutral filter) treatment) (Fig. 3). Clearly, light of a low R/FR ratio incident on the leaves can evoke stem inclination. The R/FR ratio measured below the upper leaves close to the row axis was 0.55±0.12, in contrast to 1.17±0.07 above the upper leaves close to the inter-rows. These values are consistent with those obtained using the R/FR filters.

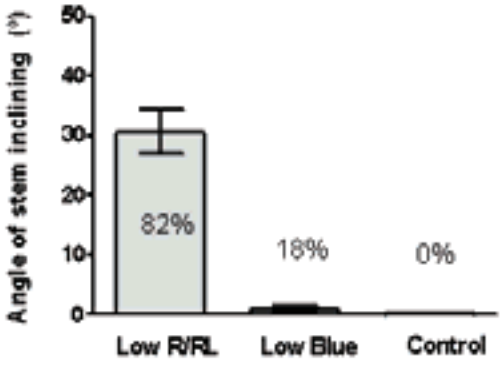

Fig. 3. Effects of light of low red/far red (R/FR) ratio, low blue (B) and normal (sunlight) R/FR and blue (control) on the angle of inclination of sunflower stems. The numerical values on or above each column indicate the proportion (%) of inclined plants (n=10) in each treatment. The vertical lines on the bars indicate ± a standard error. Experiment 3.

In Experiments 4 and 5, grain yield was significantly (Exp.4 $p \leq 0.03$, Exp.5 $p \leq 0.01$) lower in plots in which stem inclination was forcibly restricted (Exp.5 = 25.2 ±1.5; Exp. 6 =16.9 ± 1.8 g per plant) than in plots in which the natural self-organization was allowed (Exp.5 = 30.6 ±0.7; Exp. 6 =31.4 ±0.7 g per plant). These effects on grain yield were associated with significant reductions in grain number per plant in both experiments (Exp.4 $p \leq 0.01$; Exp.5 $p \leq 0.01$).

DISCUSSION

Our results indicate that the process of crop self-organization is an important response of sunflower crop to high crop population densities. This process occurs over time, is hastened at high crop population densities and the propagation of the pattern occurs in patches. Mutual shading between leaves of neighbouring plants (briefly) precedes the deviation of the stems from the vertical. The fact that low R/FR ratios in the light incident on the upper leaves can evoke stem inclination is consistent with the reduction in R/FR ratio of light transmitted through green leaves. The weak response to low blue light of the process (light transmitted through leaves is depleted in blue) suggests that phytochromes rather than cryptochromes or phototropins are the photoreceptors involved. This is the first time that it has been shown that the quality of light impinging on leaves can alter stem position (previous reports in other species involve detection/response associations limited to either leaves (Maddoni et al., 2002) or stems (Ballaré et al., 1991) The process would appear to have an effect on crop yield. It remains to be established whether the effect on crop yield is mediated by changes in resource capture (e.g., higher fractional interception); resource allocation (e.g., less competition for biomass between stem and floral structures); direct photomorphogenic effects (e.g., in soybean, the abscission of reproductive structures in the lower strata of the canopy during flowering was lower in plots with high R/FR; Heindel and Brun, 1983); or a combination of these effects.

Another issue arising from the identification of this process relates to possible intra-specific variability for the intensity of this process (and we have preliminary evidence to support this possibility). In Argentina, there is some interest in intersown sunflower/soybean crops. An important issue here is the conditions for soybean seedling establishment and early growth in the space between the rows of sunflower. Sunflower hybrids less sensitive to signals produced by neighbouring plants might produce less shade for the soybean intercrop. By contrast, in non-uniform stands of pure sunflower crops (a common circumstance in sunflower crops in Argentina), it would be desirable to have cultivars capable of responding to environmental heterogeneity and ensuring greater resource capture.

ACKNOWLEDGEMENTS

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnología and ASAGIR (PICTO ANPCyT-ASAGIR 8-13159) and the Academia Nacional de Agronomía y Veterinaria de la República Argentina. We thank Marcos Sobral for his invaluable advice on construction and use of the time-lapse photography system.

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Early sowing as a means of drought escape in sunflower: effects on vegetative and reproductive stages

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ABSTRACT

Drought occurring mainly during the flowering period is responsible for a substantial decrease in the production in sunflower. The hypothesis that early sowing of sunflower would increase the probabilities of avoiding warm and dry period during the reproductive stage was tested. Nevertheless, early sowing is associated with low temperature during the first development stages. Phenological determinations were performed to study the effect of early sowing on the vegetative and reproductive periods. A sunflower population of recombinant inbred lines (RILs) was studied in two sites (France and Algeria) during 2007 at normal sowing date (control) and one-month earlier sowing. Phenostage observations were determined from emergence to harvest. Weather data with rainfall and temperatures were daily recorded. Cumulative growing degree day requirements for each phenological stage were calculated. Earliness of flowering was observed in the two sites when sunflower genotypes were sown one-month earlier in the season. Differences in thermal time requirement for sunflower development observed between sites and between early and control sowings could be explained by variations in base temperature values and/or photoperiod effect. A significant variability between genotypes was observed for sunflower development. The genotype ranking was not affected by early sowing for vegetative stage on the two sites, but during the post-flowering stage in Algeria, high temperatures and dry conditions occurring during this period considerably reduced their variability in the phenostage (R6-R9) and modified the genotype ranking. Genetic basis of sunflower phenostages response to early sowing must be explored in terms of genetic variability for temperature x photoperiod interactions.

Key words: drought escape – early sowing – *Helianthus annuus* L. – low temperatures – phenological stage – sunflower.

INTRODUCTION

Two main strategies are considered to increase sunflower productivity of non irrigated cropping systems. The first consists of selecting genotypes tolerant to dehydration during the water deficit conditions. Poormohammad Kiani et al. (2007a, b) have studied physiological traits of sunflower under drought conditions and differential expression of water stress-associated genes in order to supply tools for drought tolerance selection. Another way is to modify cultural practices to avoid drought at flowering stage. In French cropping systems, most farmers sow around 15 April and flowering takes place under high evaporative demand and scant rainfall. Consequently, the crop is often subject to water deficit and yield decrease. A possible alternative strategy for avoiding drought at flowering is to sow earlier, at times of lower evaporative demand. Early sowing including winter planting was tested in several Mediterranean countries (Gimeno et al., 1989; De La Vega et al., 2002; Flagella et al., 2002). It was shown that this approach allowed to increase water availability (Barros et al. 2004; Soriano et al. 2004). Therefore, the yield was increased (Hadjichrisyodoulou et al., 1987; Gimeno et al., 1989; Tenteiro et al. 1994; Anastasi et al. 2000). However, a major disadvantage of growing crops during low-evaporative-demand periods is the common association between low evaporation and low temperature.

The aim of the present work was to determine the effect of early sowing on vegetative and reproductive stages in a population of 100 recombinant inbred lines (RILs) of sunflower with a large genetic variability, through two experiments conducted in contrasted pedoclimatic conditions (France and Algeria).

MATERIALS AND METHODS

Plant material and field experiments

A population of 98 RILs of sunflower (*Helianthus annuus*) and their parents RHA 266 and PAC2 (Flores Berrios et al., 2000; Poormohammad Kiani et al., 2007a) were used to investigate early sowing in term of vegetative and reproductive stages. Genotypes were tested in two locations: in France (Toulouse: 43°31'46,94" N; 1°29'59,71" E) and in Algeria (Constantine: 36°16'17.65"N; 6°40'13.01"E). In France, field experimentation was conducted at INRA station of Auzeville, and in Algeria at CNCS station of El-Khroub. For the last ten years, the French site had low temperatures in winter and the Algerian site had warm and dry conditions during summer (Fig. 1). Weather data were obtained from Meteo France.

In 2007, RIL population was sown on two dates in both sites: 14 March (early sowing: ES-F) and 19 April (control sowing:CS-F) in France and 3 March (early sowing ES-A) and 26 March (control sowing: CS-A) in Algeria. Three replications by sowing date were performed. Each replication consisted of two rows 3m long, with 50cm between rows and 25cm between plants in rows, giving a total number of about 24 plants per experimental unit.

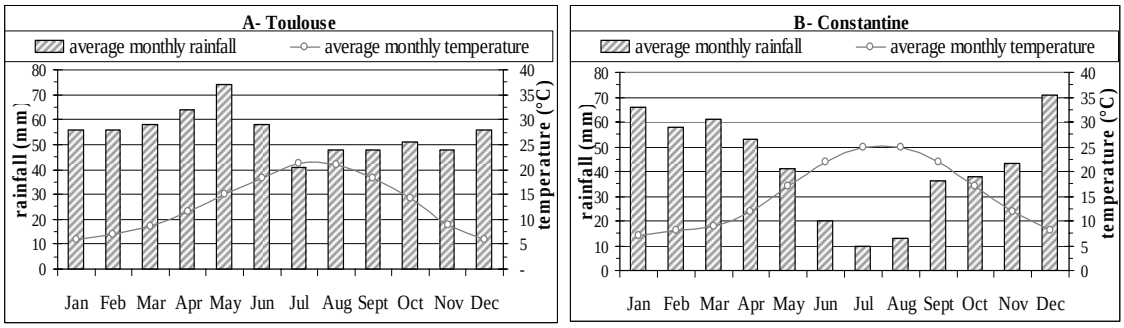

Fig 1. Average monthly temperatures and average monthly rainfalls (mean of the last 10 years) in the two experimental sites (Toulouse in France and Constantine in Algeria).

Phenological measurement

Plant development was recorded according to the definition of growth stage system of Schneiter and Miller (1981). Dates were obtained for 100% emergence (VE), 50% of plants at beginning of flowering (R5), 50% of plants at complete flowering (R6), 50% of plants at physiological maturity (R9) and 50% of plants at harvest. The results were recorded as Vegetative Period (VP) from sowing to R5 and Post-Flowering Period (PFP) from R5 to harvest.

Daily maximum and minimum temperatures and rainfall were recorded at each site. Cumulative growing degree days (°Cd) were calculated as the sum of the average daily temperature minus base temperature of 4.8°C (Granier and Tardieu, 1998).

Statistical analysis

Statistical analyses were performed with SPSS for Window (11.0.1). Sowing date, location and genotype effects were tested using ANOVA procedure. Correlation between control and early sowing for vegetative period (VP) and post-flowering period (PFP) in cumulative growing degree days were performed in France and in Algeria sites. Moreover, correlations between VP and PFP were realized for each sowing date in both sites.

RESULTS

Sunflower development (vegetative and reproductive stages)

The two sites (France and Algeria) presented substantial differences during the growing season: colder at the first phenostages and warmer during post-flowering stage in Algeria (Fig. 2A). Fig. 2B shows that the total of time cycle length was significantly different between the two controls even if differs for only 5 days with 130 days in France and 125 days in Algeria. Vegetative development period represented 62% of total duration in France vs. 72% in Algeria. In fact, time to emergence (sowing-VE) was twice longer

in Algeria compared with France. Temperatures corresponding to sowing-VE were inferior in Algeria (13°C) than France (15°C) (Fig. 2A). The time between VE and R5 stage did not differ with, respectively, 73 days and 74 days in France and Algeria. The post-flowering period (PFP) with respect to sowing-harvest duration was proportionally shorter in Algerian location. With the same flowering time (10 days), the period from R6 to harvest differed between the two sites (9 days more for R6-R9 in France, and 5 days more for R9-harvest in Algeria).

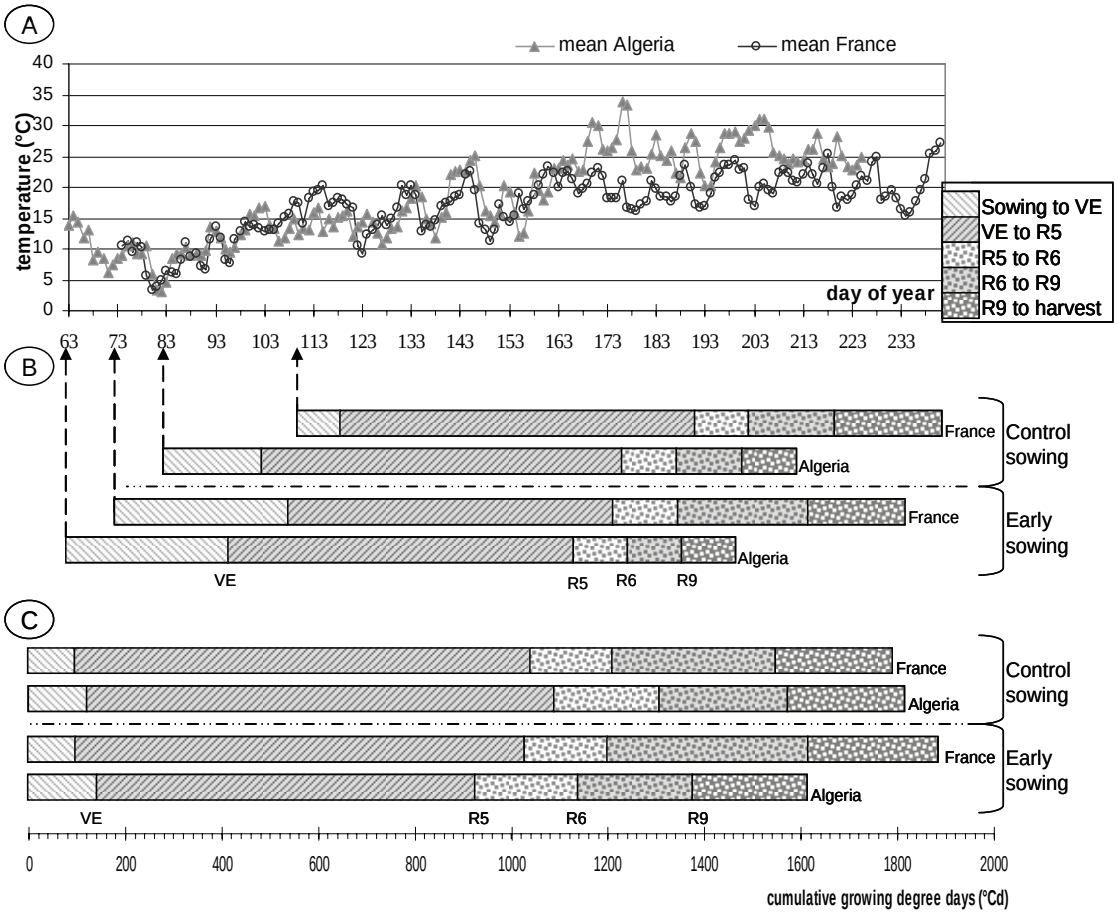

Fig 2. Mean temperature for control and early sowing in France and Algeria (A). Sunflower phenostages in relation to day of year (B) or to cumulative growing degree days with tb = 4.8°C (C).

Whereas total cycle length differed between France and Algeria, there was no significant difference for cumulative growing degree days with, respectively, 1857 and 1871°Cd (Fig. 2C). Cumulative growing degree days required for vegetative period (VP) was higher in Algeria than in France. We observed that weather conditions at sowing differed between locations: (average daily temperatures 3°C colder in Algeria vs. France). For the post-flowering period (PFP), sunflowers in Algeria needed fewer cumulative degree days, in spite of superior thermal requirement for flowering. Cumulative growing degree days required for R6-R9 period was diminished (- 86 °Cd) in Algeria vs. France.

The cumulative growing degree days of vegetative period was negatively correlated with post-flowering period in France with a Pearson correlation coefficient of -0,739. On the contrary there was no significant correlation in Algeria location between VP and PFP.

Effect of early sowing on phenostages

In France, early sowing was one month before control, and crop was harvested with only one week in advance as shown in Fig. 2B. Total cycle duration was longer for 22% on early sowing comparatively to control sowing (159 days vs. 130 days for control sowing). In Algeria, total cycle duration of early sowing was only 8% longer than control sowing. It was sown 3 weeks before the control and harvested 13 days before the control. Proportion of VP in total cycle was mildly longer than in control. In both sites sowing to emergence period increased (multiplied by four in France and by two in Algeria), and emergence to flowering period decreased in response to early sowing.

Thermal time requirement for sunflower development in early sowing compared to control sowing was different between French and Algerian locations, except for flowering duration (from R5 to R6) (Fig. 2C). In France, we observed an increase in the thermal time requirement for the total cycle in response to early sowing. Vegetative stage and PFP present a significant correlation between control and early sowing of -0.336**. On the contrary, in Algeria, early sowing necessitated less cumulative degree days for total cycle (186 °Cd less than control) despite increasing the thermal time requirement for emergence (Fig. 2C).

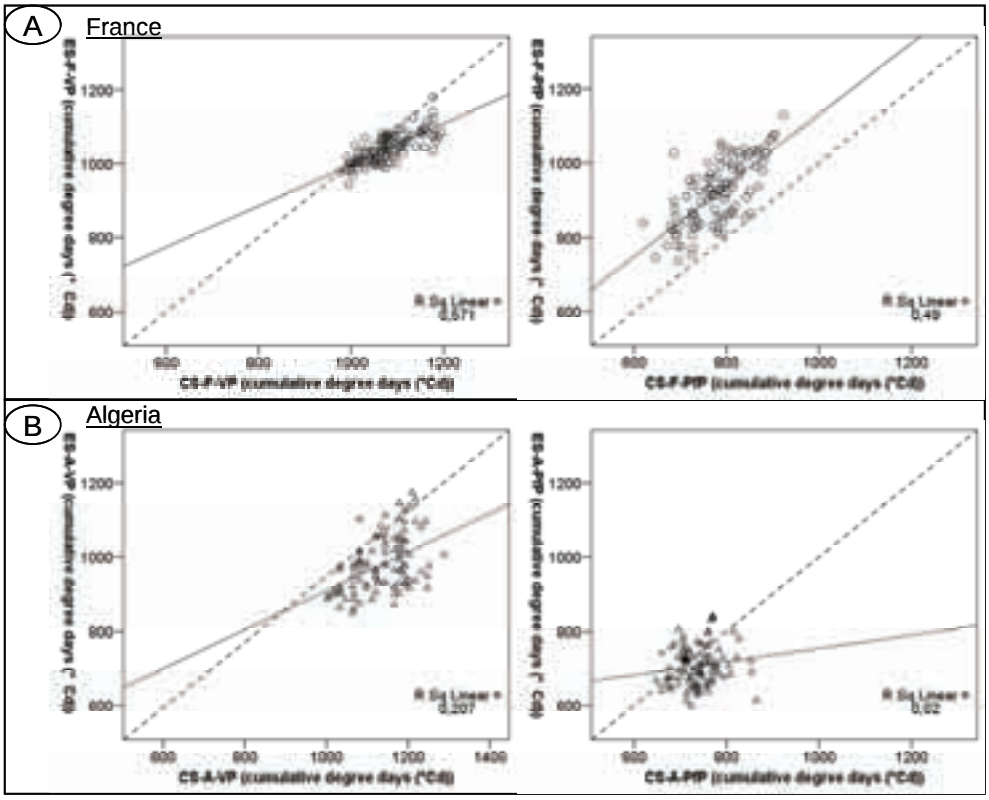

Fig 3. Correlation between control (CS) and early sowing (ES) for vegetative period (VP, left graphs) and post-flowering period (PFP, right graphs) in cumulative growing degree days: in France (A) and in Algeria (B).

Genotypic variation among the 100 recombinant inbred lines of sunflower

Differences among RILs were significant for vegetative period (VP) in all conditions (planting date and site). For example, VP in control sowing ranged from 977 °Cd to 1195 °Cd depending on genotypes in France, and similar amplitude was observed for early sowing. However, genetic differences were less pronounced for post-flowering period in Algeria (PFP ranges from 598 °Cd to 840 °Cd).

High significant correlations were observed between planting dates for vegetative period in both sites (Pearson correlation coefficient was equal to 0.571** and 0.207 in France and Algeria, respectively, Fig. 3). However, non significant correlation was observed between planting date for the post-flowering period in Algeria.

DISCUSSION

Thermal time requirements for sunflower phenostages differed between control and early planting dates. We have shown in Fig. 2C that early sowing in France required more cumulative growing degree days than control sowing, whereas in Algeria early sowing required fewer cumulative growing degree days than control sowing. Cumulative growing degree days were calculated assuming the same base temperature in control and early treatments. Different base temperatures were used in the literature. The base temperature used by Hammer et al. (1982) for VE to R1 stage was 6.6°C whereas Villalobos and Ritchie (1992) and Aiken (2005) used a base temperature of 4 °C. Casadebaig et al. (2008) used a value of 4.8°C as in this study. The value of 4.8°C we have used was probably not suitable for forecasting sunflower phenostages under early sowing conditions.

Differences in the thermal time requirement for sunflower phenostages observed between sites and between early and control sowings could also be explained by photoperiod effect. Aiken (2005) has shown that field observations support earlier reports of long-day photoperiod response for sunflower development to the bud-visible (R1) phenostage; a short day response for development to maturity (R9) was most closely correlated with daylight at the floral initiation. Goyne and Schneiter (1987) have shown that photoperiods of 11 through 13h severely delay the rate of development for most genotypes. Therefore, Goyne et al. (1989) have shown no influence of a photoperiod within the range of 14.5h to 16.2h. In our experiments, daylength differences between early and control sowing and between locations were observed (data not shown). Further investigations on temperature x photoperiod interactions have to be conducted.

A significant variability between genotypes was observed for vegetative period and reproductive period (Fig. 3). Concerning the effect of early sowing on phenostage, we have shown in Fig. 3 that genotype ranking was not affected by early sowing for vegetative stage on the two sites. However, genotype ranking was not preserved for the control sowing during post-flowering stage in Algeria. During the grain filling period (R6-R9) high temperatures were observed in Algeria. Moreover, meteorological data showed that there was no rainfall in Algeria during this period. Drought occurring during this period considerably reduced the variability of the phenostage (R6-R9) and modified the genotype ranking.

Genetic basis of sunflower phenostages response to early sowing must be explored, in terms of genetic variability for base temperature and temperature x photoperiod interactions.

ACKNOWLEDGEMENTS

The authors thank CMEP (Comité Mixte d'Evaluation et de Prospective de cooperation interuniversitaire franco-algérien) for supporting this work, Meteo France for Algerian weather data and Michel Labarrère for technical assistance.

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SUNFLO: A joint phenotyping and modelling approach to analyse and predict the differences in yield potential of sunflower genotypes

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ABSTRACT

This work was focussed on improving the description of organogenesis, morphogenesis and metabolism in a biophysical plant model. A greenhouse experiment was carried out to identify and to estimate the phenotypic traits involved in plant productivity variability of 26 genotypes. The ability of the biophysical model to discriminate the genotypes was tested on previous results of a field survey focussed on evaluating their genetic progress since 1960. Plants were phenotyped on 4 areas: phenology, architecture, photosynthesis and biomass allocation. 12 traits or genotypic parameters were finally chosen to account for the phenotypic variability. A biophysical model was especially built to integrate the genotypic parameters and to evaluate their respective contribution to the variability of yield potential. A large phenotypic variability was found for each term of the energetic approach of above-ground biomass production. The biophysical model was able to account for 80 to 90% of observed variability in yield potential. This model was an interesting tool for analyzing the phenotypic variability of complex plant characteristics such as light interception efficiency. This model showed that several ways are possible to reach high yields. Unlike a classical statistic analysis, this approach allowed to highlight some efficient parameter combinations used by the most productive genotypes. The next steps will be to evaluate the genetic determinisms of the genotypic parameters and to test the reliability of the phenotyping approach.

Key words: biophysical model – *Helianthus annuus* – phenotypic expression – phenotypic characterization – sunflower – yield potential.

INTRODUCTION

Current knowledge in biology does not presently allow to link “whole plant” and “molecular” approaches. As a result, complex plant characteristics such as crop yield cannot be grasped by using “molecular” knowledge in "bottom up" approaches. Consequently, journals are crammed with attempts to identify genes that might explain the build up of plant phenotypes in responses to environment. However, strong difficulties have been encountered in attempting to quantitatively relate the information at gene level to its expression in complex phenotypic traits at plant level (e.g. Sinclair et al., 2004). This actual gap between the identification of an allelic combination at genome level and the corresponding phenotype at plant level greatly limits the potential benefits of the bottom-up approaches in improving our understanding of the genotype-environment interactions and the phenotypic plasticity (e.g. Sinclair and Purcell, 2005). At the same time, the biophysical approaches progressed in understanding and in formalizing the interactions between physical environment and plant responses and regulations (e.g. Jones, 1992).

A possible approach to reducing the gap between the molecular and plant levels is the use of models representing the plant as a biophysical system decomposed as a set of functions determining the phenotype built up in response to environment (e.g. Jeuffroy et al., 2006). To get a coherent system, two types of equations have to be combined, the energy and mass balance equations and the biological regulation equations in response to environment. From one degree of breaking down the plant functioning in elementary processes, the parameters of the equations used to describe these elementary processes may be compared to genotypic characteristics (Yin et al., 2004). Then, it is possible to use the quantitative genetic methods, especially heritability calculations and QTL determinations, to evaluate the genetic determinism and the variability of the studied process. Depending on the way the plant response is taken into account, the use of this genetic information in a set of equations describing the plant functions may allow to account for the plant phenotypic plasticity. This approach has been explored for complex traits such as the expansion rate of a single leaf (Reymond et al., 2003). These examples are far from crop yield, in terms of complexity and time and space levels. The more suitable plant representations to tackle the yield variability would be the crop models (Sinclair and Seligman, 1996). Recent studies have attempted to integrate biochemical and physiological information in crop model to improve the heuristic performance of these models in the analysis of phenotypic plasticity (e.g. Hammer et al., 2004).

The objective of this study was to evaluate the ability of a phenotyping approach combined with a dedicated simple biophysical model to account for the genotypic variability of yield potential. Our assumptions was that the genotypic variability of seed yield could be accounted for by using a set of robust equations, well-tested in crop modelling studies, coupled with a few parameters taking into account the observed phenotypic variability of the studied genotypes. This approach included three parts (i) the development of a biophysical model taking into account the specificities of the sunflower biology, (ii) the estimation of genotypic parameters from measurements on a limited number of isolated plants grown in greenhouse and (iii) an independent set of data obtained in a field experiment for the model evaluation. A panel of 26 genotypes was studied mixing historical commercial hybrids (Vear et al., 2006), experimental hybrids and introgression lines between *Helianthus annuus* and *Helianthus mollis*. This panel was interesting for two reasons. First, the 26 genotypes displayed a wide range of phenotypic differences. Previous observations reported differences related to phenology, light interception, biomass production and allocation (Debaeke et al., 2004). Secondly, there were very large differences in seed yield between genotypes. The seed yield of the most productive genotype is five time higher than the introgession lines. Even among the commercial hybrids, differences higher than 40% were observed (Vear et al., 2003). This trait variability and seed yield scale are relevant for evaluating a modelling approach. The genotypic parameters were chosen according to their ability to integrate the specificities of the sunflower biology in the terms of the Monteith generic approach of the biomass production (Monteith, 1977). Beyond the objective to model the seed yield phenotypic variability from genotypic characteristics, this second aim was to evaluate which plant traits highly contribute to the seed yield variability.

MATERIALS AND METHODS

Model development: The model SUNFLO estimates the above-ground biomass production of a sunflower crop from incident radiation and mean air temperature. It works in daily time steps and describes the plant phenology, the plant leaf expansion, the biomass production and allocation. It takes into account the behaviour of various genotypes by the mode of some parameters which are genotype dependent.

The plant phenology is driven by the thermal time. Cumulative thermal time was calculated as the sum of the daily mean air temperature from emergence using a base temperature of 4.8°C common to all genotypes. Four key stages, expressed as thermal dates with genotypic values, were used to delimited periods of plant cycle with changes in plant physiology: the floral bud appearance (E1), the beginning of flowering (F1), the beginning of grain filling (M0) and the physiological maturity (M3) (Table 1).

Assuming the canopy is a homogeneous absorber, the daily radiation interception efficiency (RIE) was estimated from Beer's law using daily LAI and an extinction coefficient (k) determined for each genotype (Table 1). LAI was calculated from the plant density and the plant leaf area able to intercept photosynthetically active radiation. This latter was estimated as the difference between total leaf area and senescent leaf area. Because in sunflower the distribution of the leaf area along the stem showed a bell-shape (Dosio et al., 2003), plant leaf area was calculated from leaf number (N) with a logistic equation with 3 genotypic parameters, A1, A2 and A3, respectively, the maximal plant leaf area, the rank and the area of the largest leaf of the plant (Table 1). The number of leaves increases linearly with cumulative thermal time from emergence to the beginning of flowering. Then the leaf number decreases linearly from the beginning of seed filling to plant maturity as nitrogen moves from leaves to seeds during the monocarpic leaf senescence (Sinclair and deWit, 1975).

The radiation use efficiency (RUE) represents the ability of the crop to convert the intercepted energy into biomass. RUE is known to change during the plant growth cycle (e.g. Lecoeur and Ney, 2003 on pea). A single general pattern of change in RUE over crop development was used for all genotypes. RUE was equal to a minimum up to 300°Cd, then it increased linearly to reach a maximum level at the beginning of flowering. RUE remained constant until the beginning of seed filling, then it declined exponentially to zero upon the plant death. This general pattern was modulated through a genotypic parameter taking into account the different photosynthetic capacities of genotypes relative to Melody. A depressive function of non optimal temperature was applied to RUE, calculated from daily mean air temperature. The above-ground biomass production was calculated from Monteith's formula (1977) linking dry matter production to incoming photosynthetically active radiation through two radiation efficiencies.

The allocation of biomass to seeds was estimated by using two allocation coefficients. The first coefficient determined the fraction of total biomass allocated to the capitulum (HIcap). It changes with thermal time and was modelled as a single logistic function. However, its maximum value reached at physiological maturity was genotype-dependent. A second coefficient corresponded to the fraction of

capitulum biomass allocated to the seeds (HIseed). It was also genotype-dependent. Finally, the seed yield was calculated by multiplying the final biomass and the two allocation coefficients.

Estimation of the genotypic parameter: The estimation of the genotypic parameters was carried out in a greenhouse experiment (Montpellier, southern France). Plants were grown in pots of 7.5 l filled with mixtures of loamy soil and organic compost. There were 10 pots per genotype and they were arranged in order to mimic an agronomic culture density of 6 plants per m². The soil was continuously maintained at water retention capacity by irrigations at least once a day with a modified one-tenth Hoagland solution corrected with minor nutrients. Air temperature and radiative conditions were managed in order to obtain thermal conditions and photoperiod similar to those classically observed in field conditions.

The dates of occurrence of developmental stages E1, F1, M0 and M3 were determined according to the notation proposed by the CETIOM for sunflower on six plants per genotype, twice a week. In addition, the number of visible and senesced leaves were also counted. Architectural measurements were made at the end of flowering when all the vegetative organs were fully expanded. Height, length, width and distance from stem of each blade were measured on 6 plants per genotype with a ruler (± 0.5 mm). In addition, their zenithal angles were measured with a digital protractor (± 0.5°, Pro 360, Mitutoyo, Paris, France). Then, the individual leaf area was estimated from an allometric relationship with the length and the width of the leaf blade. The light interception efficiency, and, thus, the extinction light coefficient (k), were estimated using 3D virtual scenes and a radiative balance model (Rey et al., 2008). The photosynthetic parameter (PS) was estimated from leaf photosynthetic activities measured with a portable photosynthesis system (CIRAS, PP system, UK) with a control radiation level of 1500 µmol m^{-2} s^{-1}. All the genotypic values were normalized with respect to those obtained for Melody.

Yield index and model validation: For a given genotype, the yield index (I) was defined as the ratio between the seed yield of this genotype and the average seed yield of the five oldest genotypes (see Vear et al., 2003 for more details). A yield index was calculated (Imod) for each of the 26 genotypes running SUNFLO with the mean climate conditions observed in Montpellier during the last 25 years. Imod was compared to a reference yield index (Iref). For the genotypes 1 to 20, Iref was taken from Vear et al. (2003). Iref of genotypes 21 to 26 came from experiments where some of genotypes 1 to 20 were grown in addition to the considered genotypes (Vear, pers. comm. for genotypes 21 to 23; Seryes, pers. comm. for genotypes 24 to 26).

Model simulations were tested with an independent data set collected in a field experiment carried out in Montpellier in 2002 with 5 genotypes (Albena, Heliasol, Melody, Prodisol and Vidoc) (see Rey et al., 2008 for more details on experimental design and measurements).

RESULTS

12 phenotypic traits displayed statistical differences between the 26 genotypes and were then considered as genotypic parameters (Table 1). The architectural parameters presented the highest variability with a CV value higher than 20%. The thermal date of the four key developmental stages presented a similar range of variation with CV around 10%. More surprising was the high variability in parameter k, which corresponds to the efficiency of the plant leaf area to intercept the incident radiation. This parameter is generally considered as a species characteristic and close to 0.80 for cultivated sunflower. The obtained range of k values is close to what is observed in the plant kingdom. The 10% difference in photosynthetic activity (PS) was also surprising because it was observed to be among the best commercial genotypes which are considered as optimized on this trait. In term of biomass allocation, almost no variability was observed in the proportion of biomass allocated to the capitulum (HIcap). On the other hand, the capitulum biomass allocated to the seed (HIseed) displayed a high variability with a significant increase in this value among the recent commercial genotypes.

Table 1. Minimum, maximum and mean values of the 12 genotypic parameters displaying significant differences between the studied genotypes

Genotypic parameter		Minimum	Maximum	Mean	CV
E1	(CDD)	425	690	525	0.12
F1	(CDD)	863	1253	989	0.09
M0	(CDD)	1136	1460	1253	0.07
M3	(CDD)	1578	2242	1772	0.09
Nmax	(#)	18.8	42.5	27.3	0.20
A1	(cm^2)	1939	7430	5095	0.25
A2	(#)	11.0	31.5	14.9	0.25
A3	(cm^2)	138	466	343	0.21
K		0.52	0.96	0.85	0.11
PS		0.92	1.02	0.97	0.03
Hicap		0.5	0.55	0.52	0.03
Hiseed		0.40	0.70	0.59	0.14

The ability of SUNFLO to account for the crop functioning was evaluated by comparing the values of a set of simulated variables to observed independent values obtained in Montpellier in 2002. The chosen variables tested the model performance on its major parts which are phenology, architecture and biomass production and allocation (Table 2). A good consistency was seen between observed and simulated values whatever the considered variables. The mean errors on phenology, architecture and total biomass were close to 10% of the observed values. The capitulum biomass displayed the highest mean error with approximately 30%.

Table 2. Comparison of observed or simulated values of the SUNFLO model.

Variables	n	slope	R^2	Mean	RMSE	Bias
Number of leaves	62	1.118	0.949	16.18	2.14	-0.0214
RIE	90	1.011	0.744	0.83	0.088	0.003
Total biomass (g)	35	0.962	0.970	752.1	85.4	14.99
Capitulum biomass (g)	32	0.978	0.904	263.5	77.1	28.32

The yield was simulated for the 26 genotypes with the mean climate data observed in Montpellier during the last 25 years. The emergence date was set on the 15th April. The simulated yield ranged from 119 to 716 g m^{-2} of seeds with a mean value of 447 ± 48 g m^{-2}. The highest simulated yields were close to the values considered as being the biological potential of present genotypes (Connor and Hall, 1997). The simulated yield indices of commercial hybrids ranged from 85 to 167 for, respectively, Peredovik and Melody. The introgression lines had much lower yield indices of below 60. The comparison between the observed and simulated yield indices showed a good consistency between both values (Fig. 1). The slope of the linear regression between observed and simulated values is equal to 1 and the model accounted for more than 80% of the observed variability in the yield index. The mean quadratic error indicated that the model is able to distinguish, in terms of productivity, groups of 3 to 4 genotypes with close yield indices.

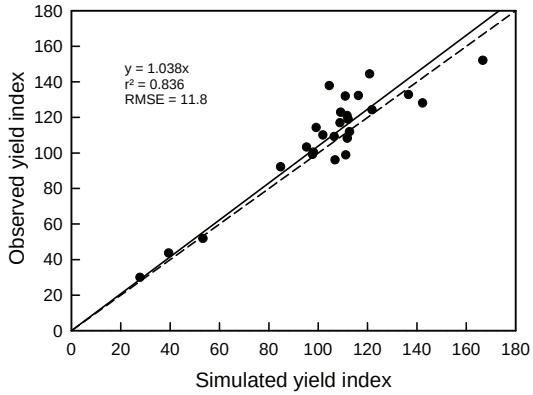

Fig. 1. Comparison of observed and simulated yield indices.

The impact of the variability of the genotypic parameters on the potential yield was estimated through a coefficient of variation (Fig. 2). To estimate this coefficient of variation, a mean value was imposed for all the genotypic parameters except one. The yield indices were then estimated by using the values observed for the 26 genotypes. This approach predicts the existing variability in *Helianthus annuus* species. As the parameter impacts are not strictly additive, the sum of the individual impacts was higher than the total observed variation in yield indices. However, this approach gave some information on the relative weight of the genotypic parameters in the yield variations.

The impact of the genotypic parameter values on the coefficient of variation of seed yield ranged from 0.5% to 14.3% for, respectively, the thermal date of E1 and the coefficient of capitulum biomass allocation to the seeds (HIseed). The other strongest individual impacts were observed for the thermal dates of F1, M0 and M3 and for the maximum plant leaf area (A1) and the position of the largest leaf (A2). When the parameters were bulked according to the major parts of the model, the ranking of the processes in term of their impact on yield variability was, first, the biomass allocation and the light interception through the plant architecture, second, the plant phenology and, far away, the photosynthesis.

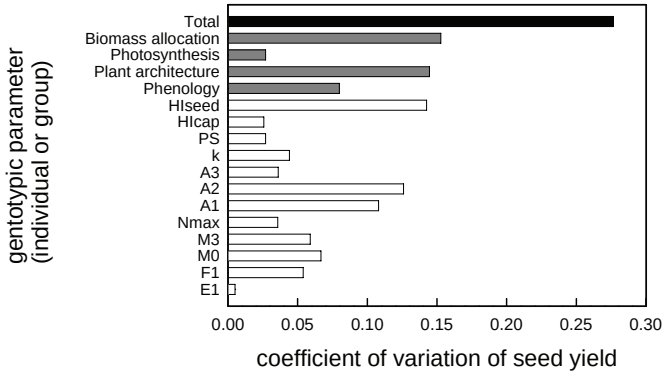

Fig. 2. Relative impact of individual or group of genotypic parameters on simulated seed yield.

DISCUSSION

The proposed approach combining phenotyping and modelling appeared to be relevant for analysing complex phenotypic traits such as seed yield. The estimation of the genotypic parameters on a few plants grown in a greenhouse gave values close to those usually observed in field conditions (Vear et al., 2003). This approach also revealed interesting traits rarely taken into account such as plant architecture, light interception efficiency and photosynthesis. The next steps would be to evaluate the robustness and the reliability of such phenotyping approaches. The more relevant traits might be the target of a more detailed analysis, especially in terms of their genetic determinism (Triboi et al., 2004). At present, this analysis would be greatly slowed down by some phenotypic measurements which are time-consuming. This suggests that simple and rapid methods in measuring phenotypic traits have to be developed. With a broader and genetically organized panel of genotypes, statistical analysis of the parameter combinations

or associations may allow to identify different ideotypes. It may also allow a subsequent analysis of the breeding strategies.

The simple biophysical model SUNFLO was able to account for approximately 80% of the variability in potential seed yields. This result was obtained with a highly contrasting panel of genotypes in terms of productivity. This is promising, although the resolving power of the model is still insufficient. A ten point uncertainty in yield indices is still too high to distinguish the genotypes of one same breeding generation. However, the modularity of the biophysical model is of interest for identifying the strong and weak points of a given genotype. For instance, some genotypes were very efficient in biomass allocation or in photosynthesis but none of them were optimized for all the processes described in the model. This suggests that some progress margins still exist in terms of productivity. These margins may be defined by looking for original combinations of the genotypic parameters corresponding to unknown virtual genotypes, which could be tested *in silico*. This last approach might be a useful tool for increasing the efficiency of breeding programs.

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Root system and water extraction variability for sunflower hybrids

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ABSTRACT

Root traits and soil water extraction of fifteen genotypes were characterized in five greenhouse experiments. The objective was to evaluate the genotypic variability and to identify possible new strategies in plant breeding for drought-stressed conditions. The root traits were characterized at the flowering stage by the root length density (RLD) and the effective rooting depth (Z). The performance in soil water extraction was characterized by the fraction of extracted soil water (EW). It was estimated from soil drying experiments conducted on plants at different stages. Z and EW were used to calculate an indicator of the amount of extractable soil water (EW_{gen}). Wide variability of those traits was observed among the genotypes. Four classes of genotypes were found with a maximal difference of 10% between the extreme values of fraction of extracted soil water. Water depletion kinetics was different between the experiments but the fraction of extracted soil water was stable for each genotype. A large genotypic variability for the indicator of the extractable soil water was also observed. This variability resulted from different combinations of effective rooting depths and fractions of extracted soil water. These traits might be of interest for breeding cultivars well adapted to water stress conditions.

Key words: drought stress – extracted soil water – genotype – rooting depth – root length density – sunflower.

INTRODUCTION

Water deficit is the most predominant abiotic stress experienced by sunflower (*Helianthus annuus* L.) especially because most sunflower crops are cultivated under rainfed conditions (Goyne et al., 1978; Yegappan et al., 1982; Connor et al., 1985). To sustain production in such limiting environmental conditions, sunflower drought tolerance should be increased. It could be done through the selection of plants able to limit the water deficit they undergo under limited soil moisture conditions. One way could be to improve the plant performance in soil water extraction, either by increasing the soil depth explored by roots (Connor and Hall, 1997) or by increasing the fraction of soil water extracted by the plant.

The objective of this study was to evaluate the genotypic variability in the root system architecture and in the soil water extraction for a panel of commercial genotypes. Five greenhouse experiments were conducted between 2005 and 2007 on 15 genotypes. They represented 40 years of currently used cultivars; 10 are old and modern hybrids currently cultivated in France and 5 are experimental hybrids, which could be the next cultivars in France (F. Vear, pers. comm.). The root traits were characterized by the root length density and the effective rooting depth. The performance in soil water extraction was characterized by the fraction of extracted soil water. It was estimated at the end of a drying cycle.

MATERIALS AND METHODS

Plant materials and growth conditions

Five experiments were conducted in a greenhouse in Montpellier (France, 43°35'N and 3°58'E) from 2005 to 2007 (Table 1). 15 genotypes with contrasted phenology, architecture, photosynthesis and productivity were studied (Table 2). Plants were grown in plastic pots in Exp. 1 to 4 and in PVC columns in Exp. 5 filled with a mixture of loamy soil, sand and compost at the same volume. Each genotype was characterized by 6 plants in Exp. 3, 4, 5 and five plants in Exp. 1 and 2. Pots were installed in order to obtain a culture density of six plants per square meter. In order to avoid water deficit, plants were irrigated four times per day with a one-tenth Hoagland solution corrected with minor nutrients. Irrigation was stopped when the plant had 6, 12 or 14 full-expanded leaves respectively in Exp. 1, 3 and 4. In Exp. 2 irrigation was stopped when the plant reached the floral bud stage E1 (CETIOM, 2004). The natural light in the greenhouse was supplemented with sodium lamp (250 µmol m^{-2} s^{-1}) giving a photoperiod of 12h. Temperature in the greenhouse was maintained between 16°C and 30°C. Environmental conditions for the experiments are summarized in Table 1.

Table 1. Mean characteristics of the five experiments

Exp N°	Sowing date	Mean value of Temperature (°C)	Mean value of Vapour Pressure deficit (kPa)	Mean of daily cumulative PPFD (mol $m^{-2}d^{-1}$)	Number of genotypes
1	21 November 2005	18.4	2.12	25.13	15
2	19 November 2006	23.1	2.84	26.19	10
3,5	15 February 2007	23.5	2.91	23.58	10
4	3 April 2007	23.3	2.87	32.69	10

Table 2. Studied genotypes in the different experiments and their registration years

Genotype	Exp N°	Registration year
Peredovik	2, 3, 4	1960
Primasol	1, 2, 3, 4	1978
Albena	1, 2, 3, 4	1988
Vidoc	1, 2, 3, 4	1989
Santiago	2, 3, 4	1993
Melody	1, 2, 3, 4	1996
Sanbro	2, 3, 4	1997
Prodisol	1, 2, 3, 4	1998
LG5660	1	1998
Pegasol	1	2001
VAQxPAR6	2, 3, 4	2003[1]
VDQxOPB4	1	2003[1]
VDQxPPR9	1	2003[1]
XRQxPPR9	1	2003[1]
XRQxPST5	2, 3, 4	2003[1]

[1]Experimental breeding year

Measurements

Environmental conditions were measured continuously for all experiments. Air temperature and relative humidity were measured with a capacitive hygrometer (HMP35A Vaisala, Oy, Helsinki, Finland). Incident photosynthetic photon flux density (PPFD) was measured with a quantum sensor (Campbell PKS 215, Campbell Scientific Ltd, Shepshed, Leicestershire, England). Data were collected every ten seconds and means were stored every 1800s in a datalogger (CR10, Campbell Scientific Ltd).

Plant leaf area was estimated just before stopping irrigation in Exp 1 to 4 and at flowering stage in Exp. 5, by measuring the length and width of leaves. In Exp. 5, soil column was stratified per 10 cm for the first 20 cm layer and per 20 cm for the next. In each layer, roots were harvested and separated in thin or "structural" roots. Roots with a diameter of less than 2 mm were considered as thin. A 2-meter thin roots sample was picked from the first 10 cm soil layer. The root dry weight of this sample (DW_{2m}) and the DW (g) of the two classes of roots were estimated after drying at 60°C for 48h. The root mass length (Lm, cm g^{-1}) was calculated as the thin root length per unit of thin root mass:

$$Lm = 200 \,/\, DW_{2m} \qquad \text{(Eq. 1)}$$

The root length density (RLD, cm cm^{-3}) is the length of thin roots per unit of soil volume explored by the root system. It was calculated for each soil layer as follows:

$$RLD = \frac{Lm \;\; DW_{thin}}{V} \qquad \text{(Eq. 2),}$$

RLD, root length density (cm cm^{-3}); DW_{thin}, dry weight of thin root in the considered soil layer; V, volume of the considered soil layer.

The effective rooting depth for water extraction (Z, cm) was estimated as the root depth for which the root length density was more than 1cm cm^{-3}. As proposed by Gregory (1994), Z was determined from linear regression between the depth of a layer (Y, cm) and the logarithmic value of the root length density.

$$Y = a \ln RLD + Z \qquad \text{(Eq. 3)},$$

Y, soil depth; a, coefficient of root length density distribution; RLD, root length density; Z, effective rooting depth.

In Exp. 1 to 4, a drought stressed treatment was applied stopping the irrigation at a determined phenological stage. The evening prior to the beginning of the treatment, all pots were fully watered and allowed to drain overnight. The following morning, pots were weighed to determine the initial soil water content (SWC_i). To prevent soil evaporation, the pots were enclosed in plastic bags. The plant transpiration rates were estimated by weighing each pot every day. The lower limit of soil water content (SWC_{min}) was assumed to have occurred when the plant transpiration remained constant during several successive days and reached 10% or less than that of well watered plants.

The soil water content (SWC, g g^{-1}) was estimated by weighing soil samples after drying at 105°C during 72 hours.

$$SWC = \frac{FW_{soil} - DW_{soil}}{DW_{soil}} 100 \qquad \text{(Eq. 4)}$$

SWC, soil water content; FW_{soil}, soil fresh weight; DW_{soil},soil dry weight

The fraction of soil water extracted by the plant (EW) was estimated as follows:

$$EW = \frac{SWC_i - SCW_{\min}}{SWC_i} 100 \qquad \text{(Eq. 5)}$$

Estimation of the amount of extractable soil water

The effective rooting depth (Z) and the fraction of soil water extracted by the plant (EW) were used to calculate an indicator of the amount of extractable soil water for each genotype (EW_{gen}, mm) relative to a standard condition. The chosen reference was a sunflower with an effective rooting depth of 1800 mm (Angadi and Entz, 2002) growing in a soil with 0.13 mm mm^{-1} of available soil water (Ratliff et al., 1983). EW_{gen} was calculated as follows:

$$EW_{gen} = \left[\frac{EW_i}{\frac{1}{n}\sum_{i=1}^{n} EW_i} 0.13 \right] \left[\frac{Z}{\frac{1}{n}\sum_{i=1}^{n} Z_i} 1800 \right]$$

(Eq. 6)

EW_{gen}, amount of extractable water for the genotype i; n, number of studied genotypes (10)

RESULTS AND DISCUSSION

Root traits: root length density and effective rooting depth

As illustrated in Fig. 1, the pattern of the evolution of the vertical distribution of root length density was similar for all the genotypes. The root length density decreased exponentially with soil depth. 85% of root length density was observed in the first 40 cm of soil depth (Fig. 1). These results obtained in pot experiments are consistent with previous works in field experiments (Sadras et al., 1989; Cabelguenne et al., 1999; Angadi and Entz, 2002) showing a conical root system. Nevertheless, a large genotypic variability in root length density was observed, especially in the first 0.40 m depth. The mean root length density in the top 1 m soil depth was significantly different between genotypes (Fig. 1 and Table 3). This value varied from 2.39 (Primasol) to 7.65 cm cm^{-3} (XRQ x PST5). Similar genotypic differences in root distribution were reported by Angadi and Entz (2002).

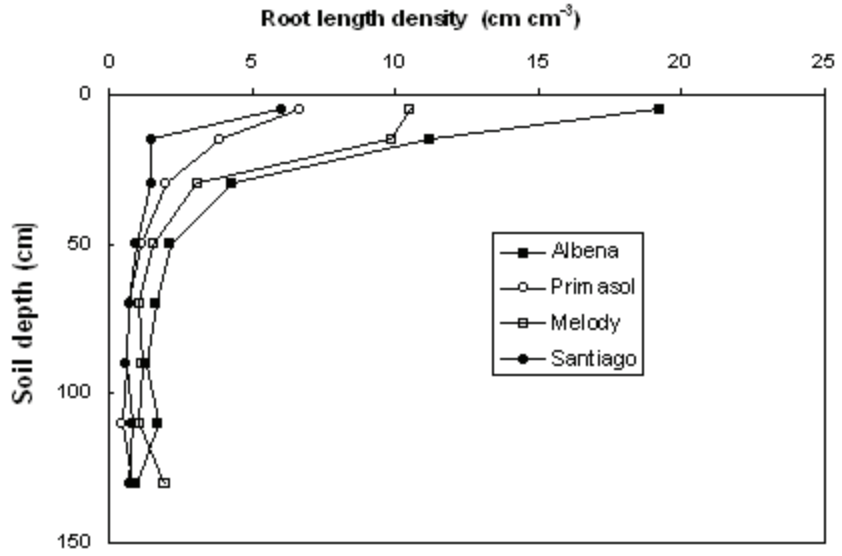

Fig. 1. Vertical distribution of root length density of four contrasted genotypes. Each point is the mean of 6 plants.

Table 3. Effective rooting depth and root length density. Values are average of 6 plants. Genotypes with the same letters did not differ significantly (α = 5%)

Genotype	Effective rooting depth (cm)	Mean root length density in the top 1 m soil depth (cm cm^{-3})
Peredovik	87 abc	5.10 abcd
Primasol	68 b	2.39 a
Albena	105 a	5.69 bcd
Vidoc	81 abc	6.17 cd
Santiago	71 bc	2.45 a
Melody	94 ac	4.52 abc
Prodisol	82 abc	2.91 ab
Sanbro	99 a	4.34 abc
VAQxPAR6	99 a	5.35 abcd
XRQxPST5	104 a	7.65 d

A large genotypic variability was observed for the effective rooting depth (Table 3). Values ranged from 68 cm (Primasol) to 105 cm (Albena). Three classes of genotypes were found, one with an effective rooting depth of below 71 cm, one with an effective rooting depth of over 99 cm and the last one with intermediate values. As all the genotypes were cultivated in identical soil columns, the differences could be attributed to genotypic plant characteristics. Nevertheless, it is worth noting that effective rooting depth in sunflower is also dependent on soil characteristics (Meinke et al., 1993). Different combinations of effective rooting depths and root length density were observed. Some genotypes with an effective rooting depth over 99 cm presented a high RLD as XRQxPST5 or a moderate one as Sanbro (Table 3). Other genotypes with an effective rooting depth of between 71 cm and 99 cm presented a low RLD like Prodisol or a high one such as Vidoc (Table 3).

Fraction of extracted soil water

The comparisons of the soil water depletion kinetics in experiments 1 to 4 revealed significant differences in the mean duration of pot desiccation between cultivars (data not shown). This resulted from differences in environmental conditions between experiments but also from differences in the initial developmental stages of the plants. But variability for soil water depletion duration did not have any influence on the

fraction of extracted soil water between the genotypes. The fraction of extracted soil water (EW) showed significant differences between genotypes (Table 4). Five classes of genotypes were found with a maximal difference of more than 10% between the extreme ones. For example, EW varied from 82.7% for the experimental hybrid VDQxOPB4 to 69.8 % for Peredovik.

Table 4. Fraction of extracted soil water. Values are the average for 5 or 6 plants.

Genotype	*Fraction of extracted soil water*[1] (%)	
Peredovik	69.8	a
Primasol	71.3	abc
Albena	75.1	bcd
Vidoc	75.7	bcd
Santiago	71.4	abc
Melody	70.4	ab
Sanbro	75.8	cd
Prodisol	73.9	abcd
LG5660	73.1	abcd
Pegasol	73.9	abcd
VAQxPAR6	74.8	abcd
VDQxOPB4	82.6	e
VDQxPPR9	76.7	d
XRQxPPR9	70.5	ab
XRQxPST5	71.7	abcd

[1]Genotypes with the same letters did not differ significantly (α = 5%)

These classes were globally the same in the four experiments (Exp. 1 to 4). This result shows that the water extraction ability in sunflower was quite stable and it might be under genetic control. The stability and the heritability of EW should be studied in further experiments.

Genotypic extractable soil water

Significant differences in the indicator of the extractable soil water (EW_{gen}) were observed between genotypes (Fig. 2). Values ranged from 169, for Primasol, to 283 mm for Sanbro. This leads to a maximum difference of 114 mm between the genotypes studied corresponding to 28 - 38% of the amount of water used for a sunflower crop in West of Europe, which is about 300 to 400 mm. In this study, EW_{gen} was estimated for a reference soil with 0.13 mm mm^{-1} of available soil water (Ratliff et al., 1983). This range could be wider under field conditions. Indeed, the amount of available water for a crop depends either on plant or soil characteristics. For one cultivar of sunflower, Meinke et al. (1993) have found a total plant available water for the root profile ranging from 77 to 210 mm for a wide range of soil types.

The variability in EW_{gen} resulted from different combinations of effective rooting depths and fractions of extracted soil water. The lower EW_{gen} was observed in Primasol (Fig. 2), which combined a low effective rooting depth (Table 3) and a low fraction of extracted soil water (Table 4). Intermediate values of EW_{gen} were observed for low or high fraction of extracted soil water as for Melody and Prodisol (Fig. 2 and Table 4). Finally, the best performing genotype for water acquisition was Sanbro, which combined a high effective rooting depth (Table 3) and an intermediate fraction of extracted soil water (Table 4). These results are consistent with those of Angadi and Entz (2002) who attributed greater soil water depletion to deeper rooting depth. No genotype presented both a high effective rooting depth and a high fraction of extracted soil water. No correlation was found between EW_{gen} and the registration year of the genotypes (Table 2). That means that an unexplored source of variability could be used by the breeders to improve sunflower productivity.

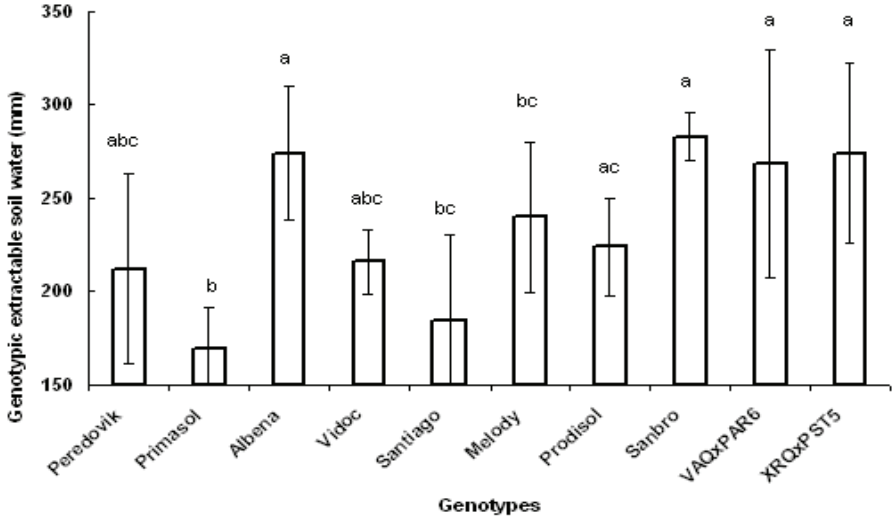

Fig. 2. Amount of extractable water of ten cultivars. Each point is the average of 6 plants. Vertical bars represent the standard deviation. Genotypes with the same letters did not differ significantly (α=5%)

CONCLUSIONS

This study showed a large genotypic variability for the root traits and the soil water extraction: root length density, effective rooting depth and fraction of extracted soil water. No correlation was found between EW_{gen} and the registration year of the genotypes, nor between effective rooting depth and fraction of extracted soil water. The modern genotypes are not better in soil water extraction than old ones. The effective rooting depth and the plant ability to extract soil water could be interesting targets for sunflower breeding programs. Ideotype with a deep root system and a low root density would be suitable under deep soil conditions. In contrast, ideotype with a small deep root system and a high root density would be suitable under shallow soil and limited water conditions.

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Effects of high water table conditions on sunflower growth and quality

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ABSTRACT

Sunflower is one of the major crops for edible oil, which is widely known as a high quality and healthy oil. Recently it was reported that the oil of high oleic acid cultivars had higher oxidation stability and better dietary properties than that of the standard linoleic cultivars. In Japan, sunflower is frequently cultivated as an upland-crop component in rice-based cropping systems. Therefore, an understanding of the effects of water conditions on the growth and oil quality is important. The aims of this paper are to evaluate the effects of water conditions on plant growth, seed yield and oil quality, and to obtain physiological information for increasing yield of sunflower in rice-based upland fields in the central region in Japan. Seed yield and the major yield components, as well as the oil content, were negatively affected by the shallower water table and lower temperature conditions. Regarding their fatty acid composition, the percentage of oleic acid was decreased, and that of linoleic acid was increased with the higher water table. In the next step, the physiological mechanisms behind the effect of higher water table should be elucidated to develop improved management practices for increasing the seed yield and improving the oil quality of sunflower in rice-based cropping systems.

Key words: fatty acid – rotational upland paddy field – sunflower – water table level.

INTRODUCTION

Regarding the effects of short-term waterlogging on sunflower, Orchard and Jessop (1984) reported that the yield was most affected by the waterlogging at anthesis. They also reported that waterlogging at the vegetative and floral initiation stages inhibited the leaf expansion (Orchard and Jessop, 1984). Regarding the soil conditions affected by waterlogging, Orchard and So (1985) reported that availability of oxygen concentration was reduced and ethylene concentration was increased. Hunt et al. (1981) reported that this increased ethylene concentration affected plant growth and reduced root growth in tobacco. Grassini et al. (2007) reported that waterlogging during grain filling caused direct and adverse physiological responses: leaf area, leaf capacity to fix carbon, water absorption, and grain yield were all decreased.

As mentioned above, there are some reports about the effect of waterlogging on sunflower growth, but there are few reports about the effect on oil quality. In the case of soybean, Shimada et al. (1995) reported that the depth of the water table affected chlorophyll contents and yield. The effects on chlorophyll contents varied with the leaf position on the main stem. When the plants were grown with a shallower water table, the lower leaves contained less chlorophyll, and the ripened pod number and 100-seed weight were decreased.

Regarding the fatty acids, there were some reports on the factors changing their compositions. Since waterlogging conditions usually delay the growth stages, the air temperature during the grain filling would be different among the treatments, so that the temperature condition was widely reported as being one of those factors. Nagao and Yamazaki (1984), Sobrino et al. (2003) reported that the oleic/linoleic acid ratio was increased with higher temperature during grain filling. Izquierdo et al. (2006) reported that night minimum temperature during grain filling was better related to oleic acid concentration and its linear increase increased oleic acid concentration. Flagella et al. (2002) reported that oleic acid and stearic acid were decreased and linoleic and palmitic acid were increased under irrigation. But not many papers have been published regarding the change in quality under high water table conditions.

In Japan, the cultivation of sunflower on rice-based upland fields for the purpose of human consumption, and for the use of bio-diesel fuel is increasing. Therefore, the objective of this paper is to elucidate the effects of a shallow water table on the growth, yield and quality of sunflower.

MATERIALS AND METHODS

Field experiments

Experiment I was conducted in 2005 in two farmers' upland fields after irrigated rice at Tsukubamirai city in Ibaraki prefecture. Soil moisture conditions were different between the two fields (Fig. 1). One field (F7) was wetter than the other (E7). Twenty-one hybrid varieties of sunflower were cultivated in them. In 21 cultivars, 9 were linoleic type, 11 were middle oleic type and one was high oleic type. They were sown on May 30 and 31 at 30 x 60cm or 30 x 80cm spacing. A compound fertilizer was applied at the rate of $N\text{-}P_2O_5\text{-}K_2O$ = 8.4-8.4-8.4 g/m^2.

Stem length, disk diameter, number of seeds in a disk, flowering date, and maturing date were measured. Soil moisture content was measured by a soil moisture probe (Profile Probe PR2, Daiki Rika Kogyo Co. Ltd., Tokyo) at depths of 10, 20, and 30cm. Yield of each plot was determined from harvest of 1.44m2 . All treatments were replicated two or three times depending on the varieties.

Experiment II was conducted in 2007 on an artificially sloped plot at Ibaraki Agriculture Institute at Ryugasaki city in Ibaraki prefecture. The slope, 8.3 m in length and 86 cm in height at one end gave a set of 10 rows with different water-tables. The ditch surrounding the sloped plot was constantly filled with water after June 7. Two hybrid varieties were used. One was traditional type; Hybridsunflower (Kaneko Seeds Ltd. Gunma), and the other was NuSun type, 63M80 (Pioneer Hi-Bred International, Inc., USA). These varieties were sown on June 7 and June 26, 2007. The sowing space was at 20 x 86cm. The ditches around the sloping plot were filled with water from June 7 until the end of the experiment. Soil moisture contents were measured as in Exp. I at a depth of 20, 40, 60 and 80 cm. Fertilization and observed parameters were the same as in Exp. I.

Sample and data analysis

Sampled seeds were air-dried. Two g seeds from each sample were crushed and the oil was extracted with *n*-butyl alcohol. The measurement of oil content and fatty acids composition in total fatty acid were determined by the Caviezel method (Pendl et al., 1998) using a gas chromatograph (B-820, Nihon Büch Co. Ltd., Tokyo). The content of fatty acids was calculated from the peak areas and calculated as the percentage of the total fatty acid content.

Statistical analysis

The results were analyzed by ANOVA. All statistical analyses were performed with SPSS 11.0 for Windows (SPSS, 2001). All values are expressed as mean values. Significant statistical differences between treatments were established by the Tukey's test at $P<0.05$. A correlation was calculated with the values of the different parameters. The significance levels ($P<0.01$) are based on the Pearson coefficients.

RESULTS

Exp. I.

Soil moisture conditions in the sloping plot

The first flowering date was July19. and the last maturing date was Sept. 13. During this period, soil water content was always higher in F7 than in E7. The largest differences were observed in the row with the water-table of 20cm in depth (Fig. 1).

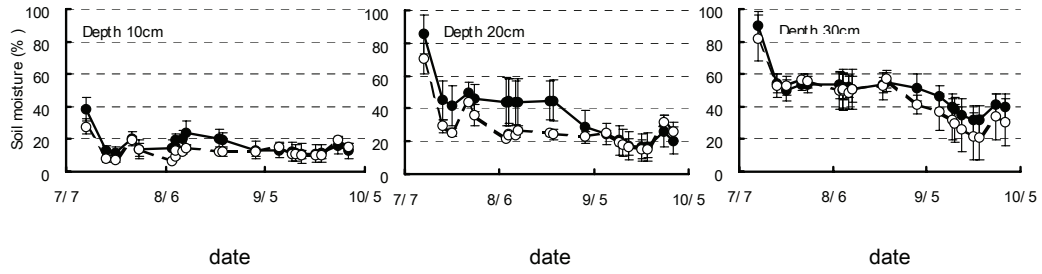

Fig. 1. Soil moisture conditions in the two fields. F7 ● , E7 ○

Effects of soil moisture conditions on growth, yield and quality

The differences in growth and yield between the two fields are shown in Fig. 2. The reductions in stem length in F7 (higher soil moisture condition) were measured in all cultivars. The responses of numbers of seeds in a disk were different depending on the cultivars: that of Hybridsunflower increased and that of 63M02was decreased in F7 field. Their yields in F7 field (higher soil moisture condition) were almost always lower than those in E7 (lower soil moisture condition) (Fig. 2). In this study, the decrease in oil content and oleic acid composition and the increase in linoleic acid composition were observed at the harvest in higher soil moisture conditions. But the order of high or low oil content and oleic acid and linoleic acid composition in cultivars was unchanged in two water table conditions. Regarding the correlations of some traits with the yields, numbers of seeds in a disk had a significant correlation with the yields in both fields (data not shown).

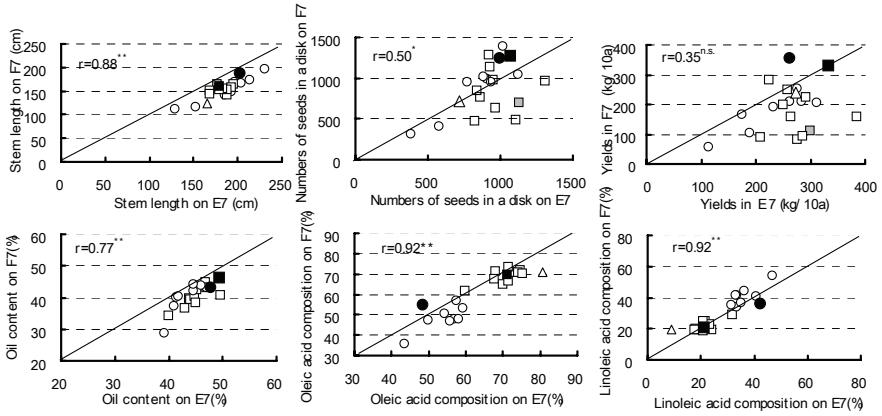

Fig. 2. Growth, yield and harvest quality in the two fields.
○:Trad. (●:Hybridsunflower),□:NuSun (■:63M80, ▣63M02), :High oleic

Exp. II.

Effects of soil moisture conditions on growth, yield and quality.

Soil moisture contents at different depths in the sloping plot are shown in Fig. 3. The largest difference in soil moisture content was found at the depth of water table of 40 cm. The mean air temperature during the ripening period of each treatment (water-table depth) is shown in Fig. 4. Except for the cases at depths of 0 cm and 9.2 cm, there were no large differences in mean temperature and accumulated temperature (data not shown) between the treatments of the same sowing date (Fig. 4).

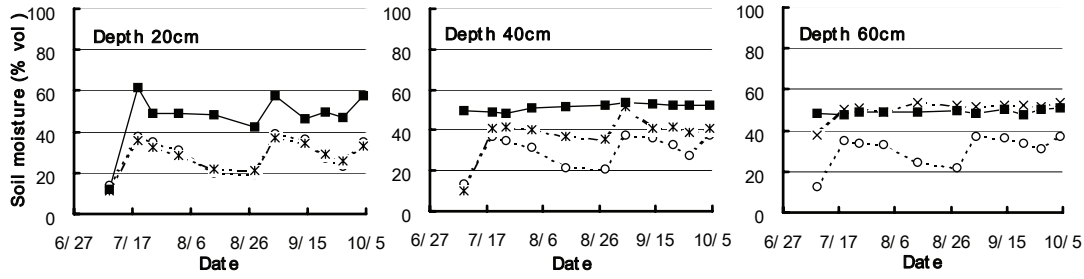

Fig. 3. Soil moisture conditions on the sloping field. Depth to water table; ○:86cm, ×:47.6cm, ■:9.2cm

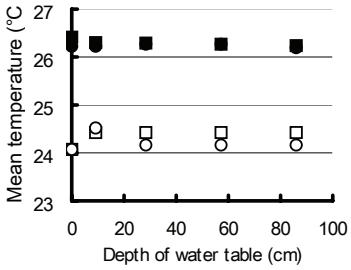

Fig. 4. Mean temperature during ripening period.
Sown on June 7.●Hybrid. (Trad.) ■63M80 (NuSun)
Sown on June 26.○Hybrid. (Trad.) □ 63M80 (NuSun)

The effect of different water table conditions on the growth of sunflower is shown in Fig. 5. Stem elongation, diameter of a disk and yields were significantly reduced with shallower water table. The stronger response to the water table rise was seen when the depth of water table was shallower than about 30 cm (Fig. 5). The growth stage when the water treatment started was different between the plants sown on June 7 and those sown on June 26. The decrease in oleic acid composition ratio and the increase in linoleic acid composition ratio with a rising water table were somewhat clearer for the plants sown on June 7 (Fig. 5).

These experiments demonstrated that the shallow water table affected not only the growth and yield but also the quality of the harvest even under the same temperature conditions.

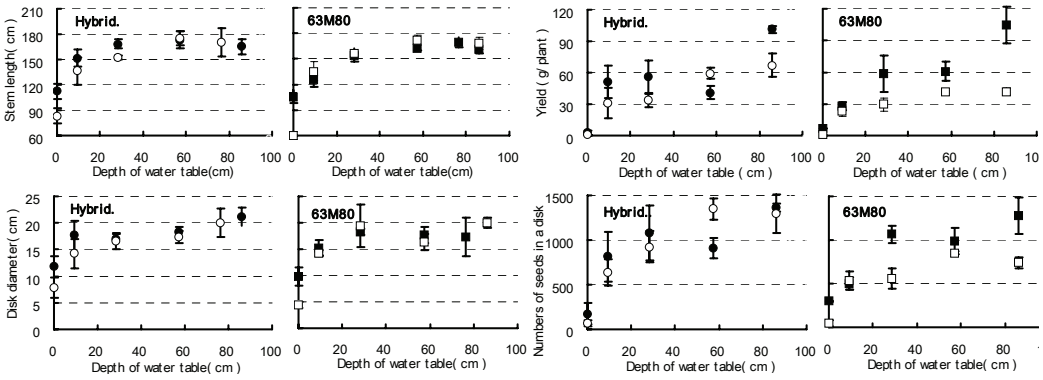

Fig. 5. Growth and yields on the sloping field.
Sown on June 7.●Hybrid. (Trad.) ■63M80 (NuSun)
Sown on June 26.○Hybrid. (Trad.) □ 63M80 (NuSun)

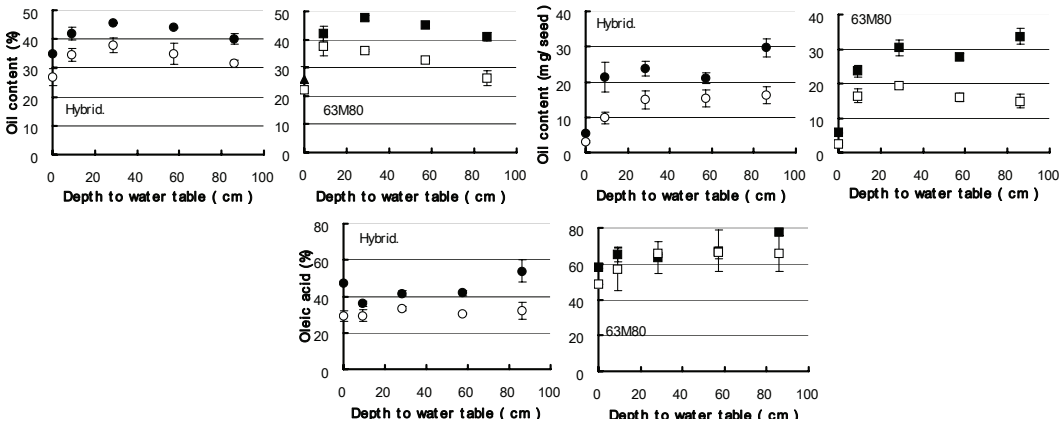

Fig. 6. Harvest quality on the sloping field.
Sown on June 7.●Hybrid. (Trad.) ■63M80 (NuSun)
Sown on June 26.○Hybrid. (Trad.) □ 63M80 (NuSun)

DISCUSSION

The increase in oleic/linoleic acid ratio and the decrease in oil content with increasing temperatures during grain filling have been widely reported. Izquierdo et al. (2006) reported that increasing night temperature resulted in higher oleic acid concentration, and Flagella et al. (2002) reported that a decrease in the oleic/linoleic acid composition ratio was observed in early sowing treatments (lower mean temperature) and under irrigation. In this study, a similar tendency was shown so that the decrease in oleic acid composition and the increase in linoleic acid composition were observed at lower temperature conditions and rise of water table. The oil content (%) was highest at the water table of 30 cm. With a deeper water-table the oil content was decreased. Oil content per one-gram seeds reached the highest at the same water-table depth. After that it remained unchanged. The oil accumulation in seed was presumably most active at around 30cm depth of water table, a comparatively shallow depth. In the case of soybean, Shimada et al. (1995) reported that the fluctuation of the water table reduced the yield. In this experiment, the water table condition was constant, which might have affected the results that even when the water table was shallow, around 30cm in depth, the oil content was the highest.

As a further step, it is necessary to gain a deeper insight into the physiological mechanisms behind the effect of excess moisture in order to increase seed yield and improve the harvest quality.

ACKNOWLEDGEMENTS

The authors would like to thank the staff of the National Agricultural Research Center and the staff of Ibaraki Prefectural Agricultural Experiment Station for their help in data collection.

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Optimizing of potassium and magnesium fertilizers in sunflower production

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ABSTRACT

A factorial experiment with a randomized complete block design (RCBD) with three replications was carried out during 2001 at Khoy Agricultural Research Station to evaluate the main and interactive effects of potassium and magnesium on the yield and quality of Golshid variety of sunflower (*Helianthus annuus* L.) crop with due considerations to the antagonistic effects between these two nutrients in the context of determining the optimum levels of fertilizer application for the best yields as is often the main objective of plant nutrition research. The treatments included four levels of potassium (K_0=0, K_1=45, K_2=90 and K_3=135 kg K_2O as potassium sulfate per hectare) and four levels of magnesium sulfate (Mg_0=0, Mg_1=50, Mg_2=100 and Mg_3=150 kg magnesium sulfate per hectare). The results showed that the sunflower seed yield increased with increasing levels of potassium up to K_3=1.5 times the SWRI′s recommended rate which, combined with magnesium sulfate at a rate of 100 kg ha^{-1}, yielded the best with no significant increases at higher rates. The best rate for magnesium sulfate turned out to be 50 kg ha^{-1}. The application of potassium and magnesium increased thousand seed weight but no effect was seen on plant height or the diameter of the plant stem. The diameter of the sunflower disc improved with the application of potassium and magnesium but the effect was not significant. However, the best seed yield was obtained with the treatment K_2Mg_1 and the best disc diameter and the weight of a thousand seeds index were obtained with the treatment K_3Mg_2. Finally, the best ratio for the rates of potassium to magnesium was determined to be about 3.

Keywords: magnesium - potassium - seed yield - sunflower

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the high nutrient demanding plants (Glas and Kassel, 1988; Lie, 1996; Sepehr and Malakouti, 2002), often responding positively to the application of chemical fertilizers; however, despite the fact that there is a good potential for high sunflower yields in Iran, its cultivation in poor. Calcareous soils along with poor fertilizer programs (imbalanced use of fertilizers) have produced low yields causing too little interest in cultivation of this needed oil crop even though some 90% of the vegetable oil must be imported into the country. During the recent years, numerous attempts have been made to increase oilseed yields through fertilizers (Tandon, 1990; Amnuaysilpa et al., 1991; Shinde et al., 1993; Anadurai and Palaniappan, 1994; Krishnamurthi and Marthan, 1996; Malakouti and Sepehr, 2003), but few studies have been carried out with regard to Mg and its interaction with K in Iran. Therefore, the objective of this research was to evaluate the effect of K and Mg and their interactions on the yield of sunflower, and to determine optimal ratios of these nutrients for obtaining maximum yields.

MATERIALS AND METHODS

A factorial field study in a randomized complete block design (RCBD) with three replications was carried out during 2001 at Khoy Agricultural Research Station to evaluate the main and interactive effects of potassium and magnesium on the yield and quality of Golshid variety of sunflower (*Helianthus annuus* L.) crop with due considerations to the antagonistic effects between these two nutrients in the context of determining the optimum levels of fertilizer application for the best yields, often the main objective of plant nutrition research. Soil was clay-loam, low in organic matter, with a pH=8.1, and a total neutralizing value (TNV) of 14.8%. Extractable K and Mg were 190 and 440 mg/kg of soil, respectively. The treatments included four levels of potassium (K_0=0, K_1=45, K_2=90 and K_3=135 kg K_2O as potassium sulfate per hectare) and four levels of magnesium sulfate (Mg_0=0, Mg_1=50, Mg_2=100 and Mg_3=150 kg magnesium sulfate per hectare). Other fertilizers were applied at uniform rates of 350 kg ha^{-1} urea-N, 100

kg P_2O_5.ha^{-1} as triple super phosphate (TSP), iron sulphate (80 kg ha^{-1}), zinc sulphate (40 kg ha^{-1}), manganese sulphate (40 kg ha^{-1}), and boric acid (20 kg ha^{-1}) on all plots.

RESULTS

The results showed that the sunflower seed yield increased with increasing levels of potassium up to the rate of K_3, which is 1.5 times the SWRI's recommended rate. When combined with magnesium sulfate, outyields of up to 100 kg ha^{-1} were reached, with no significant increases at higher rates (Table 1 and 2), The best rate for magnesium sulfate turned out to be 50kg ha^{-1} (Table 2). The application of potassium and magnesium increased the thousand seed weight, but no effect was seen on the plant height or the diameter of the plant stem (Table 1 and 2). The diameter of the sunflower disc improved with the application of potassium and magnesium, but the effect was not significant (Tables 1 and 2). However, the best seed yield was obtained with the treatment K_2Mg_1 and the best disc diameter and the weight of a thousand seeds index were obtained with the treatment K_3Mg_2 (Fig.).

Table 1. The effect of K rates on the seed yield, 1000-seed weight, and head diameter

Treatments	Rates (kg K_2O.ha^{-1})	Seed yield (kg ha^{-1})	1000-seed weight (g)	Head diameter (cm)
K0	0	3417 b	69.33 b	19.94 a
K1	45	3748 ab	71.20 ab	20.05 a
K2	90	3869 a	72.75 a	20.08 a
K3	135	3939 a	72.83 a	20.98 a

Table 2. The effect of Mg rates on the seed yield, 1000-seed weight, and head diameter

Treatments	Rates (kg $MgSO_4$.ha^{-1})	Seed yield (kg ha^{-1})	1000-seed weight (g)	Head diameter (cm)
Mg0	0	3500 b	69.88 b	19.67 a
Mg1	50	3948 a	71.17 ab	20.23 a
Mg2	100	3928 a	71.83 ab	20.93 a
Mg3	150	3597 ab	73.31 a	20.22 a

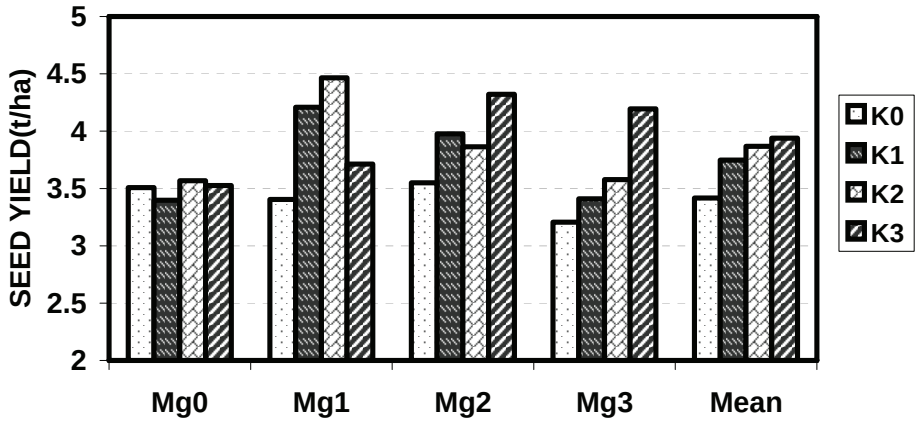

Fig. 1. The effects of potassium and magnesium on the seed yield.

DISCUSSION

According to this study, in order to obtain high yields, potassium and magnesium fertilizers must be applied in the right proportions, and the best ratio for the rates of potassium to magnesium was measured to be 3 for sunflower production in the Khoy area. Although the soils of the experiment site contained high levels of Mg, more than the total sunflower uptake during the growing season, since most of this nutrient (90%) was absorbed in the short period of flowering (Glas and Kassel, 1988), the rate of supply by the soil was not enough to meet the plant demands. Besides, our method of measuring soil Mg by acetate may not be accurate in estimating plant available Mg since some of the soil Mg may be in the form of insoluble salts like $MgCO_3$, not available to plants but extractable with acetate.

ACKNOWLEDGEMENTS

We would like to thank all the colleagues and researchers at the Soil and Water Research Institute (SWRI); West Azerbaijan Research Centre and the Agricultural Research Station of Khoy.

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Sunflower response to mineral nitrogen, organic and bio-fertilizers under two different levels of salinity

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ABSTRACT

This investigation was carried out during the two summer growing seasons of 2005 and 2006 at two locations in the north-east of the delta of Egypt. The first site (S1) is characterized by a good clay soil with fresh irrigated water while the second site (S2) has a salt-affected soil and is irrigated with a mixture of fresh and drained water. Two open pollinated cultivars of sunflower (Sakha 53 and Giza 102) were used. Seven different packages of the combinations of bio (cerealin), organic and mineral nitrogen fertilizers were used as follows: T1 is the recommended chemical nitrogen fertilize (45 Kg N/fad) (fad= Faddan = 4,200 m^2), T2 (1/2 N +30 m^3/fad of organic fertilizer) , T3 (bio fertilizer + 1/2 T1), T4 (bio fertilizer + 30 m^3 /fad organic fertilizer + 1/2 of T1), T5 (bio fertilizer + 30 m^3/fad organic fertilizer + 1/4 of T1), T6 (30 m^3 /fad of organic fertilizer + 1/2 of T1) and T7 (bio fertilizer + 30 m^3 /fad organic fertilizer). The results obtained showed that the application of farmyard manure in T4 has increased yield and yield component traits compared with the control treatment at S1 location. Head diameter, number of seeds per head, 100 seed weight, seed yield per plant and seed yield per plot were increased by 3.4, 13.4, 4.7, 12.8 and 16.8%, respectively, compared with the control treatment. T5 recorded the next rate of increase for the same traits by 2.2, 5.8, 5.8, 8.7 and 11.2%, respectively. The application of mineral nitrogen or organic manure has increased protein content in the good soil, while the mineral nitrogen alone (45kg N/fad) surpassed all other treatment in the salt-affected soil.

Key words: bio-fertilizer – mineral nitrogen – organic fertilizer – salinity

INTRODUCTION

Sunflower (*Helianthus annuus* L.) was chosen in this investigation as it is considered to be one of the most important promising oil crops in Egypt and it could be successfully grown in a great range of climatic conditions and soils. It could also play an important role in the cultivation of the new reclaimed lands, which are suffering drought, high temperatures and salinity effects. Organic and bio fertilizers were studied in this investigation as a replacement of part of the chemical nitrogen to reduce the total cost of cultivation and the chemical nitrogen pollution, and to improve the soil physical and chemical structure.

Singh et al. (1995) pointed out that oil content in sunflower seeds was reduced as the nitrogen increased from 40 to 80 kg N/ha. Singh et al. (1998) studied the content and uptake of nutrients by the sunflower crop as affected by *Azotobacter*, farmyard manure and NP levels. They showed that application of farmyard manure at 10 ton/ha, significantly improved the nitrogen and phosphorus contents in seed in both seasons and potassium content in second year only. In addition, they reported that the seed yield of sunflower was significantly higher with the farmyard manure (FYM) than with no FYM and *Azotobacter* inoculation treatments. El-Bana (2000) found a significant increase in oil yield per faddan (fad= Faddan = 4200 m^2) caused by the addition of organic matter and bio fertilizer (Cerealin). The interaction between organic matter application and inoculation with cerealin significantly increased seed oil content. Abou-Khadrah et al. (2002) pointed out that 100-seed weight, seed yield/plant and seed yield/fad were significantly increased by increasing nitrogen levels up to 45 kg N/fad.

MATERIALS AND METHODS

This investigation was carried out during the two summer growing seasons of 2005 and 2006 at Gamalia Dakahlia and at EL-Serw Agricultural Research Station in north east of the delta of Egypt. The first site (S1) has a good clay soil, while the second one (S2) is characterized by a salt-affected soil which is irrigated with a mixture of fresh and agricultural drained water.

Seven different packages of combinations of bio fertilizer, as cerealin, organic fertilizer as a farmyard manure (FYM) and mineral nitrogen (N) fertilizer besides the recommended rate of nitrogen were used as

follows: T1 (45 Kg N/fad as the recommended chemical nitrogen fertilizer), T2 (30 m^3/fad FYM +1/2 T1), T3 (Bio fertilizer +1/2T1), T4 (Bio fertilizer +30 m^3/fad FYM + 1/2T1), T5 (Bio fertilizer +30 m^3/fad FYM + 1/4T1), T6 (15 m^3 /fad FYM + 1/2T1) and T7 (Bio fertilizer + 30 m^3 /fad FYM) (fad= Faddan = 4200 m^2).

The nitrogen fertilizer used, was a form of ammonium nitrate (33.3% N). The analysis of the farmyard manure (FYM), which was used as an organic fertilizer during both seasons (2005 and 20006), were: Moisture = 30%, 34 % ;C/N ratio = 11.92, 12.04; Organic matter = 10.40, 10.63 %; N = 0.51, 0.58%; P = 0.30, 0.27%; K = 3.74, 3.96 %; EC dS/m = 3.12, 3.27 and PH = 7.51, 8.04, respectively.

The soil salinity was 832 and 858 ppm at the site of Gamalia (S1; conventional soil) in both seasons, respectively, while at EL-Serw Agric. Research Station (S2; saline-affected soil) it was of 3140 and 3789 ppm, respectively. The electric conductivity of the irrigated water for S1 was 0.40 and 0.37 dS/m, while in S2 it was 1.60 and 1.51 dS/m for the first and the second seasons, respectively.

Seven random plants from the inner rows of each sub plot were taken at harvest time to determine plant height, number of leaves per plant, stem diameter, head diameter, number of seeds per head, 100-seed weight and seed yield per plant and the whole seed yield per plot were recorded.

The experiment plot contained 4 ridges (0.60 m width x 4 m long) and seeds were sown in hills (30 cm apart) on one side of each ridge and surface irrigation was used.

All data were subjected to the appropriate statistical analysis of variance as outlined by Snedecor and Cochran (1980). Data of the two seasons were compared by using the Least Significant Difference Test (LSD).

RESULTS AND DISSCUSION

The means of vegetative growth traits, yield and yield components, and oil and protein contents of the two varieties of the combined data over the two locations are presented in Tables 1, 2 and 3.

Vegetative growth traits

At the first site (S1), which was characterized with good soil, data obtained (Table 1) indicated that plant height under the fertilization treatment T4 showed a significant superiority over the control treatment (45 kg N/fad) and, to different extents, over the other treatments. The tallest plant (236 cm) was recorded with the higher FYM application (T4 treatment) followed by T5 treatment and the shortest plants were obtained with T7. On the other hand, at El-Serw site (S2), it was found that, with the exception of T2 treatment, plant height was significantly less under all fertilization treatments than under the control (T1) treatment. Data also revealed that the number of leaves/plant was significantly affected by all treatments at site S1 only. The highest and lowest values were observed with T4 and T7, respectively, while, there was no significant effect at El-Serw site (S2). These results indicate that the differences between the treatments were not great enough to reach the significant level. Stem diameters of plants were also significantly affected at both locations. T4 treatment recorded the highest value for this trait (Table 1) and showed a significant superiority over the other treatments tested. In contrast, the treatment consisting of the sole mineral nitrogen was the superior one in the second site (S2). These results could be attributed to the negative effects of salinity on the bio and organic fertilizers. At Gamalia (S1), data presented in Table 1 indicated that the highest number of days to 50 % flowering was recorded under the fertilization treatment T4, while T7 (bio. + 30 m^3 OM) recorded the lowest number. In contrast, at El-Serw (S2) location was observed the lowest number of days to 50% flowering (53.9) under the fertilization treatment T7. This means that the response of this trait to the seven selected fertilization treatments at the two locations was not the same and revealed contradictory findings.

Yield and yield component traits

The results presented in Table 2 showed that at Gamalia (S1) the application of organic manure and/ or cerealine to this good soil significantly increased head diameter, number of seeds per head, seed yield per plant and per plot. These increases were more pronounced under T4, but the absence of bio or organic fertilizers reduced the values of the mentioned traits in relation to the control treatment as shown in T2 or T3. Moreover, the rate of these two fertilizers alone gave the lowest values of the same traits and could not compensate for the absence of mineral nitrogen (45kg N/fad). These results indicate that the used rates of the bio and organic fertilizers did not meet sunflower requirement of nitrogen. The data also showed that the highest value of 100 seed weight was obtained under T5 treatments, in which the rate of the mineral nitrogen was reduced, whereas at S2, the T1 (45 kg N/fad) was superior in all studied traits except for the 100 seed weight.

Table 1. Effect of selected fertilization treatments on some vegetative growth traits at Gamalia and El-Serw locations (combined analysis of the two seasons).

Treatments	Plant height cm.		leaves /plant (N0)		Stem diameter(cm)		Days to flowering	
	S1	S2	S1	S2	S1	S2	S1	S2
T1 (45 kg N/fad)	227	126	31.1	27.2	2.22	1.55	56.8	55.7
T2 (1/2 N + 30 m^3 fym)	222	126	31.3	26.4	2.24	1.50	56.8	54.6
T3 (1/2 N. +30m^3 fym)	224	119	31.0	26.4	2.36	1.49	55.5	56.1
T4 (1/2 T1 + bio + 30 m^3org.)	236.	116	33.6	25.0	2.50	1.43	57.8	53.9
T5 (1/4 N + 30 m^3 fym)	228	118	31.8	24.8	2.38	1.21	56.2	56.6
T6 (1/2 T1 + 15 m^3 fym)	225	114	30.9	27.2	2.41	1.32	56.8	54.6
T7 (bio. + 30 m^3 fym)	212	117	30.7	26.6	2.27	1.40	55.3	56.8
L.S.D. $_{0.05}$	6.1	5.9	1.57	-	0.211	0.149	0.684	1.01

S1 = The good clay soil at Gammalia; S2 = The salt-affected soil at El-Serw

Table 2. Combined data of 2005 and 2006 seasons on some yield and yield component traits under different fertilization treatments.

Treatments	Head diameter (cm)		Number of seeds/head		100 seed weight (gm)		Seed yield /plant (gm)		Seed yield /Plot (kg)	
	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2
T1 (45 kg N/fad)	22.0	17.4	1119.2	982.0	6.85	6.93	80.0	67.5	5.33	4.44
T2 (1/2 N + 30 m^3 fym)	21.4	17.2	1170.8	870.0	7.13	6.88	85.8	62.2	5.71	4.26
T3 (1/2 N. +30m^3 fym)	21.6	16.6	1157.3	875.2	6.91	5.79	83.2	53.1	5.69	3.65
T4 (1/2 T1+ bio+30 m^3org)	22.7	16.7	1269.2	893.3	7.17	6.53	90.2	60.6	6.22	4.02
T5 (1/4N+30 m^3 fym)	22.5	16.6	1188.2	856.7	7.27	6.53	87.6	57.1	6.00	3.79
T6 (1/2 T1+ 15 m^3 fym)	21.3	16.8	1156.2	862.5	6.89	6.27	83.2	56.6	5.65	3.75
T7 (bio. + 30 m^3 fym)	20.1	16.4	967.1	738.8	6.69	6.77	66.6	515	4.53	3.55
L.S.D. $_{0.05}$	1.01	0.68	62.8	62.6	0.562	0.359	4.74	3.92	0.151	0.164

Table 3. Combined data of 2005 and 2006 seasons on some quality traits under different fertilization treatments at Gamalia and El-Serw locations

Treatments	Seed oil (%)		Seed protein (%)	
	S1	S2	S1	S2
T1 (45 kg N/fad)	43.1	41.1	16.5	16.3
T2 (1/2 N + 30 m^3 fym)	43.3	40.2	16.9	15.9
T3 (1/2 N. +30m^3 fym)	42.2	41.4	16.1	15.6
T4 (1/2 N + bio.+30m^3fym)	43.0	40.6	17.0	16.3
T5 (1/4N+30 m^3org	41.7	39.9	16.1	16.1
T6 (1/2 N. + 15 m^3 fym)	41.3	42.9	16.0	15.8
T7 (bio. + 30 m^3 fym)	42.5	41.2	17.2	16.6
L.S.D. $_{0.05}$	0.611	0.524	0.416	0.418

The results indicated that the stimulatory effect of the combination of the three fertilizer sources, i.e mineral nitrogen, FYM and cerealine at site 1 in the previous treatments on the seed yield and yield components traits may be attributed to increasing the meristemic and enzymatic activities which encourage plant growth. Meanwhile, the release of N of the FYM and cerealine were not enough to compensate for the 50% reduction of chemical nitrogen dosage in S2 location. The salinity may also negatively affect the microorganism activity For this reason, the full dosage of N (45kg N/fad) was superior in the salty soil. These results are in agreement with those obtained by Singh et al. (1998) and Abou-Khadrah et al. (2002).

Quality traits

The results at S1, presented in Table 3, indicated that most seed oil percentages obtained under fertilizer treatments were lower than those obtained under control treatment T1, especially T4 and T6 by 0.2 and

4.2%, respectively, while T2 showed an increment over the control treatment by 0.4%. At site2, data also indicated that seed oil percentage under treatments T6, T3 and T7 showed an increase over the control treatment which was 4.5, 0.8 and 0.4% respectively. However, for fertilizer treatments T4 and T5 oil contents were lower than the obtained under control treatment T1 by 1.0 and 2.9%, respectively. These results indicated that the highest nitrogen fixation gave the highest protein content and the lowest oil content at site 1.

These results may be due to the effect of organic manure by improving the physical structure of the soil and increasing available nitrogen, which reflects the greater growth and, consequently, more absorption of nitrogen and more crude protein synthesis. These results are in line with those obtained by El-Bana (2000) and El-Sadek (2005).

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The effect of different amounts of animal manure on qualitative and quantitative traits of sunflower hybrid varieties

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ABSTRACT

A field study was conducted to investigate the effect of different amounts of animal manure on quantitative and qualitative traits of sunflower hybrid varieties in 2006-2007 at the Uromia Agricultural Research Station, Iran. The experimental design was a completely randomized block with three replications. The first factor was animal manure at 0, 15, 30 and 45 t ha^{-1} and the second factor was 3 hybrid varieties: Euroflir, Alistar and Golshid. The parameters assessed were head diameter, stem diameter, number of seeds per head, 100 seed weight, oil percentage, protein percentage, harvest index, seed yield, biomass and oil yield. Results of this study showed significant effects of different amounts of animal manure and various varieties on head diameter, number of seeds per head, 100 seed weight, seed yield, and oil yield. Comparison of mean data showed that by increasing rate of manure, the head and stem diameter, number of seeds per head, 100 seed weight, seed yield, biomass and oil yield were increased. The results revealed that the Golshid variety had the highest values for all the traits, except oil percentage and harvest index. Correlation analysis showed that seed yield had a significant positive correlation with head diameter, stem diameter, plant height, number of seeds per head, 100-seed weight, protein percentage, biomass and oil yield. According to these results, application of 45 t ha^{-1} animal manure increases the quantitative and qualitative yield of sunflower. Golshid variety was found to be suitable for cultivation in the region.

Key words: animal manure – hybrid varieties – oil yield – seed yield – sunflower.

INTRODUCTION

Sunflower has modest fertility needs, but does respond to animal manure. When following soybeans in the rotation, roughly 30 to 50 t of animal manure per ha are appropriate. Following a non-legume, about 80 to 100 t of animal manure per ha is suitable. Animal manure or a legume cover crop can reduce or eliminate need for N fertilizer (Khajehpour, 1998).

Sunflower (*Helianthus annuus* L.) has relatively proved to be a good oil seed crop in Iran. It is a potential source of high quality edible oil. Due to the increasing edible use of this oil crop, its production has been enhanced rapidly all over the world. Sunflower seed contains 48-52% of good quality edible oil and 40-50% of protein in the meal (Khajehpour, 1998). The oil cake from sunflower is also useful for cow and fish feeding. At present, sunflower is grown in many districts of Iran without proper care. The total cultivation area of this oil crop is limited. The progress in sunflower production has been slow due to the lack of proper production technologies and management practices. Among the several agro-techniques which can enhance the production of yield is the use of proper land preparation, irrigation, fertilizer application, proper plant spacing and other important related factors. So, an attempt has been made to study the effect of animal manure for obtaining a maximum yield of sunflower.

MATERIALS AND METHODS

The experiment was conducted in the experimental field of Uromia Agricultural Research Station, Iran in 2005. The soil of the experimental site was loamy, pH of 8.5, 0.9% organic matter and 0.09% total nitrogen. The unit plot size was 3 by 5 m. The varieties used for the study were 3 hybrid varieties: Euroflir, Alistar and Golshid. The experiment was carried out in a randomized block design with three replications. The row spacing was 60 m. The plant spacing was 25cm. With regard to the results of the animal manure analyses under study, the concentrations of total N, P and K were 1.54, 0.75 and 2.8% respectively. At the time of land preparation, animal manure was incorporated into the soil. At harvest time, 10 plants were selected randomly from each plot and plant height and different yield contributing characters (Table 1) were measured. Grain protein content was also determined by a grain analyzer. The data were analyzed using the SAS statistical package (SAS Institute, 1996) and the mean comparisons

were made following Duncan's multiple range test at P = 0.05 by MSTATC (version 2.10, Inc, Michigan State University). The correlation coefficients between all pairs of traits were determined by the SPSS statistical package (version 10, Chicago, USA).

RESULTS AND DISCUSSION

The data on the effect of the animal manure and the cultivar and their interaction are presented in Table 1. By increasing the rate of animal manure, the head diameter was increased. The maximum head diameter was obtained from 30 t ha^{-1} treated plots. Comparison of different cultivars showed that Golshid cultivar had the highest head diameter at any of the animal manure levels. This result is consistent with previous reports that plant growth, photosynthesis and nutrient uptake are affected (increased) under animal manure treatments (Kandil et al., 1988). Animal manure had also a significant effect ($p \leq 0.05$) on stem diameter, number of seeds per head, 100 seed weight, seed yield, biomass, and oil yield of all cultivars (Table 1). The response of the cultivars differed significantly with increasing animal manure levels ($p \leq 0.05$). Golshid cultivar had the highest value of the above measured parameters at all animal manure rates.

Table 1. Analysis of variance to test the effect of animal manure on yield and yield contributing traits of sunflower[1]

Source	d.f.	Head Ø	Stem Ø	Plant height	Number of seeds per head	100 seed weight	Seed yield	Bio-mass	Harvest index	Oil content	Protein content	Oil yield
Replication	2	ns	ns	ns	**	ns	ns	ns	ns	ns	ns	*
Animal manure	3	**	*	ns	**	**	**	**	ns	ns	ns	**
Cultivar	2	**	**	**	**	**	**	**	**	ns	**	**
Animal manure x cultivar	6	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	*

[1]ns, not significant; *, ** Significant at 0. 05 and 0. 01 probability levels, respectively.

With increasing of animal manure levels, the sunflower stem diameter increased, which may be due to increasing nutrient uptake and translocation and increasing of photosynthesis (Hassanzadeh-Gorttapeh et al., 2006).

In crop production, the most important part is the plant's reproductive stage. If photoassimilates are allocated at a suitable time, the number of filled seed per head increases. Although the important yield components are seed weight and number of seeds per head, heads with a large diameter accompanied by more seeds could result in giving a higher yield (Vannozzi et al., 1987).

The maximum number of seeds per head was found by application of animal manure at 45 t/ha. The results were in close conformity with those of Steer et al. (1984). In general, the application of animal manure improved the yield components of sunflower. The plots treated with 45 t/ha animal manure produced the highest 100 seed weight. The results are in close agreement with the findings of Hassanzadeh-Gorttapeh et al. (2006).

According to the results of this study, we can conclude that the application of animal manure can reduce the input consumption per unit area. Among the cultivars studied, Golshid cultivar gave the highest yield due to its higher head weight, number of seeds per head and 100 seed weight. No significant difference in the oil content among the cultivars studied was observed. Therefore, Gholshid cultivar is suitable and may be recommended for this region. The results are in close agreement with the findings of Ulger et al. (1993) and Singh et al. (1996).

Correlation coefficients between traits are presented in Table 2. As reflected in the literature, the number of seeds per head is the yield component most significantly correlated with grain and oil yield (Connor et al., 1997; López-Pereira et al., 1999; Steer et al., 1984). Moreover, the stronger correlation between grain yield with oil yield, compared with that between oil percentage and oil yield, shows that ultimately higher grain yields will mean higher oil yield for farmers. The highly positive correlation ($r=0.87^{**}$) between grain yield and plant height (Table 2) suggest that the seed yield is positively influenced by biomass, in addition to the number of seeds per head (Kesteloot, 1982; Lakshmanrao, 1985).

Table 2. Correlation coefficients between sunflower traits under treatment with different doses of animal manure[1]

	Head Ø	Stem Ø	Plant height	Number of seeds per head	100 seed weight	Seed yield	Bio-mass	Harvest index	Oil content	Protein content
Oil yield	0.78**	0.81**	0.81**	0.94**	0.83**	0.97**	0.92**	0.72**	0.27 ns	0.40*
Protein content	-0.38*	0.52**	0.46**	0.20ns	0.75**	0.50**	0. 53**	-0.55**	-0.55**	
Oil content	-0.45**	-0.48**	-0.39**	-0.31ns	-0.60**	-0.48**	-0.49**	0.40*		
Harvest index	-0.81**	-0.89**	-0.89**	0.61**	0.76**	0.55**-	-0.88**			
Biomass	0.48**	0.91**	0.89**	0.85**	0.89**	0.96**				
Seed yield	0.81**	0.85**	0.83**	0.92**	0.90**					
100 seed weight	0.77**	0.84**	0.79**	0.69**						
Number of seeds per head	0.78 **	0.77**	0.77**							
Plant height	0.88 **	0.96**								
Stem diameter	0.96 **									

[1]ns, not significant; *, ** Significant at 0. 05 and 0. 01 probability levels, respectively.

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Nitrogen fertilization of high oleic sunflower in wet climate

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ABSTRACT

Fertilization may help crops to yield better. To determine whether meteorological and soil conditions influence the productive response of nitrogen fertilization in sunflower a study was conducted in an Italian interregional project (BIOLI). The effects of nitrogen fertilization on two commercial high oleic varieties (Carnia and PR 64 H 61) were investigated in North East (Udine) Italy in 2005-2006-2007. Nitrogen fertilization gave the best yield at the highest level in Udine in wet and irrigated conditions. In Udine the locally selected high oleic hybrid (Carnia) had the best performance. Nitrogen fertilization is suggested only in good weather conditions and in nitrogen-poor soil. Under drought conditions nitrogen influences plant growth but not yield.

Key words: fertilization – irrigation condition – nitrogen – soil condition – sunflower.

INTRODUCTION

As in other crops, sunflower requires NPK fertilization. In Italy, trials with potassium (K) and phosphorus (P) in the last decade did not show any response in the crop due to the naturally high level of potassium in the soil, at least 160 mg/kg of available K_2O (international method), or due to the large quantity of fertilizer applied, in the effort to build up phosphorus levels. For phosphorus, the levels above 10-20 mg/kg of P_2O_5 in the soil (Olsen method) are maintained by annually applying the amount that was removed by the previous crop. In addition, sunflower has only moderate phosphorus requirements and utilizes mycorrihizas (Glass, 1988).

Nitrogen fertilization is very variable and depends on the amount of the element already present in the soil and the potential yield of the environment. Crnobarac et al. (2004) and Monotti (1978) reported that 100 kg/ha was suitable. Malligawad et al. (2004) expressed the importance of nitrogen combined with phosphorus and potassium and reported better yields when the ratio of the first two elements was between 1.5 and 2.0 (results of two experiments). Steer et al., (1994) reported that sunflower has a high nitrogen requirement. Bonari et al. (1992) associated the needs for nitrogen with available water. Laureti and Pieri (1999, 2001) reported that 40-80 kg/ha (depending on the water available) of fertilizer alone or associated with green manuring was enough. Moreover, according to Merrien et al. (1986), the nitrogen of the soil participates by up to 70% in the plant nutrition and is adsorbed particularly from 40th to 80th days from emergence. When flowering starts (60 days after emergence) the 50% of the nitrogen adsorbed is in the leaf. After that, nitrogen moves in the head and finally in the seeds. The coefficient of nitrogen fertilizer utilization in sunflower is 20-30% (60% in wheat) and that coming from fertilizers is adsorbed starting from flowering.

In an effort to contribute to the debate, under an interregional project, three levels of nitrogen were tested.

MATERIALS AND METHODS

To study the response of two high oleic sunflower hybrids (Carnia and PR 64 H 61) against nitrogen fertilization, three different levels of N (0; 60; 100 kg/ha) were used in two field experiments in two locations during 2005-2006-2007, under irrigated conditions at Udine, North East Italy. The experiments were laid in a randomized complete block design with four replicates with an individual plot size of 279 m^2 (9 x 31 m).

Weather conditions (temperature and rainfall) observed during the experiments are presented in Fig. 1. The average annual rainfall at Osimo is usually half that of Udine. In the experimental year the rainfall at Osimo was normal whereas, in May and June, the levels were below normal in Udine and was necessary to compensate with four irrigations of 30 mm each, every ten days starting from the 10th of May until the 10th June (May 10 and 20; June 1 and 10).

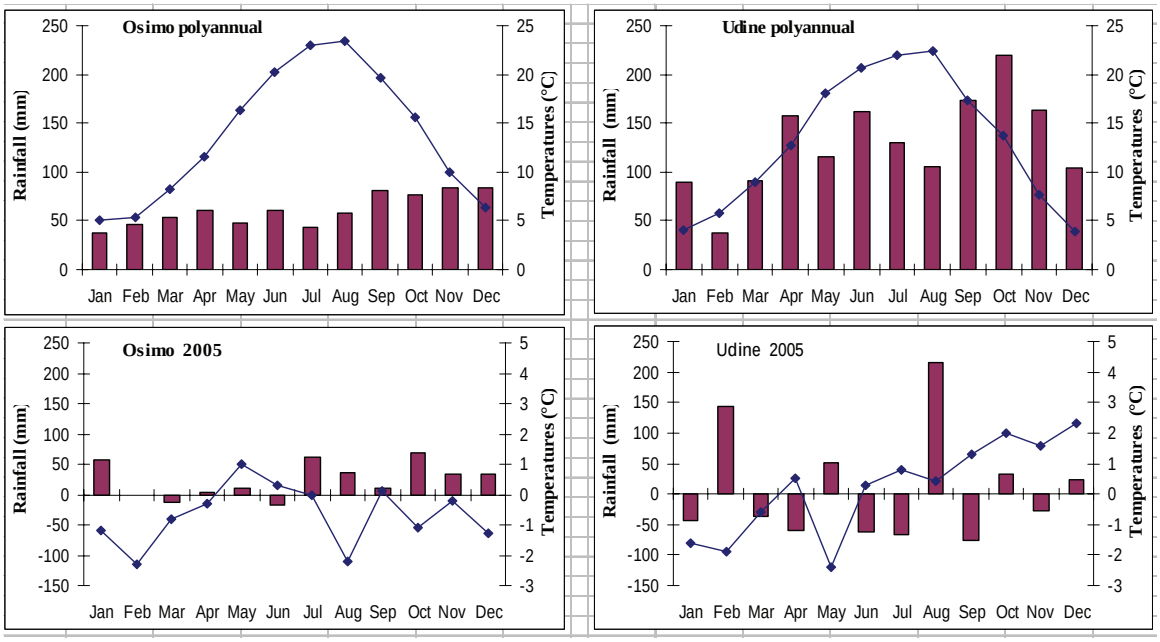

Fig. 1. Rainfall (mm) and mean temperatures (°C) in 2005 compared with the previous polyannual period of 20 years for Osimo and 10 years for Udine.

Soil tests showed high phosphorus and potash levels in both locations but low nitrogen content, especially in Udine (Table 1).

Table 1. Chemical properties of experiment field soils

	Osimo	Udine
Sand g/kg	133	400
silt g/kg	472	430
clay g/kg	395	170
nitrogen g/kg (N)	1.1	0.2
Phosphorus mg/kg (P)	11	41
potash mg/kg (K)	423	200

The soil was a Vertisol in Osimo with good water availability down to a deep level, whereas the soil was gravelly at Udine with good water availability only in the upper 50 cm and very poor water availability at deeper level. To satisfy crop water requirements, four irrigations (30 mm each) were done in May and June in Udine.

RESULTS AND DISCUSSION

Sunflower yield in Italy is greatly dependent on the amount of water stored in the soil and on the amount and distribution of rainfall during the vegetative period. In the summer of 2005 rainfall at Osimo, before blooming and seed filling, was below average so the yield was less than expected based on the plant size. In fact, during the whole cycle the better fertilized plots were always greener, taller and with larger leaves (Table 2). The only datum recorded for this aspect, plant height, was in fact influenced by nitrogen; the plants were taller with higher doses of nitrogen at both Osimo and Udine.

Table 2. Sunflower response to nitrogen fertilization

Nitrogen kg/ha	Yield t/ha		Oil content %		Oil yield t/ha		Thousand-seed weight g		Plant height cm
	Osimo	Udine	Carnia	PR64 H61	Osimo	Udine	Osimo	Udine	
0	2.14	2.27	47.4	48.1	0.91	1.01	62.3	48.2	165
60	2.24	2.27	49.0	47.8	0.96	1.02	62.8	47.6	173
100	2.24	3.28	45.8	47.9	0.94	1.42	62.6	55.6	178
LSD	0.31		1.3		0.14		4.07		4

The data recorded agree with those of Blanquet et al. (1987) who found a weak response whenever water availability was less than 200 mm during the crop cycle. In Udine, on the contrary, the highest nitrogen dose gave the best yield, but the intermediate dose (60 kg/ha) did not differ from the control (Table 2).

The highest yield was due to improved seed weight and number of seeds per plant. The positive response of nitrogen in Udine could be related to the very low nitrogen level in the soil. The improvement in Osimo was not evident because seed set was negatively influenced by the scarcity of rainfall during blooming; the subsequent good meteorological conditions of above average rainfall only produced an increased seed size.

The seed oil content changed as a function of fertilization only in Carnia (Table 2), whose value decreased at the highest nitrogen rate, but not in PR 64 H 61. Oil yield showed the same figures as seed yield, with the higher value only in Udine at the highest nitrogen fertilization.

In spite of good water availability the crop in Udine did not reach the same thousand seed weight (TSW) due to the large number of seeds set.

Yield differences were not observed in the hybrids used in the experiment at Osimo (Table 3) whereas at Udine the locally selected hybrid (Carnia) was significantly more productive than PR 64 H 61 probably due to its higher capacity to set seed. Carnia had also the best oil content at Udine and consequently the best oil yield, whereas at Osimo no differences were found.

Table 3. Variety differences

Varieties	Yield t/ha		Oil content %		Oil yield t/ha		Thousand-seed weight g		Plant height cm	
	Osimo	Udine	Osimo	Udine	Osimo	Udine	Osimo	Udine	Osimo	Udine
CARNIA	2.39	2.81	45.2	49.6	0.89	1.27	60.4	47.7	153	185
PR 64 H 61	2.24	2.17	48.3	47.5	0.99	1.07	64.7	53.2	177	175
LSD	0.25		1.1		0.11		4.07		5.0	

For plant height, PR 64 H 61 was little influenced by water availability, whereas Carnia was more sensitive

CONCLUSIONS

According to the literature, the response of sunflower to nitrogen fertilization is influenced by weather conditions during the season and the natural nitrogen level in the soil.

Under drought conditions and medium natural soil nitrogen content, the response of the crop was evident in the size of the plant but not in its yield. On the contrary, excessive growth could cause lower water use efficiency, but this was not evident in the trials.

Under good water conditions and low nitrogen content in the soil, sunflower responded positively to fertilization; the highest dose improved the amount of seed set, seed size, and, consequently, yield.

The results among the varieties tested were similar in Osimo and significantly different in Udine where the most productive local variety was used.

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Efficiency of modeling sunflower and *Amaranthus retroflexus* L. competition

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ABSTRACT

In order to evaluate the efficiency of empirical models of sunflower (*Helianthus annuus* L.) and redroot pigweed (*Amaranthus retroflexus* L.) competition, factorial experiments were established on randomized complete block design during 2005-2006. Treatments were three weed densities (8.3, 25 and 41.7 plants m^{-2}), three times of weed emergence (15 and 30 days after sunflower emergence) and three sunflower cultivars (Azarghol, Hysun and Allstar). Three weed-free sunflower plots were used as control. Yield was analyzed by three non-linear regression models. Results showed that in any sunflower cultivar, leaf area index (LAI) decreased significantly when weed density increased and redroot pigweed emerged with sunflower, and in full-season competition of 41.7 plants m^2, reduction of LAI in Allstar was two-fold compared with Azarghol. Reduction in Allstar LAI at interference with redroot pigweed took place earlier, compared with Azarghol and Hysun. Azarghol could tolerate 8.3 weeds/m^2 from 15 days and 41.7 weeds/m^2 from 30 days after sunflower emergence. In the short height cultivar Allstar, yield loss was higher than in Azarghol and Hysun. The model of Cousens (1985) was suitable for yield estimation of Hysun and Allstar, while the Model of Cousens et al. (1987) was the best description for the yield of Azarghol.

Key words: interference time – leaf area index – models of competition – redroot pigweed – sunflower – yield estimation.

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the most important oil crops, considerably affected by weed interference. Herbicide weed control is usually expensive. On the other side, redroot pigweed (*Amaranthus retroflexus* L.) is a one of the most troublesome weeds in sunflower in Iran.

The aim of weed management is changing of competitive relationships between weeds and crops (Aldrich, 1984). High competition power increases light interception efficiency (Assemat and Allirand, 1995). In McLachlan et al. (1993) opinion, the inhibitory effect of one plant species in relation to light received by another plant is a main factor in weed-crop competition modeling. As reported by Oliver et al. (1976), leaf area index (LAI) is a suitable physiological trait for evaluation of the amount of competition and production in plants. Between crop yield loss and weed density there is a sigmoidal relationship, which has an asymptote. So that, in low weed density, crop yield loss rate is lower, but with increasing of density, the rate of yield reduction increases, while at higher densities, because of high intra-specific competition between weed plants, yield loss rate decreases again (Beckett et al., 1988). Cousens et al. (1987), Kropff and Lotz (1992) and Cousens (1985) have used hyperbolic equations for modeling the relationships between weed density and crop yield loss. With the application of weed density and interference time-based models, the accuracy of the model will increase (Knezevic et al., 1997; Bosnic and Swanton, 1997). Cousens et al. (1987) developed hyperbolic equations in which crop yield is estimated in relation to density and relative interference time of weeds. Harper (1983) reported that some variables such as density, growth rate and emergence time of weeds are the most important factors in weed-crop competition for light interception.

The objective of this study was to evaluate sunflower vs. redroot pigweed competition using several empirical models previously developed.

MATERIALS AND METHODS

This study was conducted during 2005-2006 in Research Station of Tabriz University (Latitude 38º53'; Longitude 46º17', elevation 1360m), located in the north-west of Iran, with a semiarid and cold climate. The experimental design was a factorial combination of three weed densities (8.3, 25 and 41.7 plants m^{-2}), three times of weed emergence (15 and 30 days after sunflower emergence), and three sunflower cultivars

(Azarghol, Hysun 33 and Allstar RM). Three weed-free sunflower plots were used as control. The fertilizer used before planting was 150 kg ha^{-1} potassium sulfate, 150 kg ha^{-1} ammonium phosphate and 75 kg ha^{-1} urea. Data were analyzed with MSTAT-C software. Comparison of means was done using the Duncan's Multiple Range Test.

To determine the relationship of weed density and sunflower yield, an hyperbolic model (Equation 1) was used, as described by Cousens (1985):

$YL=(I.d)/[1+(.Id)/A]$ where YL is yield loss of sunflower, d is weed density and I and A are coefficients of the model.

For determination of relationship between weed density and emergence time with sunflower yield the model of Cousens et al. (1987), was used (Equation 2):

$YL= (I.d)/[c.t+(I.d)/A]$ where YL is yield loss of sunflower, d and c are weed density and emergence time and I, A and c are coefficients of the model.

One parameter model (Equation 3) was used for determination of relationship between sunflower yield loss and weed relative leaf area.

$YL=(q.Lw)/[1+(q-1)Lw]$ where YL is yield loss of sunflower and q is coefficient of relative damage. Lw is relative leaf area of weed, which was calculated by equation 4.

$Lw=LAI_r/(LAI_s + LAI_r)$ where r and s indicate redroot pigweed and sunflower, respectively.

In order to evaluate the validity of abovementioned yield estimation models, four statistics were used, as follows (Thornley and Johnson, 1990):

Correlation between estimated and observed yield values (equation 5):

$$r = \frac{\sum_{i=1}^{n}(O_i - \overline{O})(P_i - \overline{P})}{\sqrt{\sum_{i=1}^{n}(O_i - \overline{O})^2 \times \sqrt{\sum_{i=1}^{n}(P_i - \overline{P})^2}}}$$

where O_i=observed yield loss, $\overline{O}$ mean of observed yield loss, P_i estimated yield loss and P mean of estimated yield loss.

Mean Percentage Error (Equation 6):

$$MPE=\left[\sum_{i=1}^{n}\left(\frac{|obs_i - sim_i|}{obs_i}\right)\times 100\right]/n$$

Where obs_i and sim_i are observed yield and estimated yield, respectively, and n is number of treatments.

Root Mean Square Error (Equation 7):

$$RMSE=\left[(\sum_{i=1}^{n}(sim_i - obs_i)^2)/n\right]^{0.5}$$

Mean Bias Error (Equation 8):

$$MBE=\left[\sum_{i=1}^{n}(sim_i - obs_i)\right]/n$$

RESULTS AND DISCUSSION

In the three sunflower cultivars, LAI decreased significantly as weed density increased and redroot pigweed emerged simultaneously to sunflower, and in full-season competition of 41.7 plants m^{-2}, reduction of LAI in Allstar was double compared with Azarghol (Table 1). Also, in high densities and early time of weed emergence, LAI and canopy light transmission of redroot pigweed increased significantly, and canopy closure happened earlier (data not shown). In full season competition of 41.7

weeds/m^2, LAI in sunflower decreased from 4.57, 4.20 and 3.90 in the controls to 3.69, 3.12 and 2.56 in Azarghol, Hysun and Allstar, respectively (Table 1). In Allstar, weed density was more effective than weed interference time. For each day delaying weed emergence time, sunflower LAI in i_0-i_{15} and i_{15}-i_{30} decreased 180 and 160 cm^2 per unit area, respectively (Fig. 1). On the other hand, in spite of same interference time duration (15 days) in the above mentioned treatments, reduction value in LAI at i_0-i_{15} was higher than i_{15}-i_{30}, as reported by Knezevic et al. (1994). In that study, when redroot pigweed emerged with corn, LAI in corn decreased by 36%, but in delayed interference time (3-5 leaves stage of corn), LAI reduction value was not significant. It seems that, between physiological characteristics in crops, LAI is more effective in compatibility of crops and influences the amount of light interception by canopy and availability of weeds to light.

Studying the effect of weed density on sunflower LAI at 30-90 days after emergence, it was observed that the difference between redroot pigweed density levels in the three cultivars, especially in Allstar, starts from early growth stages, and difference value is gradually increased. Reduction in Allstar LAI happened earlier, compared with Azarghol and Hysun. In 45-75 days after sunflower emergence, increasing rates of sunflower LAI at i_0, i_{15} and i_{30} were 750, 810 and 850 cm^2/day in Azarghol, 630, 700 and 770 cm^2/day in Hysun, and 550, 590 and 600 cm^2/day in Allstar, respectively (Table 1). Negative growth of LAI in Allstar at interference with redroot pigweed was in advance from 75 DAE, compared with control. This condition which arose from leaf senescence resulting from weed shading, caused a LAI reduction of 410 cm^2/day at 80-85 DAE in Allstar cultivar, while the negative growth of LAI in the control had not yet started. As reported by Hall et al. (1992), Tollenaar et al. (1994), Knezevic et al. (1994), and Bosnic and Swanton (1997), LAI is one of the most important characteristics indicating the competition power of plants and could be used in estimation of crop yield loss at interference with weeds. With regard to the effect of LAI on plant photosynthesis and the effect on yield of the latter, it is expected that redroot pigweed causing a reduction of sunflower LAI, causes also a significant reduction in yield.

Increasing of redroot pigweed density increased weed LAI, but the increased value in delayed weed emergence time was reduced, especially in Azarghol and Hysun (Table 1). Therefore, when increased weed density from 8.3 to 41.7 plants/m^2 at i_0, weed LAI increased from 0.66 to 0.76 (13% increase) in Azarghol, from 0.9 to 1.06 (15% increase) in Hysun and from 1.01 to 1.19 (15% increase) in Allstar. But at i_{30}, the effect of a similar density increase on LAI was not significant in Azarghol, whereas it increased from 0.75 to 0.78 (4% increase) in Hysun and from 0.95 to 1.07 (19% increase) in Allstar. These results showed that the weed interference time was more effective than density in Azarghol and Hysun, but weed density was more effective than interference time in Allstar, and canopy condition was suitable for development of weed LAI in Allstar (Fig. 2).

The three studied cultivars indicated different interactions of densities and interference times of redroot pigweed with grain yield (Table 1). Azarghol could tolerate 8.3 weeds/m^2 from 15 DAE and 41.7 weeds/m^2 from 30 DAE, without significant reduction in yield. Redroot pigweed could decrease sunflower yield of Azarghol only at high densities (>25 weeds/m^2). In Hysun, any of the studied treatments could produce similar yield to the control plot. Significant difference in sunflower yield arising from early emergence time of redroot pigweed was expected, as weed interference time in relation to crops is a main factor in crop yield loss (Kropff et al., 1992; Rajcan and Swantom, 2001).

Allstar experimented higher yield loss compared to Azarghol and Hysun. This was explained on the basis of its shorter stature, which favored the competitive power of redroot pigweed with this cultivar. Knezevic et al. (1997) reported that yield loss values in tall and short sorghum cultivars at interference with redroot pigweed were 16% and 75%, respectively, because of higher LAI in the taller cultivar. In the present study, a higher oil yield was obtained from treatments with greater grain yield, and the lowest oil yield was observed in treatments of full season interference of 41.7 weeds/m2 in the three cultivars.

In Azarghol and Hysun hybrids, correlation coefficients between redroot pigweed relative leaf area and sunflower yield loss were 0.99 and 0.92 and distinction coefficients of the model were 0.41 and 0.75, respectively; Also, higher RMSE values (378.32 and 342.62, respectively) indicated that this model has a low efficiency in estimating yield loss in these two hybrids. We obtained similar results in Allstar (data not shown).

In the comparison of observed and estimated yield values by the model in Hysun and Allstar hybrids, it was observed that MBE, RMSE and MPE values were +0.003, 28.87 and 8.01%, respectively in Hysun, and +0.003, 41.47 and 4.64%, respectively in Allstar. This model was suitable for yield estimation of these two cultivars (Fig. 3, 4).

Correlation between observed and estimated yield values in Azarghol hybrid was 0.99. Values of MBE, RMSE and MPE were calculated as being +0.09, 18.58 and 2.16%, respectively. With regard to reduction in RMSE value in this model as compared with the model of Cousens (1985), it seems that, this

model was the best description for the yield of Azarghol (Fig. 5). Besides, as reported by Bosnic and Swanton (1997), if SE values of model parameters were lower than half the parameter main value, the model has a higher validity for the estimation of crop yield. The accuracy of Cousens et al. (1987) model in the estimation of yield loss in Hysun and Allstar hybrids was lower.

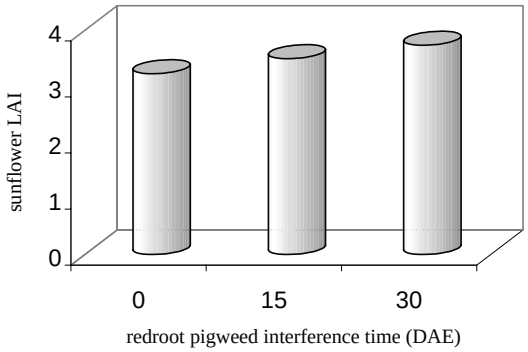

Fig. 1. Effect of redroot pigweed interference time on sunflower LAI at 90 days after emergence

Table 1. Mean comparisons for some of variables studied.

Treatments[1]	LAI of sunflower at 75 DAE[2]	LAI of redroot pigweedat 75 DAE[2]	Sunflower grain yield (kg/ha)[2]
$V_1D_1I_0$	4.07 k	0.66 w	3343 cd
$V_1D_1I_{15}$	4.35 e	0.57 z	3960 ab
$V_1D_1I_{30}$	4.49 b	0.53 z	4061 ab
$V_1D_2I_0$	3.96 i	0.72 v	3109 de
$V_1D_2I_{15}$	4.26 f	0.59 x	3884 b
$V_1D_2I_{30}$	4.39 c	0.55 z	4036 ab
$V_1D_3I_0$	3.69 q	0.76 t	2716 fg
$V_1D_3I_{15}$	4.09 i	0.61 x	3578 c
$V_1D_3I_{30}$	4.37 d	0.56 z	4081 ab
$V_2D_1I_0$	3.52 t	0.90 n	2151 h
$V_2D_1I_{15}$	3.91 m	0.80 q	2726 fg
$V_2D_1I_{30}$	4.11 h	0.75 u	3507 c
$V_2D_2I_0$	3.40 v	0.99 i	2032 hi
$V_2D_2I_{15}$	3.81 o	0.81 q	2556 g
$V_2D_2I_{30}$	4.08 j	0.77 r	3238 d
$V_2D_3I_0$	3.12 y	1.06 h	1722 j
$V_2D_3I_{15}$	3.40 v	0.85 o	2078 h
$V_2D_3I_{30}$	3.77 p	0.78 r	2960 ef
$V_3D_1I_0$	3.42 u	1.01 j	1581 j
$V_3D_1I_{15}$	3.58 s	0.95 m	1793 ij
$V_3D_1I_{30}$	3.62 r	0.90 n	2039 hi
$V_3D_2I_0$	2.92 z	1.09 f	830 lm
$V_3D_2I_{15}$	3.16 x	1.02 i	1032 kl
$V_3D_2I_{30}$	3.24 w	0.99 k	1226 k
$V_3D_3I_0$	2.56 z	1.19 c	535 n
$V_3D_3I_{15}$	2.80 z	1.11 e	651 mn
$V_3D_3I_{30}$	2.91 z	1.07 g	752 mn
V_1D_0 (control)	4.57 a	-	4171 a
V_2D_0 (control)	4.20 g	-	3858 b
V_3D_0 (control)	3.90 n	-	3153 de
LSD	0.005	0.2277	239.7

[1]V indicates sunflower variety; D and I indicate density (plants/m^2) and time of emergence (days after sunflower emergence) of redroot pigweed, respectively.

[2]Values within columns followed by the same letter have no significant difference at the 0.01 probability level.

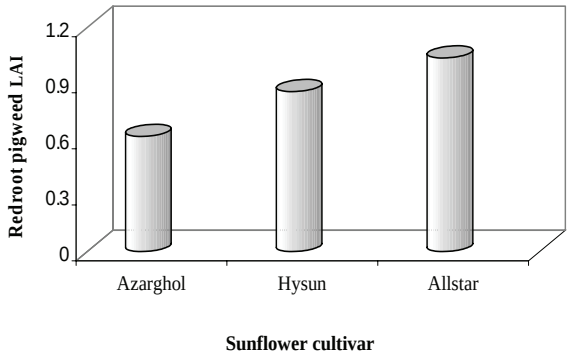

Fig. 2. Effect of sunflower cultivar on redroot pigweed LAI at 90 days after sunflower emergence

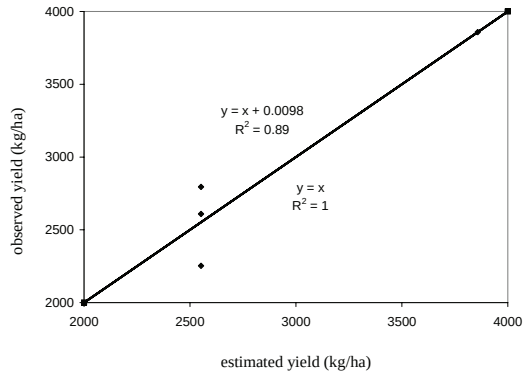

Fig. 3. Comparison of observed yield vs estimated yield by the model of Cousens (1985) at Hysun cultivar. YL=(1.1d)/[1+(1.1d)/33.84], where YL= estimated yield loss and d=weed density.

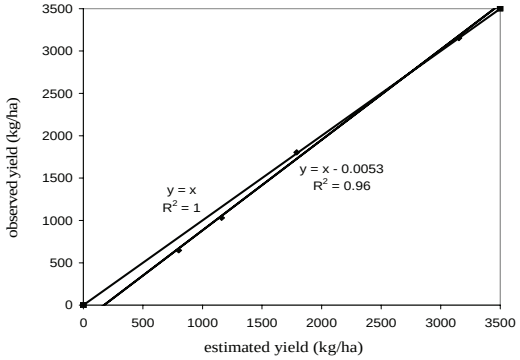

Fig. 4. Comparison of observed yield vs estimated yield by the model of Cousens (1985) at Allstar cultivar. YL=(1.4d)/[1+(1.4d)/23.22], where YL= estimated yield loss and d=weed density.

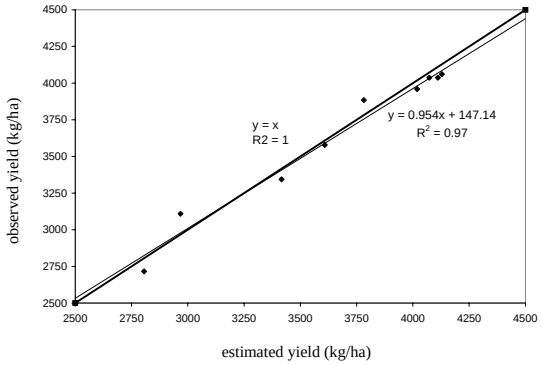

Fig. 5. Comparison of observed yield vs estimated yield by the model of Cousens et al. (1987) at Azarghol cultivar. YL= 4171[1-3.89d/100(Exp(0.14*t)+(3.89d)/41.00)], where YL= estimated yield loss and d=weed density, and t= relative interference time of weeds.

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Sunflower protection from negative effects of 2,4-D

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ABSTRACT

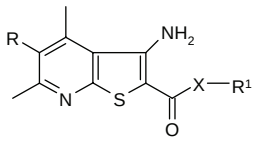
Twelve novel chemical derivatives of thieno[2,3-*b*]pyridines were synthesized and tested, aiming to find new compounds to protect/increase crop selectivity from herbicide impacts. Among these compounds, those with positive effect in reducing the toxicity of 2,4-D on sunflower are herein described.

Key words: 2,4-D – antidote – sunflower – thieno[2,3-*b*]pyridines.

INTRODUCTION

The use of herbicides to control weeds faces the problem of protecting some crops with low selectivity/ tolerance to some of them. Occasionally some compounds are very aggressive to some particular crops, and therefore, could occur a risk of damaging neighboring highly sensitive crops (Pitina et al., 1986; Strelkov et al., 1997). In addition, inaccuracy and mistakes of operators are possible while applying herbicides. Up to date, some approaches to protect crops from damage from herbicides have been developed, including selection of cultivars, agricultural methods, application of sorbing materials, etc. (Pitina et al., 1986, 1994). One promising approach includes a search for and application of chemical antidotes. Earlier, the possibility to use some pharmaceuticals and plant growth regulators to protect sunflower plants from 2,4-D during their growing season was shown by us (Strelkov et al., 1995a,b, 1997).

The objective of this work was to continue a search for new effective substances to protect crops from herbicides similar to 2,4-D. For this purpose, a number of novel chemical compounds have been synthesized by us, which belong to the derivatives of thieno[2,3-*b*]pyridines and have the following common formula:



where R = H, CI; X = O, NH, N; R^1= substituted phenyl, alkyl.

The compounds having this type of structure are known as biologically active compounds with a broad spectrum of activity (Litvinov, 1989); therefore, it seemed very useful to study their plant growth-regulating and antidote activity on sunflower seedling and adult plants.

MATERIALS AND METHODS

Germinating sunflower seeds with the embryo root of 2-4 mm in length were placed in the 2,4-dichlorophenoxiacetic acid (2,4-D) solution at the concentration of 10^{-3} % for one hour to inhibit the hypocotyls growth by 40-60%. After the herbicide action, the seeds were rinsed with water and put into solutions of the compounds tested for their antidote (safener) activity at the concentrations 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} % (herbicide + antidote treatment). An hour later, the seeds were washed with water and laid out on the filter paper bands (10 x 75 cm in size), 20 seeds per band. The bands were rolled up and placed into the beakers containing 50 ml of water. The further seed germination happened in the thermostat at 28°C for three days. The solution and rinsing water temperature was 28°C. The seeds from the "herbicide" treatment (comparison standard) were incubated in the solution of 2,4-D at the concentration of 10^{-3} % for one hour and then in water for the following one hour. The seeds from the control treatment were soaked in water for 2 hours.

The experiment included three replications; 20 seeds per replication were used. A protective (antidote) effect was determined by comparing an increase in hypocotyls and root lengths in the herbicide + antidote treatment with the same values in the herbicide treatment (standard).

The antidote field activity was evaluated in the ARRIBPP experimental field located in the central zone of the Krasnodar Region with moderate continental climate conditions. The soil in the field is a super-deep low-humic leached chernozem. The tests were conducted using the following methods: the sunflower plants of the cultivar Flagman, which were at the 10-16 leaf stage, were sprayed with butyl ether of 2,4-dichlorophenoxiacetic acid at the rate of 18 g/ha and, 5 days later, the tested antidote solution was applied at the rate of 200 g/ha by using the working fluid at the rate of 500 l/ha.

The experiment included the following treatments:

- Control – untreated plants;
- Herbicide (standard) – plants treated with herbicide;
- Herbicide + antidote – plants treated with herbicide and antidote.

The experiments were conducted in the 2.8 m^2 plots, with five replications. The plants were harvested with a Xere – 125 combine at the time of full seed ripeness.

An antidote effect was determined as a percentage by calculating the absolute yield gain value against the herbicide standard according to the formula:

$$A_x = \frac{A - E}{E} \text{ x } 100,$$

A_x – antidote effect, %;

A – yield in the herbicide + antidote treatment;

E – yield in the herbicide (standard) treatment.

The data obtained were statistically processed using Student's t-test at the probability level P = 0.95.

RESULTS

Twelve novel compounds derivatives of thieno[2,3-*b*]pyridines were synthesized using conventional methods (Shestopalov et al., 1988). Under the laboratory experiment conditions it was determined that the synthesized compounds had no growth-regulating effect on sunflower seedlings. At the same time, some compounds having an antidote effect were identified (Table 1).

The compounds deploying the most activity during the laboratory experiment were tested under the conditions of a field small-plot experiment. The results are given in Table 2.

Table 1. Antidote activity of the derivatives of thieno[2,3-*b*]pyridines having a common formula: against 2,4-D in sunflower seedlings (numerator:hypocotyl, denominator:root)

No.	R	X	R^1	Treatment								
				Control A[1]	Standard A	Herbicide + antidote at the below concentrations, %							
						10^{-2}		10^{-3}		10^{-4}		10^{-5}	
						A	B	A	B	A	B	A	B
1	H	NH	2-bromphenyl	65	40	45	113	48	120*	46	115	40	100
				110	44	46	105	56	127*	57	130*	36	82
2	CI	NH	2- bromphenyl	65	40	47	118*	44	110	36	90	40	100
				110	44	57	130*	50	114	38	86	41	93
3	H	NH	3-fluorophenyl	77	36	43	119*	39	100	28	78	36	100
				115	32	47	147*	36	122*	27	84	32	100
4	CI	NH	3- fluorophenyl	74	51	59	116*	57	112	59	116*	55	108
				143	69	73	106	83	120*	81	117*	72	104
5	H	N	dipropyl	70	46	51	111	46	100	46	100	43	93
				125	61	69	113	63	103	61	100	53	87
6	CI	N	dipropyl	70	46	50	109	47	102	49	107	50	109
				125	61	67	110	55	90	61	100	57	93
7	H	NH	2,5-dimethoxy-4- chlorophenyl	73	40	44	110	49	123*	49	123*	44	110
				116	42	56	133*	54	129*	68	162*	60	143*
8	CI	NH	2,5- dimethoxy-4- chlorophenyl	73	40	41	103	34	85	40	97	46	115
				125	61	68	111	67	110	59	100	57	93
9	H	O	benzyl	76	37	49	132*	42	114	42	114	44	119
				120	47	51	109	51	109	38	81	38	81
10	H	O	allyl	76	37	52	141*	44	119	50	135*	49	132*
				120	47	42	89	44	94	42	89	44	94
11	CI	O	benzyl	77	44	56	127*11	54	123*	51	116*	53	120*
				91	44	63	43*	54	123*	55	125*	60	136*
12	CI	O	allyl	77	44	54	123*	45	102	43	98	45	102
				91	44	46	105	47	107	41	93	43	98

[1] A – average hypocotyl length, mm; B – increase in hypocotyl length compared to the standard, %.; * Reliable differences.

Table 2. Antidote activity of the derivatives of thieno[2,3-*b*]pyridines applied at the rate of 200 g/ha against 2,4-D in sunflower plants (field experiment)

Compound (Number given in the Table 1)	Treatment			
	Control (untreated)	Standard (herbicide)	Herbicide + antidote	
	Seed yield, g/ha			Gain against standard, %
2	2.83	0.89	1.43	161*
3	2.83	0.89	1.16	130*
7	2.04	0.75	1.11	148*
9	3.30	1.07	1.20	124
10	3.30	1.07	1.23	126*
11	3.19	0.94	0.99	105

* Reliable differences

DISCUSSION

As a result of screening novel compounds for their antidote activity against 2,4-D in sunflower, it was determined that a clearly marked and statistically reliable protective effect was produced by the derivatives of thieno[2,3-*b*]pyridines number 2, 3, 7, and 10. They contributed to the negative effect reduction of 2,4-D and increase in yield compared to the standard by 61, 30, 48, and 26 %, respectively (Table 2).

Under the laboratory experiment conditions, it was determined that the tested compounds did not have any growth-stimulating activity in sunflower seedlings; therefore, reduction in the phytotoxicity of 2,4-D could not be caused by its influence. It may be supposed that the herbicide action leveling was caused by one of the mechanisms described in the overview of Pitina et al. (1986).

The results obtained should be considered as the development of previous work and will expand the spectrum of protective means reducing negative effects of herbicides containing 2,4-D as part of their compositions. This is especially important in view of high sensitivity of sunflower to the herbicides of the 2,4-D group and insufficient research done in this field.

The results of the primary screening of novel antidotes (safeners) belonging to a series of thieno[2,3-*b*]pyridines led us to expect that, after adjusting their rates of application and spraying terms, they could be used to reduce negative impacts on 2,4-D on sunflower crops.

ACKNOWLEDGEMENTS

This work was supported by the International Science and Technology Center (#3035 ISTC Project). Authors express their gratitude to their collaborators from USDA-ARS Natural Products Utilization Research Unit (Oxford, MS) Stephen Duke, Scott Baerson, Franck Dayan and Agnes Rimando for their methodological assistance and advice.

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Análisis del crecimiento de genotipos de girasol resistentes y susceptibles a herbicidas imidazolinonas

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RESUMEN

En la región central de los Estados Unidos de América especies autóctonas de *Helianthus* se comportan como malas hierbas en el cultivo de la soja. En 1996 se descubrió que dichos biotipos de girasol común son resistentes a herbicidas imidazolinonas, lo que dificultaba su control como mala hierba. Sin embargo, lo anterior abrió la posibilidad de transferir esa resistencia al girasol cultivado. A partir del cruzamiento de líneas resistentes a herbicidas imidazolinonas con los parentales del híbrido BRS 191, se obtuvo un genotipo resistente. Dicho genotipo se utilizó en el presente trabajo para realizar la comparación fenotípica con el híbrido BRS 191 susceptible, por medio del análisis de crecimiento. Los parámetros evaluados fueron: materia seca total, área foliar, materia seca de las hojas, materia seca de las raíces, materia seca del tallo, materia seca del capítulo, altura de las plantas, diámetro del capítulo, peso de mil aquenios, productividad, contenido de aceite, así como los índices de crecimiento relativo, asimilación líquida y área foliar. No hubo diferencia significativa entre ninguno de los parámetros evaluados, demostrando así que la incorporación del gen de resistencia a herbicidas imidazolinonas en los parentales del híbrido BRS 191, ha resultado en un genotipo con patrón de crecimiento similar al del BRS 191 susceptible. Este resultado abre la posibilidad de obtención de cultivares resistentes, que pueden ser importantes en el control de malas hierbas que afectan al cultivo del girasol.

Palabras-clave: *Helianthus annuus* - índice de crecimiento - resistencia genética.

Growth analysis of sunflower cultivars resistant and susceptible to imidazolinone herbicides

ABSTRACT

In USA Midwest, common sunflower is one of the main weeds of soybean. In 1996, a biotype of the common sunflower resistant to imidazolinones herbicides has caused much concern in the management of this weed. However, it also opened up the possibility of transferring this characteristic of resistance to the susceptible profitable cultivated sunflower. Starting from the crossing of American lines resistant to these imidazolinones herbicides with the parents of the hybrid BRS 191, a resistant genotype was obtained. This genotype was used in this study for phenotypic comparison with a normal BRS hybrid, through a growth analysis. The parameters evaluated were: total dry weight, foliar area, dry weight of leaves, root dry weight, stems dry weight, head dry weight, plant height, head diameter, weight of 1,000 achenes, productivity, and oil content. The relative growth rate, liquid assimilation rate, and the foliar area ratio were also estimated. There was no statistically significant difference between any of the parameters evaluated, demonstrating that the incorporation of the gene for resistance to herbicides of the imidazolinones group to the progenitors of the hybrid BRS 191 resulted in a genotype with a growth pattern similar to the susceptible BRS 191 hybrid. This finding opens up the possibility of obtaining resistant cultivars, becoming a highly important tool in the control of sunflower crop weeds.

Key words: genetic resistance – growth rate – *Helianthus annuus* – sunflower.

INTRODUCCIÓN

El girasol (*Helianthus annuus*) es una especie nativa de los Estados Unidos de América y sus poblaciones espontáneas, denominadas autóctonas o salvajes, se comportan como malas hierbas de cultivos tales como la soja y el maíz. Con el desarrollo de herbicidas inhibidores de la enzima acetolactato sintase (ALS),

selectivos para la soja, el control del girasol salvaje se ha llevado a cabo con éxito, por herbicidas de dicho grupo (Baumgartner et al., 1999). Sin embargo, la presión de selección provocada por el uso continuado de esos herbicidas ha proporcionado el desarrollo de biotipos de girasol resistente a los inhibidores de la ALS. En la mayoría de los casos, la resistencia de malas hierbas provoca mayor dificultad en el manejo de las infestantes y aumento en los costos de control. No obstante, en el caso del girasol salvaje, fue una oportunidad que se abrió para transferir esa característica genética para las variedades e híbridos cultivados, como relatan Miller y Al-Khatib (2001).

En Brasil, todavía no existe cultivo comercial de girasol resistente a los inhibidores de la ALS. Sin embargo, ello sería deseable, pues las malas hierbas dicotiledóneas son mayoría en las áreas de explotación de esa oleaginosa (Brighenti et al., 2003). De esa manera, en el año 2001 se inició en Embrapa Soja la introducción del gen de resistencia a los herbicidas del grupo de las imidazolinonas en genotipos de girasol de su banco de germoplasma, obteniendo en la cosecha de primavera/verano del 2003 tres genotipos F_4R_2 del cruzamiento entre líneas estadounidenses resistentes a las imidazolinonas y líneas nacionales susceptibles.

El objetivo de este trabajo ha sido comparar los genotipos de girasol resistente y susceptible a los herbicidas del grupo de las imidazolinonas, desarrollados por la Embrapa Soja, por medio del análisis de crecimiento de las plantas y sus características derivadas.

MATERIALES Y MÉTODOS

El experimento se desarrolló en condiciones de campo, en la Embrapa Soja. Se comparó el híbrido de girasol BRS 191 con un genotipo híbrido con las mismas líneas parentales, pero que recibieron la incorporación de la resistencia a las imidazolinonas, a través del cruzamiento con líneas estadounidenses seleccionadas por Al-Khatib y Miller (2000). También se utilizó el diseño enteramente aleatorizado, con cinco repeticiones. La siembra fue realizada el 10/03/04 y las evaluaciones fueron realizadas en intervalos de 14 días después del surgimiento de las plantas (DAE), que ocurrió en el 16/03/04. En cada evaluación se ha medido la altura de diez plantas por tratamiento, seleccionándose tres plantas por parcela al azar, que fueron cosechadas enteras, inclusive con el sistema radicular. Las raíces fueron lavadas en agua corriente para la retirada del suelo e impurezas, siendo, posteriormente, separados los órganos, el tallo, las hojas, las raíces y, después del florecimiento, también los capítulos. Cada órgano de las plantas fue puesto en fundas de papel y llevados para secar en estufa de circulación forzada de aire a 70 ± 1ºC, hasta alcanzar el peso constante, y posteriormente, pesado en balanza de precisión. Antes del secado, todas las hojas fueron utilizadas en la determinación del área foliar por medio de medidor fotoeléctrico de mesa, marca LI-COR, modelo 3100.

Los resultados de la altura de las plantas, del área foliar, de la materia seca de los órganos y de la materia seca total fueron sometidos al análisis de variancia, utilizándose el test F, a 5% de probabilidad y al análisis de regresión. En la cosecha del girasol se hizo la medición del diámetro medio de los capítulos, peso de mil aquenios, productividad y contenido en aceite. Los resultados fueron sometidos al análisis de varianza por el test F, siendo las medias comparadas por el test de Tukey, a 5% de probabilidad.

RESULTADOS Y DISCUSIÓN

Las curvas de acumulación de la materia seca total (MSt) de los genotipos fueron similares, prácticamente se solapan hasta los 47 días después de la aparición (DAE), y esa similitud se mantuvo hasta los 98 DAE (Fig. 1). La mayor acumulación de MSt ocurrió a los 86 días para el genotipo resistente y a los 87 días para el genotipo susceptible, con valores de 205, 73 y 196,52 g.planta^{-1}, respectivamente.

Como no hubo diferencia significativa en la MSt, la tasa de crecimiento relativo (Rw) también fue semejante para los dos genotipos, observándose gran ganancia de crecimiento hasta alrededor de los 30 DAE. Eso es normal en el girasol, pues ese periodo coincide con la fase vegetativa de este cultivo, que es aquella donde ocurre la formación y alargamiento de las hojas, iniciando con la germinación y terminando con la formación del pimpollo floral (Schneiter y Miller, 1981), siendo más expresiva en cultivares precoces, como es el caso del BRS 191.

Según Oliveira y Vieira (2000), el girasol BRS 191 inicia el florecimiento aproximadamente a los 53 DAE, cuando prácticamente cesa la formación y alongamiento de las hojas, alcanzando, por lo tanto, la máxima área foliar (Af). En este experimento, ambos los genotipos, resistente y susceptible, alcanzaron ese punto a los 53 DAE, con valores de 60,69 y 60,61 dm^2 planta^{-1}, respectivamente, mostrando diferencia mínima, no significativa, que se repitió en todas las evaluaciones.

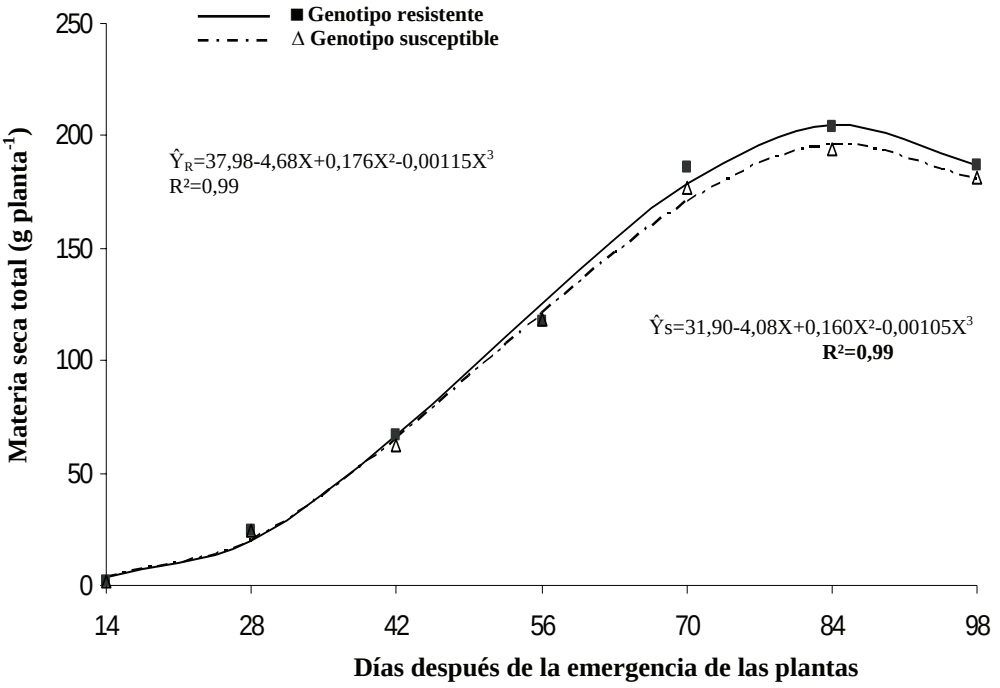

Fig. 1. Materia seca total de plantas de girasol de genotipos resistentes y susceptibles a las imidazolinonas.

La tasa asimilatoria líquida (TAL) es el parámetro que mide la producción de materia seca por unidad de área foliar, y tiene por objetivo analizar la eficiencia fotosintética de la planta. Ha habido una pequeña diferencia, no significativa, en esa tasa al inicio del desarrollo de las plantas, con mayor TAL de genotipo resistente con relación al susceptible, lo que no sucedió más a partir de los 28 DAE. Lo mismo observaron Brighenti et al. (2001), comparando biotipos resistentes y susceptibles de *Euphorbia heterophylla* a inhibidores de la ALS. Resultado inverso se obtuvo por Christoffoleti (2001) estudiando biotipos de *Bidens pilosa*, donde el susceptible obtuvo una TAL inicial superior al resistente. Sin embargo, en los dos trabajos los valores se aproximaron en las evaluaciones posteriores en las que el desarrollo de las plantas era mayor, asemejándose a lo observado en los genotipos de girasol.

Debido a los similares resultados del área foliar y de la MSt, la relación entre estos parámetros, representada por la razón del área foliar (Fa), tampoco ha resultado significativa la comparación de los genotipos. Los valores máximos de 1.82 y 1.80 $dm^2\ g^{-1}$ para el resistente y susceptible, respectivamente, se obtuvieron a los 29 DAE, disminuyendo en las evaluaciones posteriores con las curvas comportándose semejantemente a las de la TAL, pues a partir de la diferenciación floral ocurre la disminución progresiva de los fotoasimilados en dirección de las hojas (Vrânceanu, 1977). Después de la floración la senescencia y la caída de las hojas, contribuyendo para la reducción todavía mayor de la Fa. De esa forma no ha sido posible realizar la evaluación del área foliar y por consecuencia de la Fa en el proceso de la cosecha del experimento a los 98 DAE.

La materia seca de cada órgano del girasol, de las hojas (MSf), de las raíces (MSr), del tallo (MSc) y del capítulo (MScp), ha mostrado que no hubo diferencia significativa para ninguno de ellos. La acumulación de la materia seca de las hojas aumentó hasta los 66 DAE para los dos genotipos de girasol, resistente y susceptible, alcanzando valores máximos de 29.03 y 28.96 g $planta^{-1}$ respectivamente. El resultado demostró que el punto de máxima MSf ocurrió 14 días después de la máxima Af. Eso sucedió porque aún pasado el florecimiento pleno el girasol continúa manteniendo balance positivo de acúmulo de fotoasimilados en la hoja, por lo tanto, acumulando materia seca (Vrânceanu, 1977).

La materia seca acumulada de las raíces aumentó con la edad de las plantas y alcanzó los valores máximos de 21.68 y 20.86 g $planta^{-1}$, a los 81 DAE, para los genotipos resistente y susceptible respectivamente. Con relación a la materia seca del tallo, no hubo diferencia estadísticamente significativa. Los mayores acúmulos de materia seca fueron de 74.21 g $planta^{-1}$ para el genotipo resistente y de 69.29 g $planta^{-1}$ para el genotipo susceptible, ambos a los 79 DAE. Analizando la translocación de

imazetapir en biotipos de girasol resistente y susceptible a las imidazolinonas, Al-Khatib et al. (1998) concluyeron que no existía diferencia entre los biotipos en la translocación del herbicida en el tallo hasta siete días después de su aplicación.

El peso de la materia seca de los capítulos, recolectado a partir de los 56 DAE, mostró la misma tendencia de acúmulo, con alto crecimiento de los 60 a los 84 DAE, pues es la fase en la que ocurre gran translocación de fotoasimilados para la formación y llenado de los aquenios. Esa fase ocurre, según Castiglioni et al. (1997), entre el final de florecimiento hasta la maduración fisiológica. La máxima MScp fue de 96.16 g planta^{-1} para el genotipo resistente y de 92.43 g planta^{-1} para el genotipo susceptible.

El crecimiento de las plantas de girasol tuvo mayor incremento en altura hasta los 28 DAE, resultado semejante al obtenido por Amabile et al. (2003) con la variedad Embrapa 122 también de ciclo precoz. El punto estimado de máximo crecimiento de los genotipos ocurrió a los 85 DAE, con altura de 175 cm del genotipo resistente y 179 cm del susceptible. No hubo diferencia significativa en ninguna época de evaluación.

Los similares resultados de los parámetros de crecimiento fueron además observados en los parámetros de rendimiento evaluados. No hubo diferencia significativa en el diámetro del capítulo, peso de mil aquenios, productividad y contenido de aceite entre los genotipos resistente e susceptible.

Por los resultados de este trabajo se concluye que la incorporación del gen de resistencia a los herbicidas de grupo químico de las imidazolinonas en los progenitores del híbrido BRS 191 resultó en un genotipo con semejante patrón de crecimiento al híbrido BRS 191 normal y susceptible, sin diferencias fenotípicas significativas. De esta forma se abre la posibilidad de obtención de cultivares, variedades o híbridos con la característica de resistencia a los herbicidas pertenecientes al grupo químico de las imidazolinonas, que puede transformarse en tecnología viable en el control das plantas dañinas en el cultivo del girasol en Brasil.

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Assessment of sunflower yield maps and discrimination of late-season weed patches by using field spectroradiometry and remote sensing: the case of *Ridolfia segetum* Moris

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ABSTRACT

Weed control strategies are commonly applied over the entire agricultural fields, although weeds are spatially distributed in patches. To reduce the consumption of herbicides applying them only where weed patches are present, it is necessary to develop accurate maps of weed patches. These weed maps can be obtained in late-season through high spatial resolution remote sensing and can be used for site-specific control next season. This is especially helpful taken into account that most weeds are stable in time and location. The main objective of this contribution is to describe the remote sensing requirements for predicting yield and mapping weeds, and to outline some results of our group in this research area by explaining the case study of *R. segetum* in sunflower. We have chosen this weed as it is one of the most widely distributed, hard to control and competitive broadleaf weeds in sunflower.

Key words: multitemporal aerial imagery – site-specific weed management – weed patch discrimination.

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the most important crops in Andalusia (southern Spain) with over 240.000 ha grown annually (MAPA, 2006). It is normally grown under dry land conditions; sowing-time is February-March, and harvesting-time late July-mid August. One of the most frequent broadleaf weed species is the umbelliferous *Ridolfia segetum* Moris. It occurs in 25% of the sunflower surface in this region (Peña-Barragán et al., 2007) and two *R. segetum* plants per m^{-2} reduce crop yield by about 32% (Carranza-Cañadas et al., 1995). This weed is hard to control due to it not being controlled by pre-emergence and pre-plant incorporated herbicides used in sunflower, and, consequently post-emergence strategies such as tillage or hand weeding are commonly used, otherwise this weed obstructs the harvester due to it still having a partly green stem during the sunflower harvesting. Uncontrolled *R. segetum* plants also infest other crops included in the rotation, *e.g.* oilseed rape (*Brassica napus* L.) and medicinal-aromatic crops like anisette (*Pimpinela anisum* L.) generating serious contamination problems for oilseed rape oil and the anisette seeds for human consumption. Reduced and no-tillage production have increased in Spain in the last 10 years, and now account for 2.4 million of hectares of the annual crops (AESC/SV, 2005), many of them in Andalusia. So, *R. segetum* has become more troublesome since it cannot be reduced in abundance by repeated tillage or cultivation.

Patchy distribution of broadleaf weeds in sunflower fields is well documented (Jurado-Expósito et al., 2003). However, herbicide or other control strategies are not addressed to the infested zones, but they are usually broadcast over entire fields. The potential for overuse or application and corresponding eco-environmental problems is evident. One aspect of overcoming the possibility of minimizing the impact of herbicide on environmental quality is the development of Site-Specific Weed Management (SSWM). Timmermann et al. (2003) concluded that costs savings were 90% and 60% for broadleaf and grass weeds herbicides, respectively. A key component of SSWM is that accurate and appropriate weeds maps are required to take full advantage of site-specific herbicide applications. Mapping weed patches based on ground survey techniques on field scale is time consuming, expensive and unapproachable in field areas with difficult access. Remote sensing of weed canopies may be more efficient and suitable than field surveys and the majority of studies on discriminating weeds in cultivated systems have involved discrete broadband remote sensing (multispectral sensors) (Brown & Noble, 2005).

To detect and map weeds it is necessary for suitable differences to exist in spectral reflectance between weeds and crop or bare soil. Spectral reflectance differences can be enhanced by using Vegetation Indices, which are mathematical (ratio or linear) combinations between bands. Detection of late-season weed infestation has been demonstrated to have tremendous possibilities when spectral differences between crops and weeds prevail at a certain phenological stage (López-Granados et al., 2006). Taking into account that weed infestations are stable and persistent in location from year to year

(Jurado-Expósito et al., 2004), late-season weed detection maps can be used to design site-specific control methods in the coming 2 to 4 years. Thus, it is crucial to explore the variations in the spectral signatures of crop and weed, indicating suitable wavelengths for species discrimination and classification.

On the other hand, it is well known that crop yield varies spatially within the field since factors affecting crop growth such as soil properties, water availability, disease, weed and insect pressure, crop management practices, among others, vary spatially. Yield variability estimation during crop development could help farmers to make decisions (*e.g.* fertilization, irrigation, weed control) some time before harvest. Remote sensed imagery has been demonstrated to provide spatial and temporal georeferenced field information related to some field factors to predict yield estimation (Yang et al., 2006). As happens in weed mapping, one of the main challenges of remote imagery analysis in agriculture is to determine how variations in spectral information are related to differences in the crop phenological state, in order to obtain accurate yield maps long before the harvest, so that, crop management can be designed accordingly.

The main objective of this contribution is to describe the remote sensing requirements for predicting yield and mapping weeds and to outline some results of our group in this research area by explaining the case of *R. segetum* in sunflower. Our specific objectives were: 1) to determine the spectral signatures of bare soil, and different phenological stages of sunflower and *R. segetum*; 2) to select bands and Vegetation Indices for multispectral discrimination within-between phenological stages of sunflower and *R. segetum*, 3) to determine the ability to discriminate *R. segetum* patches on sunflower crops using aerial photographs, and 4) to assess the spatial relationship of sunflower yield to *R. segetum* weed presence.

MATERIALS AND METHODS

Study area: The study was conducted on two 40 ha sunflower fields located in Córdoba province (Andalusia, southern Spain), named Matabueyes and Santa Cruz, naturally infested by *R. segetum* and representative of infested areas in Andalusia. Sunflower crop Jalisco *cv*. was seeded at 4 kg ha^{-1} in rows 0.7 m apart in mid-March and harvested in mid-August. The field site was farmer-managed using shallow tillage production methods. Glyphosate was applied at pre-emergence at 0.7 l ha^{-1} for the control of annual weed seedlings. At this rate, this herbicide had no significant activity on *R. segetum*. Spectral reflectance signatures of bare soil, sunflower and *R. segetum* were measured from mid-May to mid-July according to the sunflower and weed phenological stages explained below.

Sunflower and R. segetum phenological stages: Sunflower and weed phenological stages were determined according to those adapted to our field conditions by Peña-Barragán et al. (2006).
A) Sunflower. 1) *mid-May, vegetative phase*: a) vegetative 5-10 leaves (SunV5-10), and b) reproductive head growing (SunHG); 2) *mid-June, reproductive phase*: c) reproductive head flowering (SunHF), and d) initial desiccation of lower leaves and reproductive head turning down (SunID); 3) *mid-July, senescent phase:* e) reproductive head partly desiccated and browning (SunRHPD); and f) plant completely desiccated and darkish/ black (SunPD).
B) R. segetum. 1) *mid-May, vegetative phase*: a) seedling < 5-10 cm, (RidSe); b) vegetative stage without floral stem (RidVe), and c) inflorescence (or umbella) still closed (RidInC); 2) *mid-June, flowering phase*: d) inflorescence yellowing, (RidInY); 3) *mid-July, senescent phase*: e) plant desiccated (RidPD).

Spectroradiometer data measurements and multispectral analysis: In mid-May, mid-June and mid-July, twenty hyperspectral measurements were collected for bare soil and each sunflower and *R. segetum* phenological stage using an ASD Handheld FieldSpec Spectroradiometer (Analytical Spectral Device, Inc., Boulder, USA) placed at 80-100 cm above each plant canopy or soil. Each measurement was georeferenced using the sub-meter differential GPS TRIMBLE PRO-XRS (Trimble, Sunnyvale, USA), to be located in the aerial images later. The spectral data were calibrated with a standard panel (Spectralon®) before each measurement. Measurements were made under sunny conditions between 12 and 14 h, and were collected between 400 and 900 nm (bandwidth of 1.5 nm).

Spectroradiometer data were averaged to represent the aerial imagery broad wavebands (blue, B: 400-500 nm; green, G: 500-600 nm; red, R: 600-700 nm; and near-infrared, NIR: 700-900 nm). The following vegetation indices (VI) were also calculated and analysed: Normalized Difference Vegetation Index NDVI = (NIR-R) / (NIR+R) (Rouse et al., 1973), Ratio Vegetation Index RVI = NIR / R (Jordan, 1969), R / B index (Everitt & Villarreal, 1987), VNVI = (NIR-G) / (NIR+G), and ANVI = (NIR-B) / (NIR+B). Multispectral data were subjected to analysis of variance, and means were separated at the 5% level of significance by LSD test using the SPSS software.

Aerial photographs: Conventional-colour (400–700 nm) and colour-infrared (500–900 nm) aerial photographs of the fields studied were taken in mid-May, mid-June and mid-July. Average flight height was 1525 m to obtain photographs at a scale of 1:10000. Selected photographs were digitised using the AGFA Horizon A3 scanner (635 dpi corresponding to pixels of 40 x 40 cm) and georeferenced (using 40 ground control points). ENVI 4.3 software was used to process images.

Image analysis: Two methods widely explained in Peña-Barragán et al. (2007) were applied to classify the images and to discriminate between the *R. segetum*-infested and the non-infested zones:
A) Class Separation Method: This was used in the four wavebands and the five Vegetation Indices previously described. Each image was classified by grouping the digital values according to the value ranges that characterized *R. segetum* training patches. Boundary digital values were established according to the statistical value obtained from 220 *R. segetum* training pixels, adding and reducing the standard deviation to the average.
B) Spectral Angle Mapper (SAM) Method: used for multispectral band images. It is based on an n-dimensional angle to match pixels to reference spectra made up of the digital signature of each training zone. The algorithm determines the similarity between two digital signatures by comparing their angles, treating them as vectors in a space with dimensionality equal to the number of bands. Smaller angles represent closer matches to the reference signature. The photo-interpreter has to specify the Maximum Angle Threshold in radians that set each land-use, so no pixels outside this threshold were classified.

During the field visits, 550 ground-truth pixels (infested and non-infested zones) were used to create the ground-truth images for every classification method. A numerical analysis called *Confusion Matrix* was then performed to quantify the accuracy of the coincidence between classification images and real patches for each classification method. The confusion matrix was used to obtain the overall accuracy (OA), which is the percentage obtained by dividing the pixels correctly classified among all ground-truth images. OA has been standardized in at least 85% for minimum accepted values (Thomlison et al., 1999).

Sunflower Yield Data: The fields were harvested on 8th August using the farm´s MF34 combine harvester equipped with a differentially-corrected global positioning system (DGPS) receiver and a yield monitor with the Fieldstar® system (Massey Ferguson®, AGCO Corporation, Duluth, GA, USA). The yield data set used was of 2541 points, ranging from 0.30 to 2.30 t ha$^{-1.}$ A contour yield map of the complete field was then generated using the SURFER software (Golden software, Inc., Golden, Colorado, USA). Yield data set and yield map were grouped to six yield intervals (Very-Low=[0.30-0.60], Low=[0.61-0.95], Medium-Low=[0.96-1.30], Medium-High=[1.31-1.65], High=[1.66-2.00], and Very-High=[2.01-2.30] t ha^{-1}), to perform the statistical analysis. The four wavebands B, G, R, and NIR, and two vegetation indices (NDVI and NDYI) were considered to predict the yield map. Vegetation indices were calculated as follows: NDVI = (NIR - R) / (NIR + R); NDYI = (R - G) / (NIR + G).

RESULTS AND DISCUSSION

Hyperspectral signatures of bare soil and phenological stages of sunflower and R. segetum: Reflectance curves of bare soil, and sunflower and *R. segetum* phenological stages corresponding to Mid-May (vegetative phase), mid- June (flowering phase) and mid-July (senescent phase) are indicated in Fig. 1. Phenological stages of crop and weed consistently affected the magnitude and amplitude of spectral reflectance values. Vegetative and flowering phases showed their characteristic higher reflectance in G (Green peak, 550 nm) and NIR (from 700 to 900 nm) parts of the spectrum. Senescent phase and bare soil presented their typical spectral signatures, *i.e.* reflectance values increased as wavelengths increased.

Multispectral analysis of bands and Vegetation indices: Results from analysis of variance and corresponding LSD test are shown in Table 1. B, NIR, NDVI, RVI, VNVI and ANVI values were statistically different between bare soil, and *R. segetum* and at least one of the sunflower phenological stages, suggesting that there is a potential for a successful discrimination between bare soil, sunflower and *R. segetum* using remote sensing.

Image classification method and weed map: OAs calculated throughout the confusion matrix for every classification method in mid-June (flowering phase) are listed in Table 2. Most classification methods studied, such as B, G, R, R/B, ANVI and SAM, discriminated *R. segetum* patches from bare soil and sunflower with OAs $\geq$ 85%. In particular, the best classifications of weed patches were obtained using SAM and R/B vegetation index, resulting in OAs of 95% and 98% in Matabueyes and Santa Cruz,

respectively. Results obtained in mid-May and mid-July are not shown because they were generally poor (lower than 80%) and did not reach the commonly accepted requirement of at least an 85% classification of the overall accuracy. Therefore, it is not recommended to take any image in phenological stages corresponding to these dates to discriminate *R. segetum* patches.

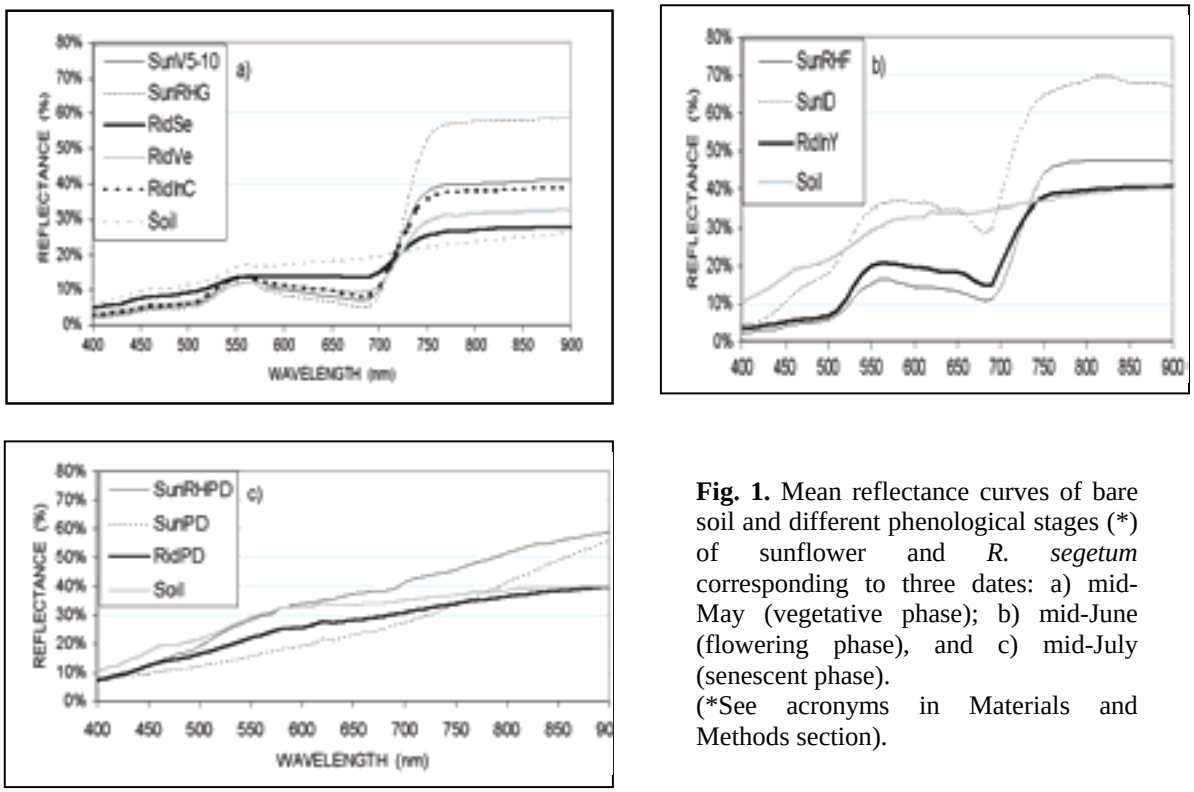

Fig. 1. Mean reflectance curves of bare soil and different phenological stages (*) of sunflower and *R. segetum* corresponding to three dates: a) mid-May (vegetative phase); b) mid-June (flowering phase), and c) mid-July (senescent phase).
(*See acronyms in Materials and Methods section).

Table 1. Mean reflectance values of bare soil, and sunflower and *R. segetum* phenological stages on three dates for multispectral bands and vegetation indices. (Best results are shaded in grey).

Dates	Phenological stages[2]	Mean values[1]								
		Multispectral Bands				Vegetation indices[2]				
		Blue	Green	Red	NIR	NDVI	RVI	RB	VNVI	ANVI
Mid-May	SunV5-10	0.04 b	0.10 ab	0.09 b	0.36 d	0.62 e	4.36 d	1.96 a	0.55 e	0.78 d
	SunRHG	0.03 a	0.09 a	0.07 a	0.50 e	0.76 f	7.61 e	1.93 a	0.69 f	0.87 e
	RidSe	0.07 d	0.13 c	0.14 d	0.26 a	0.30 b	1.86 a	1.93 a	0.34 b	0.56 b
	RidVe	0.05 c	0.10 ab	0.10 c	0.29 b	0.49 c	3.05 b	1.89 a	0.48 c	0.69 c
	RidInC	0.04 b	0.11 b	0.10 c	0.34 c	0.56 d	3.69 c	2.11 b	0.52 d	0.76 d
	Bare soil	0.09 e	0.15 d	0.18 e	0.23 a	0.13 a	1.30 a	2.01 ab	0.22 a	0.45 a
Mid-June	SunRHF	0.04 a	0.13 a	0.13 a	0.43 a	0.53 c	3.49 c	3.65bc	0.54 c	0.84 c
	SunID	0.10 a	0.31 b	0.33 b	0.64 b	0.36 b	2.40 b	3.14 b	0.37 b	0.74 b
	RidInO	0.05 b	0.16 a	0.18 a	0.37 c	0.36 b	2.16 b	3.86 c	0.39 b	0.76 b
	Bare soil	0.16 c	0.28 b	0.34 b	0.38 a	0.06 a	1.13 a	2.06 a	0.15 a	0.40 a
Mid-July	SunRHPD	0.12 b	0.27 c	0.37 c	0.51 b	0.17 c	1.40 c	3.01 c	0.31 c	0.61 c
	SunPD	0.10 a	0.16 a	0.23 a	0.41 a	0.29 d	1.80 d	2.42 b	0.45 d	0.62 c
	RidPD	0.12 b	0.22 b	0.28 b	0.36 a	0.13 b	1.30 b	2.40 b	0.26 b	0.51 b
	Bare soil	0.16 c	0.28 b	0.34 b	0.38 a	0.06 a	1.13 a	2.06 a	0.15 a	0.40 a

[1]Mean values followed by the same letter within a column for a single date do not differ significantly at the P [0.05%] according to LSD test.
[2]See Vegetation Indices and Phenological stages in Materials and Methods section.

Remote imagery vs. yield: Data presented are only from Matabueyes due to Santa Cruz's analysis still being in progress. The averaged wavebands and vegetation index data as affected by sunflower yield intervals and crop development stage are shown in Table 3. R waveband digital values and the NDVI index at the vegetative crop stage (mid-May) showed significant differences in all yield intervals. Furthermore, the NDVI mean value was negative for the three lowest yield intervals and positive for the three highest ones, and their values increased as the yield intervals increased. Mean values for the 6-sunflower yield interval map for NDVI index in mid-May are shown in Fig. 2a.

Table 2. Overall accuracy values (%) of every classification method in mid-June (Flowering phase). (Best results are shaded in grey).

Locations	Classification methods									
	SAM*	Blue	Green	Red	NIR*	NDVI[§]	RVI[§]	R/B[§]	VNVI[§]	ANVI[§]
Matabueyes	95	79	88	84	--	--*	--*	86	--*	--*
Santa Cruz	83	85	88	87	777	83	79	99	80	84

(*) SAM, Spectral Angle Mapper; NIR: Near-infrared band of Matabueyes field in mid-June could not be obtained due to technical problems and, thus, Vegetation Indices with NIR were not calculated .
([§]) See Vegetation Indices in Materials and Methods section.

The NDYI index was only significantly different in the four highest yield intervals, and their mean values were positive and very similar for the three lowest yield intervals and negative for the three highest ones. In the flowering crop stage (mid-June), there were significant differences in B and G wavebands and in NDYI index (Table 3). On this date, NDYI mean values were negative in all yield intervals and inversely correlative with the increase in the yield intervals, ranging from -0.02 to -0.17. Higher NDYI values were found in mid-June than in mid-May due to greater differences between R and G values being obtained in mid-June. In mid-July (senescent stage), data are not shown because no significant differences were found between yield intervals and any bands or vegetation indices.

Considering the three dates, NDVI and NDYI corresponding to vegetative and flowering stages (mid-May and mid-June images) produced the best results, and their mean values correlatively increased as the yield increased, and decreased as the yield increased, respectively.

Table 3. ANOVA analysis of means for sunflower yield map, elevation, and bands and vegetation index data according to airborne imagery collected in mid-May and mid-June.

Yield Interval	Airborne image in mid-May						Airborne image in mid-June			
	Blue	Green	Red	NIR	NDYI	NDVI	Blue	Green	Red	NDYI
Very-Low	179 e	191 e	204 f	112 a	0.03 d	- 0.37 a	68 a	82 a	81 b	-0.02 f
Low	178 e	191 e	198 e	162 b	0.02 d	- 0.15 b	80 d	98 d	94 e	-0.04 e
Medium-Low	159 d	175 d	179 d	179 c	0.02 d	- 0.01 c	76 c	93 c	84 c	-0.08 d
Medium-High	142 c	157 c	155 c	180 cd	- 0.01 c	0.07 d	74 b	88 b	77 a	-0.10 c
High	129 a	135 b	130 b	181 d	- 0.03 b	0.14 e	96 e	102 e	89 d	-0.12 b
Very-High	136 b	123 a	116 a	206 e	- 0.08 a	0.33 f	107 f	105 f	89 d	-0.17 a

R. segetum pressure vs. sunflower yield intervals: The presence of *R. segetum* according to each yield interval and the weed influence on the reduction in the total yield are shown in Fig. 2b. This figure shows that weed presence diminished where yield increased. At the lowest yield intervals, R. segetum infested between 15 to 20 % of the total surface, but from medium-high yield to up upwards, the infestation was reduced from 14 to 3 %, suggesting that the zones within the high sunflower yield were sensitive to the absence of weed infestation.

CONCLUSIONS

Our results demonstrated that remote sensing images taken in mid-June can be a useful tool for both: 1) to estimate sunflower yield variability that can be used to generate a yield map for within-season identification of problematic or stressed areas for further site-specific management some time before harvest, and 2) to estimate late-season *R. segetum* patch maps that can be used in subsequent years for site-specific control strategies due to uncontrolled weeds being stable in location over the years in the field. The key question in predicting yield maps and mapping weed patches in crops is related to the time interval in which weed patches and crops show consistent and significant spectral differences. This paper concluded that aerial images taken in mid-June, which corresponds to the flowering phase of *R. segetum*

and sunflower plants in our climate conditions, is the appropriate time to take aerial images for successfully completing the map of *R. segetum* patches and the expected sunflower yield.

a) b)

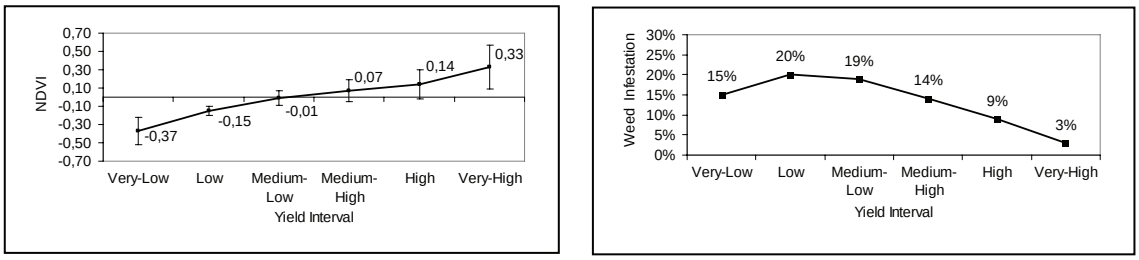

Fig. 2. a) Mean values of the 6-yield interval map for NDVI index in mid-May; b) Percentage of field surface infested by *R. segetum* according to 6-sunflower yield intervals.

ACKNOWLEDGEMENTS

This work was partially funded by the Spanish Ministry of Education and Science through the projects AGL-2005-06180-CO3-02 and AGL-2007-06926-AGR.

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Control of *Cirsium* and *Xanthium* in sunflower hybrids resistant to the herbicide Express 50 SX

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ABSTRACT

Field research was carried out in four locations and during two years to study the selectivity and efficiency of the herbicide Express 50 SX applied in post-emergence to sunflower hybrids resistant to sulfonylurea herbicides.

Key words: dicots – herbicide – post-emergence – sulfonylurea – sunflower –weeds.

INTRODUCTION

Sunflower is one of the most important crops, strongly competed for by weeds from the first vegetation stages, especially in spring with low temperatures, which lead to slight growth of plants (Jonson, 1971). The sowing time is also of a special importance for early weed species, which could influence, at a high infestation, the yield level (Vannozzi et al., 1990).

On sunflower, several herbicides were first developed to be applied before and after sowing (trifluraline, linuron, metobromuron) for the control of annual weeds (Monotti, 1980). Afterward, attention was given to the association of herbicides such as trifluralin, alachloro, metolachloride, pendimethaline, linuron, prometrin, fluorochloridone, oxifluorfen (Lauretti, 1985; Tei et al., 1991; Millet et al., 1992). The research results have shown that a high efficiency in sunflower weed control is achieved by the association of chemical and mechanical treatments (Pintilie, 1986; Sarpe, 1987). In Romania (1970-1992), sunflower weed control was only partially solved because most dominant weeds (*Xanthium, Cirsium, Abutilon, Datura*) were not controlled by any herbicide. This problem was solved by the development by the Pioneer company, in collaboration with Cyanamid researchers (after 1990), of the first "genetically unmodified" sunflower hybrids "IR", resistant to imidazolinone systemic herbicides, with effect in resistant dicots control: *Xanthium, Abutilon* and, partially *Cirsium*. This research performed world-wide after 2000 contributed to the development of the first "genetically unmodified" hybrids, resistant to tribenuron (Express 50 SX) of great importance under Romania conditions, due to the efficiency of Express 50 SX herbicide in post-emergence application, to control "problem" weeds: *Xanthium, Cirsium, Abutilon*.

The main aim of the research performed in Romania was to establish the optimum strategy to control mono- and dicots (including *Xanthium, Cirsium*) in sunflower crop with hybrids resistant to sulfonylurea (Express 50 SX).

MATERIALS AND METHODS

The experiments were performed during 2004-2005, at NARDI Fundulea, ARDS Lovrin, Oradea and Teleorman, with various weed infestations depending on the pedoclimatic conditions. The experiments were organized as randomized blocks, with plot area of 25 m^2, in four repetitions. Each plot was sown with 4 rows, with distance between rows of 70 cm. The cultivated hybrid was XF 4419, belonging to the Du Pont company. In the experiment plots, the herbicides mentioned in Table 1 were applied in post-emergence (sunflower: 4-6 leaves and 6-8 leaves stages), Also, the "split application" was employed (sunflower, 2-3 leaves and 6-8 leaves at re-infestation). For herbicide treatment, 250-400 l water/ha were used.

RESULTS AND DISCUSSION

The paper presents the results obtained during 2004-2005 at the research stations Lovrin, Teleorman, Oradea and NARDI Fundulea, placed under various climatic conditions, especially with a highly diversified infestation degree, weed spectrum and dominance. On average, the experiments presented strong infestations (80-95%) with annual and perennial mono- and dicots, with dicots prevalence (65%).

Table 1. Experimental details

Year	No.	Treatment	Rate a.i g/ha	Time of application	Content a.i g/l	Company
2004	1.	Untreated	-	-	-	
	2.	DPX.$_{75}$WG+Trend**	15 + 0.1%	Postem (4-6 lves)	75% tribenuron+Adj.	
	3.	DPX$_{75}$WG+Trend**	15+ 0.1%	Postem (6-8 lves)	75% tribenuron+Adj.	
	4.	DPX$_{75}$WG+Trend** + DPX$_{75}$WG+Trend	7.5+0.1%+ 7.5+0.1%	EPO (2-3 lves) +Reinf.(6-8 lves)	75% tribenuron+Adj.	Du Pont
	5.	DPX$_{75}$WG+Trend + Reset	15 + 0,1% + 37,5	Postem (4-6 lves)	75% tribenuron+Adj. + 50 g/l quizalofop P-etil	
	6.	Raft 400* (standard)	600	Postem (4-6 lves)	400 g/l oxidiargil	Bayer
2005	1.	Untreated	-	-	-	-
	2.	Raft 400 (standard)*	600	Postem (4-6 lves)	400g/l oxidiargil	Bayer
	3.	Express 50 SX**	15	Postem (4-6 lves)	50% tribenuron	Du Pont
	4.	Express 50 SX+ Trend**	15 + 0,1%	Postem (4-6 lves)	50% tribenuron + Adj.	Du Pont
	5.	Express 50 SX+Trend+ Fusilade s.	15 + 0,1% + 187	Postem (4-6 lves)	50% tribenuron + Adj.+ 125g/l fluazifop	Du Pont, Syngenta
	6.	Express 50 SX**	15	Postem (6-8 lves)	50 % tribenuron	Du Pont
	7.	Express 50 SX + Trend**	15 + 0,1%	Postem (6-8 lves)	50 % tribenuron + Adj.	Du Pont
	8.	Express 50 SX+ Trend+ Fusilade s.	15 + 0,1% + 187	Postem (6-8 lves)	50 % tribenuron + Adj.+ 125g/l fluazifop	Du Pont , Syngenta

*Graminicide herbicide pre-emergently applied
**Graminicide herbicide post-emergently applied

As dominance, the most representative ones were: *Cirsium*, *Xanthium*, *Sinapis*, *Raphanus*, *Chenopodium*, *Amaranthus*, *Hibiscus*, *Polygonum persicaria*, *Anthemis*, *Convolvulus* and only 35% annual mono-: *Echinochloa*, *Setaria*. In 2005, at Fundulea, with an infestation level of 90%, mono-species were predominant (65%): *Sorghum* (seed and rhizomes), *Echinochloa*, *Setaria*, while the dicots (35%) had a lower level of infestation: *Amaranthus*, *Chenopodium*, *Xanthium*, *Sinapis*, *Cirsium*, *Convolvulus*.

During two years of research, with enough rainfall after treatment, (30,2-128,8 mm in 20 DAT) at the application of herbicides DPX 75 (20 g/ha) and Express 50 XS (30 g/ha) associated with adjuvant Trend (0.1%) at two stages (4-6 and 6-8 leaves stages), phytotoxic symptoms were not recorded, as compared to standard treatment which showed leaves necrosis (averaged EWRS quotation 2.1 in 2004 and 2.3 in 2005), as we noticed in Table 2.

In 2005, at Fundulea (Fig. 3) in the variants treated by Express 50 SX alone or in association -Trend, Fusilade -in optimum stage (sunflower 4-6 leaves), the dicots control (including Cirsium, Xanthium) was higher (90-98% in 14-28 DAT) than 2004 due to lower dicots infestation (35%). In late application (sunflower 6-8 leaves) of the Express 50 SX herbicide, it obtained a lower effect in dicots control (85-93%, in 14-28 DAT) compared to the treatments applied in optimum stage.

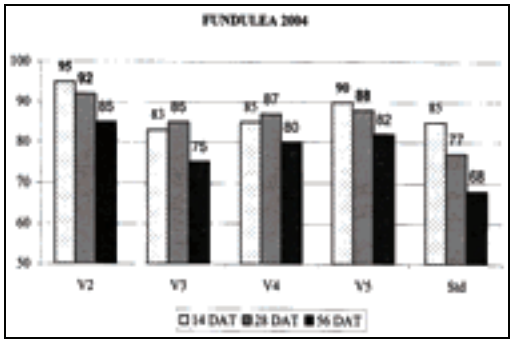
In 2004, at four stations (Fig. 1 and 2), at the application of the above mentioned herbicides to control dicots (especially the resistant ones: *Xanthium*, *Cirsium*), a superior efficiency (92-96%) was recorded as compared to standard variant (Raft 400) with control effect of 85-68% due to non control of resistant dicots (*Xanthium, Cirsium*). During the two experimentation years, the highest results in 14-28 DAT (92-96% -2004 and 96-97%, 2005), in dicots controlling (including the resistant ones: Xanthium, Cirsium) were recorded in the variants treated by DPX 75 WG (20 g/ha) or Express 50 SX (30 g/ha) + Adjuvant, applied in optimum stage for weeds and sunflower plants.

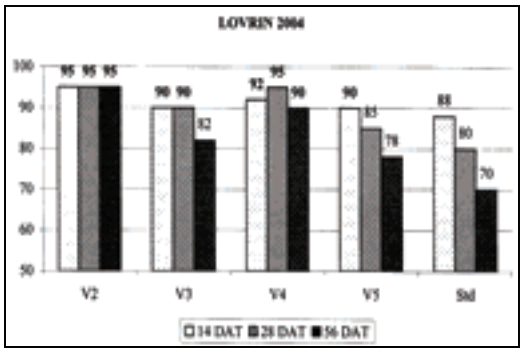
Table 2. The selectivity (EWRS quotation) of herbicides, postemergently applied, to control dicots in sunflower with hybrids resistant to Express 50 SX

Year	No	Variants	Rate a.i g/ha	Time of application	Selectivity (EWRS quotation) 2004				
					7 DAT	14 DAT	28 DAT	56 DAT	Mean
2004	V1	Untreated	-	-	1	1	1	1	1
	V2	DPX.$_{75}$WG+Trend**	15 + 0.1%	Postem. (Sunfl., 4-6 lves)	1	1	1	1	1
	V3	DPX$_{75}$WG+Trend**	15 + 0.1%	Postem. (Sunfl., 6-8 lves)	1	1	1	1	1
	V4	DPX$_{75}$WG+Trend** + DPX$_{75}$WG+Trend	7.5+ 0.1%+ 7.5 + 0.1%	EPO (2-3 lves) Reinf. (6-8 lves)	1	1	1	1	1
	V5	DPX$_{75}$WG+Trend + Reset	15 + 0.1%+ 37.5	Postem. (Sunfl., 4-6 lves)	1	1	1	1	1
	V6	Raft 400* (standard)	600	Postem. (Sunfl., 4-6 lves)	3	2^{5}	1^{5}	1	2^{1}
2005	V1	Untreated	-	-	1	1	1	1	1
	V2	Raft 400* (standard)	600	Postem. (Sunfl., 4-6 lves)	3^{5}	2^{7}	1^{8}	1	2^{3}
	V3	Express 50 SX**	15	Postem. (Sunfl., 4-6 lves)	1	1	1	1	1
	V4	Express 50 SX + Trend**	15 + 0.1%	Postem. (Sunfl., 4-6 lves)	1	1	1	1	1
	V5	Express 50 SXTrend + Fusilade	15+ 0.1%+187	Postem. (Sunfl., 4-6 lves)	1	1	1	1	1
	V6	Express 50 SX**	15	Postem. (Sunfl., 6-8 lves)	1	1	1	1	1
	V7	Express 50 SX + Trend**	15+ 0.1%	Postem. (Sunfl., 6-8 lves)	1	1	1	1	1
	V8	Express 50 SX + Trend + Fusilade	15+ 0.1+187	Postem. (Sunfl., 6-8 lves)	1	1	1	1	1

* Graminicide herbicide pre-emergently applied
** Graminicide herbicide post-emergently applied

Also, the best effect, 85-95% (2004) and 90-98% (2005) to control annual and perennial mono- and dicots was achieved in "tank mix" variant, using DPX 75, Express + Trend + graminicide (Reset or Fusilade), applied post-emergence, in optimum time (sunflower 4-6 leaves), being superior to standard treatment efficiency. The results show that the high efficiency to control mono- and dicots (especially the resistant ones) is directly correlated with rainfall before treatment, infestation degree, weed spectrum and prevalence as well as weed stage at treatment application.





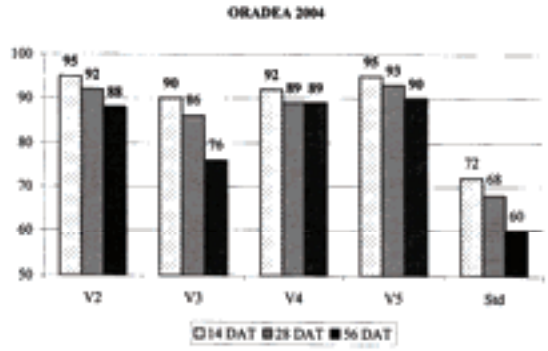
Infest. degree 85%
M/D 40/60
(Dp 35)
Weeds:

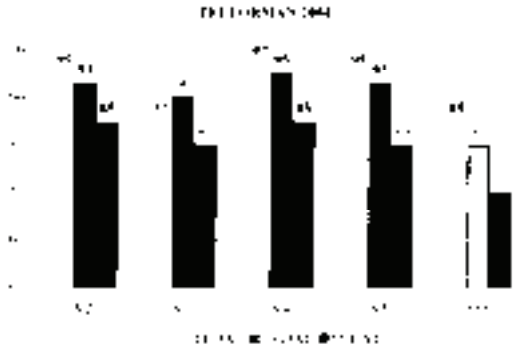
CIRAR	ANTAR
GALAP	PAPRH
XANST	ECHCG
AMARE	SETSP

Infest. degree 90%
(D/P 35/65)
Weeds:

SINAR	POLPE
CIRAR	CHEAL
CONAR	ECHCG
HIBTR	SETGL

Fig. 1. Efficiency (%) of herbicides, postemergently applied, to control dicots in sunflower with hybrids resistant to Express 50 SX in Fundulea and Lovrin in 2004.





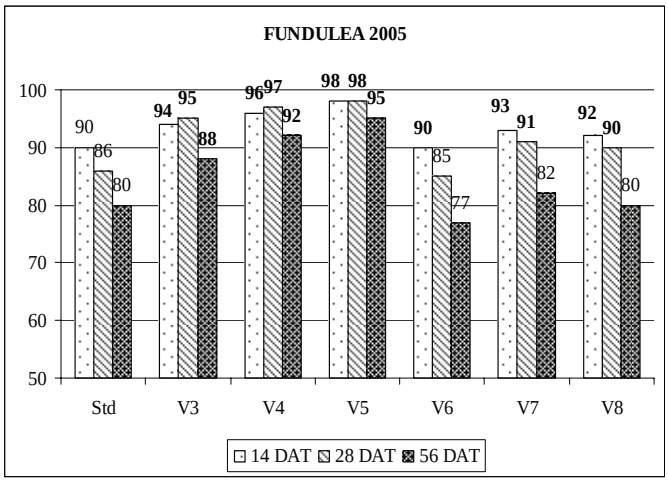
Infest degree -90%
M/D 20/80
Weeds:

XANST	POLSP
CIRAR	ANTAR
RAPRA	AMARE
CHEAL	ECHCG

Infest degree -90%
(M/D 35/65)
Weeds:

SOLNI	HIBTR
SINAR	AMARE
CIRAR	XANST
VIOAR	CONARCONAR
CHEAL	ECHCG

Fig. 2. Efficiency (%) of herbicides, postemergently applied, to control dicots in sunflower with hybrids resistant to Express 50 SX in Oradea and Teleorman in 2004.



Infest degree - 90%
M/D 65/35%
Weeds:

SORHA (s+r)	AMARE
ECHCG	CHEAL
SETGL	XANST
	SINAR
	CIRAR
	CONAR

Fig. 3. Efficiency (%) of herbicides, postemergently applied, to control dicots in sunflower with hybrids resistant to Express 50 SX

CONCLUSIONS

1. Herbicides DPX 75 and Express 50 SX, applied post-emergence (in both stages – 4-6 and 6-8 leaves) presented a very good selectivity (EWRS quotation =1) for sunflower "resistant" hybrids (XF 4419).
2. The selectivity degree recorded in variants treated with the above mentioned herbicides was superior (EWRS quotation=1) to classical treatment with herbicide based on oxydiargil (EWRS quotation=2^1-2^3).
3. Superior effect (over 90% in 14-28 DAT) was achieved in dicots control (including *Xanthium, Cirsium*) by post-emergence application of herbicides DPX 75, Express 50 SX + adj., at rate of 15 g. a.i./ha, sunflower 4-6 leaves stage.
4. The tested herbicide (in wet conditions) could be applied in association with herbicide based on fluazifop p-butil (in wet conditions), to control mono- and dicots, in "resistant" sunflower.
5. The application of herbicide based on tribenuron (single or with adjuvant), in late stage (sunflower 6-8 leaves) registers a diminution in its control of dicots (below 90%), especially on resistant species (*Xanthium, Chenopodium, Cirsium*), a re-growth taking place after treatment as compared to herbicides applied in optimum stage (sunflower 4-6 leaves; dicots 2-4 leaves).
6. The results obtained regarding the selectivity and efficiency to control dicots, at application of sulfonylurea systemic herbicides were superior to standard treatment – with contact herbicides (based on oxydiargil), which had no effect on "hard to control" species (*Xanthium, Cirsium*).
7. The establishment of an optimum strategy to control weeds in "resistant" sunflower was performed depending on climate conditions (before and after treatment), infestation level, weed prevalence and their development stage at the moment of application.

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Development of CLHA-Plus: a novel herbicide tolerance trait in sunflower conferring superior imidazolinone tolerance and ease of breeding

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ABSTRACT

A novel imidazolinone (IMI)-tolerance trait, CLHA-Plus, was developed through EMS mutagenesis and subsequent selection with imazapyr. The objective of this work was to determine the relative IMI tolerance level of this new mutation with respect to the current commercial IMISUN, by: (a) challenging genetic materials containing the new mutation and/or the old IMISUN with different doses of imazamox and imazapyr under a range of different environmental field conditions and (b) testing the *in vitro* acetohydroxyacid synthase (AHAS) activity of the CLHA-Plus and IMISUN hybrids at increasing levels of imidazolinone herbicides. Lines and hybrids homozygous for the CLHA-Plus mutation demonstrated better tolerance to imidazolinone herbicides than commercially available IMISUN sunflowers which are homozygous for the already known resistant gene (*Imr1*) and an uncharacterized modifier/enhancing factor (*Imr2*). Hybrids heterozygous for the combined mutations CLHA-Plus/IMISUN demonstrated similar field tolerance levels as well as similar AHAS enzyme IMI dose responses to hybrids homozygous for the novel CLHA-Plus mutation. Thus, a higher level of tolerance to imidazolinones can be achieved by allelic substitution of IMISUN by CLHA-Plus in only one of the parental lines of a CLEARFIELD® hybrid, which –in turn- permits a more rapid deployment of this new allele in the hybrid sunflower crop.

Key words: acetohydroxyacid synthase (AHAS) mutation – acetolactate synthase (ALS) mutation sulfonylurea – CLEARFIELD sulfonylurea – CLHA-Plus sulfonylurea – herbicide tolerance sulfonylurea – imidazolinone tolerance.

INTRODUCTION

The imidazolinone family of herbicides control weeds by inhibiting a key enzyme in the branched chain amino acid biosynthetic pathway, acetohydroxyacid synthase (AHAS) or acetolactate synthase (ALS) (Shaner et al., 1984; Tan et al., 2004). Imidazolinones, such as imazapic, imazethapyr, imazapyr and imazamox, are key herbicide components in the CLEARFIELD® production system, which provides effective and extended weed control when used in combination with elite non-GM, imidazolinone tolerant, seed varieties. The CLEARFIELD production system is used commercially in North America, Europe, South America, Asia, Australia and Africa in combination with the following crops: canola (oilseed rape), maize (corn), lentils, rice, wheat, and sunflowers (Pfenning et al. 2008).

The development of CLEARFIELD sunflowers started in 1996, when imidazolinone-tolerant (Pursuit®) wild sunflowers were discovered in a field in Kansas, USA. Seed of these plants were sent to the USDA in Fargo (North Dakota, USA) for subsequent crossing to cultivated sunflower lines (Al-Khatib et al., 1998)

The commercial imidazolinone tolerance trait, IMISUN, which arose from this original USDA introgression work, was commercially launched in the USA, Argentina and Turkey in 2004. From its initial launch up to the present, the IMISUN trait has seen growth in both the number of countries that have adopted this technology and in market share. Sunflower hybrid varieties are currently being commercialized under the CLEARFIELD trademark in 15 major sunflower growing countries in the European Union (EU), Eastern EU, North America (NA), and South America (SA).

The inheritance of IMISUN appears to be additively controlled by two genes, where one (*Imr1*) is a partially dominant gene and the other (*Imr2*) is a modifier or enhancer gene/factor (Miller and Al-Khatib, 2002; Bruniard and Miller, 2001). To produce IMISUN sunflower lines that express commercial tolerance levels to imidazolinone herbicides, both factors need to be homozygous in the final variety (*Imr1Imr1/Imr2Imr2*). Since there are no diagnostic methods available for detecting the presence of the

modifier/enhancer factor *Imr2*, all breeding selections rely on the phenotypic evaluation of the plants that have been sprayed with the imidazolinone herbicide. This phenotypic selection process can be qualitative, depending on the segregation of the IMISUN genes, making the selection of homozygote lines often tedious and time consuming.

In an effort to develop a herbicide tolerance trait which does not require a modifier/enhancing factor, Nidera S.A. together with BASF initiated a seed mutagenesis program in sunflowers to discover new AHAS mutations that would simplify the breeding process. As a result, a new tolerance trait named CLHA-Plus was discovered (Sala et al., 2008). The objective of this work is twofold: (a) to determine the relative imidazolinone tolerance level of this new herbicide tolerance mutation with respect to the current commercial IMISUN and (b) to test the *in vitro* AHAS activity of the CLHA-Plus and IMISUN hybrids at increasing levels of herbicides.

MATERIALS AND METHODS

A sunflower line, BTK47, specifically selected for lack of an E-factor (*imr1 imr1 / imr2 imr2*), was subjected to EMS seed mutagenesis (Sala et al., 2008). An $M_{2:4}$ line which survived imazapyr field selection, was selected for subsequent crossing and enzyme activity studies. This line was named GM40.

Field Evaluation of the CLHA-Plus Trait

The CLHA-Plus mutant allele was introgressed into different maintainer, restorer and sterile inbred lines. Homozygous CLHA-Plus inbreds were crossed with either WT inbreds (containing no herbicide tolerance mutation), homozygous CLHA-Plus inbreds, or homozygous IMISUN inbreds to produce different F_1 mutant allele zygosity combinations (Table 1). These entries, along with several regionally adapted CLEARFIELD® IMISUN commercial variety checks, were field tested for imidazolinone tolerance at numerous locations in North America, South America and EU from 2005 to 2008 (Table 2).

Table 1. Entry list for herbicide tolerance field evaluations (2007)

Entry	Line Description	AHASL1 Allele Zygosity
1	GM40	CLHA-PLUS Homozygous
2	cmsGM40 x R733	CLHA-PLUS Homozygous
3	cmsBTK47 x R731	CLHA-PLUS Heterozygous
4	IA9 x R733	IMISUN / CLHA-PLUS Heterozygous
5	IA9 x RHA426	IMISUN Homozygous
6	B7imi (IMISUN1)	IMISUN Homozygous
7	cmsB7 x RHA426	IMISUN Heterozygous
8	B7	WT

Table 2. Location list for herbicide tolerance field evaluations (2005 - 2007)

Year	Country	Nearest Town Location, State or Province
2005	USA	Velva, North Dakota
2005/2006	Argentina (AR)	Venado Tuerto, Santa Fe
2006	USA	Velva, North Dakota
20062007	Argentina	Venado Tuerto, Santa Fe
20062007	Argentina	Balcarce, Buenos Aires
2007	Argentina	Laguna Blanca, Formosa
2007	USA	Velva, North Dakota
2007	USA	Hickson , North Dakota
2007	France (FR)	Angers
2007	France	Saintes
2007/2008	Argentina	Venado Tuerto, Santa Fe
2007/2008	Argentina	San Jeronimo, Santa Fe
2007/2008	Argentina	Balcarce, Buenos Aires

Table 3. Imidazolinone treatment list for herbicide tolerance field evaluations (2007)

Treatment Number	Herbicide Treatment	Herbicide Product Formulation
1	Untreated	
2	50 g ai/ha imazamox + 0.25% (v/v) NIS*	Beyond 120 g/l LC
3	100 g ai/ha imazamox + 0.25% (v/v) NIS*	Beyond 120 g/l LC
4	200 g ai/ha imazamox + 0.25% (v/v) NIS*	Beyond 120 g/l LC
5	160 g ai/ha imazapyr + 0.25% (v/v) NIS*	Arsenal 240 g ai/L
6	320 g ai/ha imazapyr + 0.25% (v/v) NIS*	Arsenal 240 g ai/L

*NIS = non-ionic surfactant = Induce 90SC (90%)

The entries at each location in 2007 and 2007/2008 were arranged in a randomized two factorial split plot design consisting of 3 replications for each treatment combination. Factor A was the herbicide treatment (Table 3), and factor B was the sunflower entry (Table 1). The plot size was 2 rows x 7 m and the seeding rate was consistent with local agronomic practices. The herbicide treatment was applied at the 2-4 leaf stage with a tractor mounted boom (20 gallons/acre or 200 litres/ha). Treatment 2 was only applied at 2 locations in France.

Crop injury (% phytotoxicity) ratings were evaluated at 6 - 10 days after treatment and at 16 - 21 days after treatment. Percent phytotoxicity was recorded as the average amount of plant damage in a given plot, where a rating of '0%' indicated no damage to plants relative to the untreated plot. A rating of 10% to 40% indicated increasing levels of chlorosis (where 40 would be complete yellowing of the leaves). A rating of 50% or higher indicated that the plants demonstrated complete yellowing as well as increasing levels of leaf necrosis. A rating of '100%' indicated complete necrosis (death) of the plants.

The emergence, days to flower, days to end of flower and maturity were also assessed for each plot at each location (data not shown). The data were subjected to an ANOVA analysis.

Enzyme Assay for AHAS Activity

Twelve greenhouse grown sunflower plants from each of the lines depicted in Table 4 were bulked and subjected to an AHAS enzyme activity assay (Singh et al., 1988). Each activity assay was repeated twice. Due to the large number of samples, the experiment was split into two sets (Table 4).

Table 4. Line descriptions and corresponding AHASL1 mutation allele zygosities

Set	Line Description	AHASL1 Allele Zygosity
1	cmsGM40 x R733	CLHA-PLUS Homozygous
1	IA9 x R733	IMISUN/CLHA-PLUS Heterozygous
1	IA9 x RHA426	IMISUN Homozygous
1	B7	WT
2	GM40	CLHA-PLUS Homozygous
2	cmsBTK47 x R731	CLHA-PLUS Heterozygous
2	B7imi (IMISUN1)	IMISUN Homozygous
2	cmsB7 x RHA426	IMISUN Heterozygous
2	B7	WT

Protein extracts from young, actively growing leaves from four week old plantlets were prepared and subjected to an AHAS inhibition assay (Singh et al., 1988). Assays were conducted in a 96-well format. Fifty µl of inhibitor was added to each well containing 50 µl of soluble protein extract to give final concentrations of 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50 and 100 µM imazamox or 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50 and 100 µM imazapyr. Zero herbicide controls were also included for each line. Reactions were processed as outlined by Singh et al. (1988). Absorbance was measured at 530 nm. AHAS activity, expressed as the mean of the absorbance values for each treatment, was presented as a percentage of the mean of the zero-herbicide controls.

RESULTS AND DISCUSSION

To assess HT genes for their relative tolerance level, two approaches were used. The first approach measured herbicide injury in the field under a range of environmental stringencies (locations and years in combination with different herbicide doses), and the second approach tested the target enzyme (*in vitro*) with increasing levels of herbicide.

In the field, the crop injury phenotype can be attributed to the interaction between genotype and environment (GxE). The genotypic factor in a herbicide tolerant (HT) plant is the sum of the HT gene(s) plus the remaining genetic background, and the interaction between the two. The environmental component (E) is a sum of abiotic (i.e. weather, soil) and biotic factors (i.e. insect, disease and weed pressure) coupled with the effect of the herbicide dose. An example of this environmental effect is seen in Fig. 1, where a variation in phytotoxicity of the same genotype grown in four different locations (Velva ND, Angers FR, Saintes FR, Formosa AR) at the same dose rate (200 g ai/ha imazamox) is observed. To better understand the environmental factor associated with this trait, we calculated the mean phytotoxicity index (PI) of the current commercial, regionally adapted, IMISUN checks at 6 – 10 days after herbicide treatment across many locations over 3 years. PI values for different hybrids carrying the CLHA-Plus mutation were plotted against the mean PI values of the IMISUN checks to evaluate the relative resistance level of the new mutation across a range of Es (Fig. 2). As can be seen in the x axis of Fig. 2, the combination of locations with herbicide doses produced a diverse array of Es, which ranged in PI mean values from 5.9 to 78 for the imazamox treatments (not shown); and 2 to 100 for the imazapyr treatments (Fig. 2). The y=x line represented the mean PI value for the IMISUN checks across all Es.

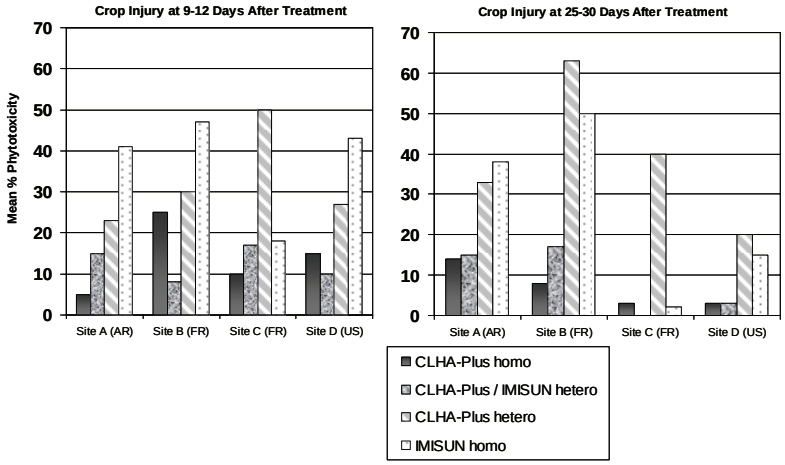

Fig. 1. Crop Injury (Mean % Phytotoxicity) at 200 g ai/ha Imazamox at 4 Field Locations in 2007 for 4 Different Types of Hybrids (see legend)

The results obtained following imazamox treatments are described in the following paragraph. The CLHA-Plus homozygous hybrids showed an increase in PI as the environmental component became more severe. However, the slope of the regression line ($b = 0.149 \pm 0.0667$, $P<0.0375$) indicated that the level of crop injury, as a function of environmental stringency, increased at a lower rate than the IMISUN checks. Hybrids which were heterozygous for the double CLHA-Plus / IMISUN stack showed a similar response to environmental stringency ($b=0.39\pm0.05$, $P<0.0001$) as the homozygous CLHA-Plus hybrids. On the other hand, hybrids containing the CLHA-Plus mutation in a heterozygous state (CLHA-Plus/WT) demonstrated higher crop injury ratings than the IMISUN checks at lower levels of environmental stringency, as shown by the higher y-intercept value of the regression line ($a=15.3\pm2.67$). When the severity of the environmental component was increased, these CLHA-Plus heterozygous hybrids showed a better performance than the IMISUN checks, as was shown by the slope of its linear equation ($b=0.45\pm0.062$, $P<0.0001$). The same was observed in Fig. 2 when the same entries, in the same environments, were challenged with imazapyr (environmental stringencies for each genotype are summarized by the regressions in the Fig. 2 Legend).

To substantiate the herbicide tolerance effect observed in the field, the same herbicide tolerance gene combinations were subjected to AHAS enzyme inhibition studies. These studies were conducted on the bulk of 12 individuals from each entry in Table 1. The mean of two replications are represented in Fig. 3 for Set 1 (Table 1) and in Fig. 4 for Set 2 (Table 1). An untreated control sample was included to provide a baseline for 100% AHAS enzyme activity. The AHAS activity in the CLHA-Plus homozygous hybrid

treated with 100 µM imazamox was 69% of the untreated control, and for the 100 µM imazapyr it was 64% of the untreated control (Fig. 3). The activity of the AHAS enzyme in the CLHA-Plus/IMISUN heterozygous hybrid was 59% and 60% for extracts treated with 100 µM imazamox and 100 µM imazapyr respectively (Fig. 3). The IMISUN homozygous hybrid line, current commercial CLEARFIELD® product, demonstrated AHAS activities of 36% of untreated control and 42% of untreated control at 100 µM imazamox and 100 µM imazapyr respectively (Fig. 3), which is lower than the activities of both the CLHA-Plus homozygous hybrid and the CLHA-Plus/IMISUN heterozygous hybrid.

In the second set of data, the IMISUN homozygous hybrid (current commercial CLEARFIELD product) performed similarly to the CLHA-Plus heterozygous hybrid (Fig. 4). More specifically, the IMISUN hybrid demonstrated 26% activity at 100 µM imazamox and the CLHA-Plus heterozygous hybrid had 30% activity at 100 µM imazamox. In contrast, the AHAS enzyme extract from the CLHA-Plus homozygous hybrid demonstrated the least amount of inhibition with increasing levels of imazamox, demonstrating activities of 63% and 60%, relative to the untreated control, at 50 µM and 100 µM imazamox respectively (Fig. 4). The WT line (B7) was genotypically identical in both experimental sets and demonstrated a variance of 6% activity at the 100 µM imazamox level between the two experiments (17% AHAS activity relative to the untreated control in Set 1 (Fig. 3) and 11% AHAS activity relative to the untreated control in Set 2 (Fig. 4).

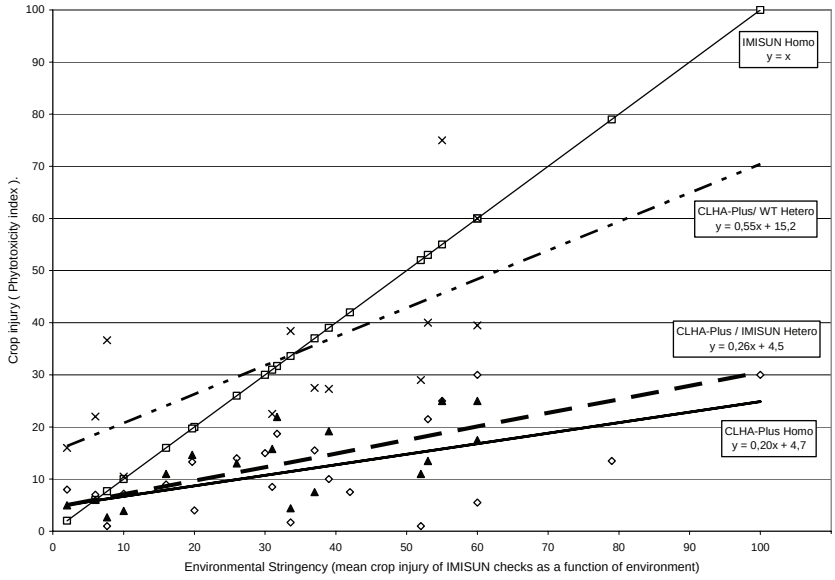

Fig. 2. Crop Injury of different types of sunflower hybrids carrying the CLHA-Plus mutation after imazapyr application ((CLHA-Plus homozygous: b= 0.20 ± 0.06, P<0.048 CLHA-Plus /IMISUN heterozygous: b: 0.26 ± 0.07, P<0.0019; CLHA-Plus /WT: b: 0.55±0.18, P< 0.0109)

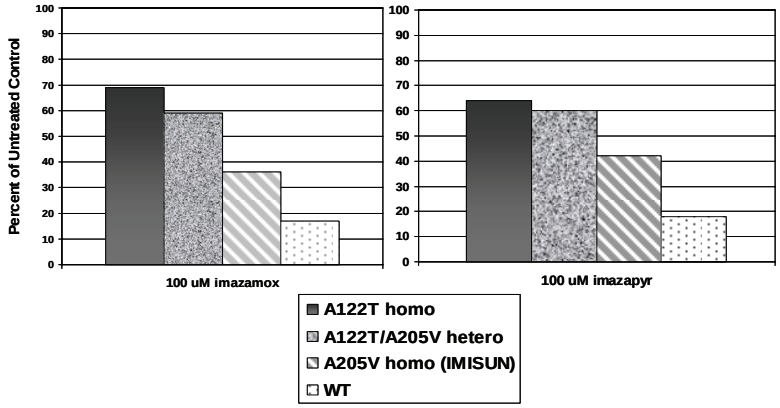

Fig. 3. AHAS Enzyme Activity (as Percent of untreated controls) of Four Sunflower Lines at 100 micromoles of Imazamox and 100 micromoles of Imazapyr

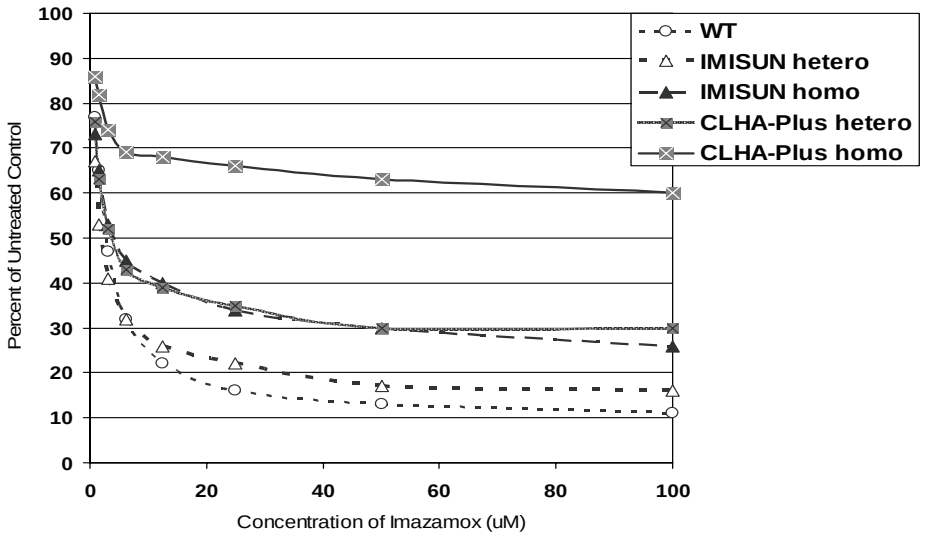

Fig. 4. AHAS Enzyme Activity (Percent of untreated controls) of Five Sunflower Lines with Increasing Levels of Imazamox

Based on field and AHAS enzyme activity data, it was determined that the novel CLHA-Plus mutation provides superior herbicide tolerance to imidazolinones versus the current IMISUN mutation. Commercial levels of herbicide resistance in IMISUN sunflowers require the combination of two genetic factors in a homozygous state due to the moderate level of resistance conferred by *Imr1*. In contrast, by using the CLHA-Plus mutation alone, the *Imr2* enhancer is no longer necessary to achieve commercial levels of tolerance. Most importantly, the results demonstrate that CLHA-Plus can be used either as a homozygous single gene HT trait or as a heterozygous stack together with the IMISUN HT trait, providing enhanced levels of tolerance, greater flexibility in weed control and facilitating the deployment of this new mutation in the CLEARFIELD® Production System.

ACKNOWLEDGEMENTS

The authors would like to thank the following employees at Nidera S.A.: Guillermo San Román, Leandro Amela, Juan Pablo Bizzera and Néstor Duarte and at BASF: Thierry Guillet, Patrice Cabiac, Daniel Rolon, Vince Ulstad and Julie Roland for their expert advice and assistance with this project.

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Selection of sunflower hybrids for Banja Luka area in Bosnia and Herzegovina

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ABSTRACT

Banja Luka area in Bosnia and Hezegovina does not have a regular sunflower production, although this area has favorable climate and soil conditions for profitable sunflower production. Based on three-year results of sunflower NS-hybrids developed at Novi Sad Institute of field and Vegetable Crops evaluated in micro experiments in the area of Banja Luka, we can establish the following conclusions: the highest average yield in a three-year period was accomplished by hybrids NS-H-411 and HN-H-45; the highest oil content in a three-year period was accomplished by hybrids Banaćanin and Kraišnik; the highest oil yield was accomplished by hybrids NS-H-411 and NS-H-45. In Banja Luka area of Bosnia and Herzegovina it is possible to achieve profitable production of sunflower on wider sowing areas, which could supply necessary quantity of oil as well as protein for livestock feeding.

Key words: Bosnia and Herzegovina – NS hybrids – oil content – oil yield – seed yield.

INTRODUCTION

Thanks to long-term selection work of Novi Sad Institute of Field and Vegetable Crops, today we have a great selection of high-yield sunflower hybrids for production in our agro-ecological conditions in Bosnia and Herzegovina. Our objective is to select high-yielding sunflower hybrids well adapted to agro-ecological areas in Bosnia and Herzegovina through long-term evaluation.

MATERIALS AND METHODS

We performed field evaluations in the period of 2004-2006 on a soil with acid reaction (pH=4.1) and low organic matter content (2.1%) at an altitude of 154 m. We evaluated 11 hybrids in 2004, 15 hybrids in 2005, and 13 hybrids in 2006. Evaluations were performed using randomized block design with four replications. The area of the plot was 14 m^2, with a distance between rows of 70 cm and a distance of plants in the row of 35 cm. Cultural practices were the standard for sunflower commercial production.

Climate parameters (Table 1) showed that the trials were conducted under optimal climate conditions. Average monthly temperatures in the three years included in the study were similar to long-term average temperatures (Table 1a). Precipitation during the vegetation period was greater than the long-term average (Table 1b).

Table 1. Meterological Parameters.

a) Average monthly temperatures (^oC).

Year	Month					
	April	May	June	July	August	September
2004	11.9	14.8	19.6	21.6	21.4	16.0
2005	11.8	16.3	19.4	22.0	19.4	17.0
2006	12.4	16.0	20.0	22.9	19.5	17.4
Average:	12.0	15.7	19.7	22.2	20.1	16.8
Long-term average:	10.9	16.1	19.3	21.4	21.1	16.7

b) Precipitation (mm).

Year	Month					
	April	May	June	July	August	September
2004	166.4	86.1	104.3	129.6	45.0	46.3
2005	80.5	79.2	135.6	129.7	124.9	79.7
2006	151.6	95.0	126.7	80.00	220.0	47.4
Average:	132.8	86.8	122.2	113.1	129.9	57.8
Long-term average:	80.3	95.0	113.7	87.1	71.6	90.6

RESULTS AND DISCUSSION

Long-term evaluation of NS-hybrids showed the great potential of sunflower production in Banja Luka area in Bosnia and Herzegovina. The high seed oil yield achieved demonstrated that it is possible to perform profitable production of sunflower in Bosnia and Herzergovina, which confirms previous results of Kondić (2004; 2005), Crnobarac et al. (2007), and Miklić et al. (2007).

The results showed that the hybrids with the highest yield potential were NS-H-411, NS-H-45, NS-H-43 and Somborac. Seed yield of these hybrids was between 3,775-4,402 kg/ha (Table 2). Oil content was found between 32.9% (Labud) and 46.81% (Baća). The hybrids with the highest seed oil content were Baća, Somborac, Banaćanin, and Kraišnik (Table 3). Oil yield ranged from 1,251 kg/ha (Labud) to 1,866 kg/ha (NS-H-411) (Table 3). The highest oil yield was recorded in the hybrids NS-H-111, NS-H-45, Kraišnik, NS-H-43, and Baćanin (Table 3).

Table 2. Seed yield of sunflower hybrids evaluated in Banja Luka area of Bosnia and Herzegovina.

Hybrid	Grain yield with 11% humidity (kg/ha)			Average
	2004	2005	2006	
NS-H-111	4,070	4,455	4,700	4,408
NS-H-45	4,190	4-312	3,700	4,401
Krajišnik	3,700	3,350	4,440	3,830
Bačvanin	3,290	3,895	3,760	3,658
Banaćanin	3,370	3,160	4,540	3,690
Velja	3,150	2,665	3,960	3,258
Perun	3,540	3,405	4,020	3,655
Olivko	3,100	3,200	3,510	3,270
Pobednik	4,050	3,510	2,970	3,510
Labud	3,660	3,945	-	3,802
NS-H-43	3,800	3,770	-	3,785
Šumadinac	-	3,300	3,200	3,250
Baća	-	3,230	3,110	3,170
Somborac	-	3,750	3,800	3,775
Sremac	-	3,605	3,400	3,502
Average:	3,656	3,570	3,778	
LSD 5%	553	391	244	
1%	735	519	326	

Table 3. Oil content (%) and oil yield (kg/ha) of sunflower hybrids evaluated in Banja Luka area of Bosnia and Herzegovina.

Hybrid	Oil content (%)			Average	
	2004	2005	2006	Oil content %	Oil yield kg/ha
NS-H-111	42.19	45.96	38.86	42.34	1,866
NS-H-45	40.57	46.33	39.72	42.21	1,858
Krajišnik	43.13	49.77	43.20	45.37	1,738
Bačvanin	45.05	45.23	40.46	43.58	1,590
Banaćanin	42.78	50.83	44.07	45.89	1,693
Velja	45.74	45.84	41.06	44.21	1,440
Perun	38.71	49.84	41.06	43.20	1,579
Olivko	40.57	45.44	45.04	43.68	1,428
Pobednik	38.86	49.44	45.89	44.73	1,570
Labud	36.13	29.70	-	32.91	1,251
NS-H-43	41.97	48.23	-	45.10	1,707
Šumadinac	-	46.60	41.81	44.20	1-436
Baća	-	49.11	44.51	46.81	1,484
Somborac	-	48.26	43.65	45.95	1,735
Sremac	-	45.61	42.20	43.90	1,537

The three-year evaluation of NS-hybrids of sunflower in the area of Banja Luka in Bosnia and Herzegovina led us to the following conclusions:

1. Climate and soil conditions are satisfactory for sunflower production in Bosnia and Herzegovina.
2. All examined hybrids gave average yield larger than 3 t/ha.
3. It is possible to increase yield potential by further evaluation of hybrids and through adoption of modern production techniques.

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Principal component analysis as a reflector of combining abilities

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ABSTRACT

In this study Principal Component Analysis was used to reveal two dimensional structures based on general (GCA) and specific combining abilities (SCA) in a sunflower crossing program. GCA and SCA of new sunflower inbred lines accompanied by genetic variance components were estimated using Line x Tester analysis method. Six new restorer lines were crossed with four CMS lines as tester in 2006. 24 combinations were planted in randomized block design with four replications in Khoy Agricultural and Natural Resources Research Station in 2007. All traits except head diameter and plant height were under control of both additive and dominant effects. Seed yield was mainly under control of dominant effects. For the growth period, and in other traits, additive effects had a major influence. Overdominant effects for growth period and seed yield, complete dominance for plant height and partial dominance for other traits were evident. Principal component analysis revealed a close positive association between seed yield, oil yield and seed number, whereas there were negative associations between these traits and 1000 seed weight. Principal components located entries based on their whole performance in the biplots provided. Restorer line R21 and CMS line CMS356 had a strong effect on their corresponding hybrids. Ordination biplots provided simplicity of selection based on agronomic means instead of SCA data. R23 x CMS78 was a superior early matured hybrid with higher oil content and yield. We could use PCA based on agronomic data alone instead of combining abilities for determining real performance of entries considering multivariate traits.

Key words: combining ability sulfonylurea – principal component analysis – sunflower sulfonylurea – variance components.

INTRODUCTION

Sunflower is one of the most important oilseed crops of Iran but its planting area has been very variable and recently decreased to 30000ha. Breeding programs in seed and plant investigation institutes are carried out for producing new hybrids to replace open pollinated (OP) varieties. Iranian Hybrids have not been very successful to date but a new generation of F1 hybrids is now being produced. A basic research program in this field has been focused on producing parental lines and many CMS and restorers have been produced. In these programs, estimation of combining ability of lines to identify superior parents for hybridization is essential. Besides an estimation of combining abilities and identifying the type of gene action governing yield-related traits, this study was made to establish a two dimensional structure between agronomic performance and combining abilities. Predominant of dominance gene action was reported for plant height, head diameter, oil content, 100 seed weight and seed and oil yield (Gangappa et al., 1997). However, additive gene action for these traits has also been reported (Singh et al., 1989) Estimates of GCA and SCA (Bedov, 1985) indicated that additive effects were more important for oil content. Additive gene action has the greatest influence on flowering (Alvarez et al., 1992). A significant relationship between morphological differences between inbred lines and SCA effects was found for oil yield and seed number per plant (Luczkiewicz and Kaczamarek, 2004). These relationships could be used to evaluate lines directly based on agronomic values instead of on combining abilities. Biometrical methods such as principle component analysis allow us to recognize structures between genotypes based on multivariate traits. Tersac and et al. (1993) used PCA based on SCA to show a structure of sunflower populations by country of origin. De la Vega et al. (2001) used PCA for revealing two dimensional structures among genotypes and environments based on their interactions. They reported the effectiveness of PCA for demonstrating genotype x environment interactions. In this study we have investigated the efficiency of PCA in screening genotypes due to GCA and SCA of multivariate traits.

MATERIALS AND METHODS

This experiment was conducted in Khoy-Iran Agricultural and Natural Resources Research Station in 2007. The station is located in 38º 32' north latitude and 44º 58' east altitudes. Six new restorer lines were crossed with four cytoplasmic male sterile (CMS) lines in line x tester fashion to generate 24 single cross hybrids in first year and twenty four F1 hybrids planted in randomized block design with four replications in 2007. Each experimental plot consisted of 3 rows of 4 m in length with 60cm spacing between rows and 25cm within rows. Fertilizers were applied at the rate of 100:70:90 NPK kg/ha. Field practices were followed according to the regional sunflower planting handbook (Ghaffari, 2006). Data of measured traits for hybrids subjected to Line x Tester analysis (Kemptorne, 1957) to estimate general combining ability (GCA), specific combining ability (SCA), effects and their respective variances were collected. Principal Component Analysis (PCA) was used to arrange the entries in two dimensional biplots (Kroonenberg, 1997) based on agronomic performance and combining abilities.

RESULTS AND DISCUSSION

There were significant differences between crosses for all measured traits but differences between CMS lines were greater than those between restorers (Table 1).All traits except head diameter and plant height were under control of both additive and dominant effects. Seed yield was mainly under the control of dominant effects. For growth period, the dominant effects were more important than additives and in other traits additive effects had a major influence. Over dominant effects for growth period and seed yield, complete dominance for plant height and partial dominance for other traits were evident (Table 2).

Table 1. Analysis of variance for agronomic traits

Sources	D.F	Growth Period	Plant Height	Head Diameter	1000 Seed weight	Seed Number /head	Seed yield	Oil Content	Oil Yield
Replication	3	44.9**	339.26	48.244**	314.2**	35628.6	1162745.4*	50.32**	182658.6
Crosses	23	32.4**	349.49**	5.445	154.7**	79052.1**	1155345.3**	18.12**	278046.6**
Lines	5	39.8**	550.8**	2.932	171.9**	34051.2	470879.6	33.48**	123211
Testers	3	68.2**	605.8*	5.653	610.33**	349693.9**	3627592.4**	58.64**	1043106**
Lines x Testers	15	22.8**	231.1	6.241	57.8*	39923.9*	889051.3**	4.89**	176646.7*
Error	69	6.6	160.9	5.373	28.7	20324.9	361540.3	7.039	86292.98
C.V.		2.42	6.86	12.47	7.42	21.73	19.29	5.87	20.80

* and ** significant at 0.05 and 0.01 level of probability respectively

Table 2. Variance components for agronomic traits

Variance	Growth Period	Plant Height	Head Diameter	1000 Seed weight	Seed Number /head	Seed yield	Oil Content	Oil Yield
Additive	3.13*	34.72*	-0.19	33.33**	15194.86*	116018.49+	4.12**	40651.16*
SE	1.24	12.40	0.12	9.94	5568.92	58699.98	1.03	16712.78
Dominance	4.03**	17.53	0.22	7.28*	4899.76*	131877.70**	0	22588.43*
SE	0.99	10.47	0.29	2.55	1764.04	38865.08	0.26	7787.033934
Dominance rate	1.61	1.00	0.66*	0.66*	0.80	1.51	0.00	1.05

* and ** significant at 0.05 and 0.01 level of probability respectively

Restorers R50, R21 and R23 had the highest GCA for seed yield but none of them was significant (Table 3). R21, R23 and R56 had significant positive GCA for 1000 seed weight, seed number and oil content, respectively. This indicates that these restorers seemed to possess increasing alleles with additive effects for the mentioned traits. R26 and R50 had significant negative GCA for growth period indicating presence of alleles with additive effects for early maturity. Therefore, R26 seemed to possess additive alleles for dwarfness. Single branch restorer RG50 distinguished itself as being a good line for using in crossing programs because of having the desired GCA for seed yield and growth period.

Testers CMS52 and CMS148 had the highest GCA for seed yield but only CMS52 had significant GCA in the desired (positive) direction (Table 4). CMS 356, CMS52 and CMS78 lines had significant positive GCA for 1000 seed weight, seed number and oil content, respectively. CMS356 seemed to have alleles with additive effects for increasing the growth period, while CMS52 had alleles for decreasing it. It would seem that CMS52, because of having the desired GCA for seed yield and growth period, could be used as a valuable A-line in crossing programs.

Table 3. GCA of restorer lines for agronomic traits

Restorer	Growth Period	Plant Height	Head Diameter	1000 Seed weight	Seed Number /head	Seed yield	Oil Content	Oil Yield
R19	0.07	-6.11	-0.32	-0.62	-50.91	-254.66	-1.28*	-158.63*
R21	-0.61	7.96*	0.58	-3.37	72.74*	114.51	-0.34	44.43
R23	2.51**	1.97	-0.43	5.41**	-28.18	82.43	1.14	82.07
R26	-1.55*	-7.72*	0.39	0.94	-31.40	-112.57	0.29	-41.77
R50	-1.49*	1.54	0.13	0.94	30.39	217.85	-1.82*	36.76
R56	1.07	2.37	-0.35	-3.31	7.38	-47.57	2.01**	39.20
SE	0.59	2.90	0.53	1.22	32.54	137.22	0.61	67.04

* and ** significant at 0.05 and 0.01 level of probability respectively

Table 4. GCA of CMS lines for agronomic traits

CMS	Growth Period	Plant Height	Head Diameter	1000 Seed weight	Seed Number /head	Seed yield	Oil Content	Oil Yield
CMS78	-0.70	2.19	-0.35	-2.41	21.10	23.96	1.31*	54.65
CMS52	-1.32*	3.47	0.69	-2.05	87.16**	318.68*	0.60	154.93*
CMS148	-0.45	-7.46**	-0.33	-3.09	67.96	211.18	0.35	97.01
CMS356	2.47**	1.81	-0.01	7.53**	-176.21	-553.82**	-2.26	-306.57**
SE	0.46	2.24	0.41	0.95	25.20	106.29	0.47	51.93

* and ** significant at 0.05 and 0.01 level of probability respectively

Hybrids R23 x CMS78 and R56 x CMS356 had high SCA for both seed yield and growth period (Table 5). These hybrids had seed yield of 4105 and 3022 kg/ha, respectively, and growth period of 107 days (Table 6). Crossing of R21, R26, and R50 with CMS52 and R21 with CMS148 resulted in higher seed yield (over 3500 kg/ha) with short growth period (107 days) and makes them high yielding early maturing hybrids for summer cropping.

Table 5. SCA of crosses for agronomic traits

Restorer	CMS	Growth Period	Plant Height	Head Diameter	1000 Seed weight	Seed Number /head	Seed yield	Oil Content	Oil Yield
R19	CMS78	-0.11	-1.51	1.18	1.13	-79.13	-308.12	-1.38	-172.54
R19	CMS52	-0.99	10.06*	-0.91	0.52	18.04	60.49	0.86	50.80
R19	CMS148	3.64**	1.31	0.53	1.94	59.81	406.32	-0.79	152.33
R19	CMS356	-2.53*	-9.89	-0.79	-3.56	1.28	-158.68	1.32	-31.98
R21	CMS78	-1.93	-3.96	-1.02	-7.25	-23.97	-403.96	1.46	-139.56
R21	CMS52	0.95	-2.46	1.92*	-1.60	149.51*	649.66*	-1.12	250.58*
R21	CMS148	0.57	2.09	-1.54	2.94	30.39	257.16	-0.46	109.74
R21	CMS356	0.41	4.30	0.64	5.94*	-155.94*	-502.85*	0.12	-222.15
R23	CMS78	-2.05*	-7.74	0.65	2.35	145.60*	881.47**	0.15	411.84**
R23	CMS52	-1.18	10.14*	-1.69	-0.88	-120.49*	-598.26*	0.74	-252.21
R23	CMS148	-2.05*	0.01	0.22	-0.22	-55.88	-222.43	0.11	-89.18
R23	CMS356	5.28**	-2.44	0.83	-1.22	30.76	-60.76	-1.00	-71.83
R26	CMS78	0.51	2.45	0.42	-0.56	-65.97	-350.21	0.80	-149.18
R26	CMS52	-0.11	2.32	-0.47	-0.54	44.58	235.07	1.07	148.78
R26	CMS148	0.26	-2.55	-0.35	-2.37	68.75	200.91	-0.35	71.64
R26	CMS356	-0.66	-2.25	0.41	3.50	-47.39	-85.76	-1.52	-72.63
R50	CMS78	2.45*	7.86	-1.07	1.94	-8.67	6.04	-0.48	-17.65
R50	CMS52	-0.43	-16.36**	0.54	-0.66	13.36	29.66	-0.40	3.84
R50	CMS148	-2.05*	-0.01	1.91*	-3.00	-37.44	-337.85	0.60	-132.12
R50	CMS356	0.03	8.49	-1.38	1.75	32.75	302.16	0.28	144.53
R56	CMS78	1.14	2.86	-0.16	2.44	32.12	174.80	-0.55	65.00
R56	CMS52	1.76	-3.74	0.62	3.21	-105.02	-376.60	-1.16	-203.87
R56	CMS148	-0.36	-0.89	-0.76	0.75	-65.63	-304.10	0.90	-114.49
R56	CMS356	-2.53*	1.74	0.30	-6.37	138.52*	505.91*	0.81	251.98*
SE		1.02	5.02	0.92	2.12	56.35	237.68	1.05	116.12

* and ** significant at 0.05 and 0.01 level of probability respectively

Ordination with PCA was used to determine if there is any structure related to agronomic performance, GCA and SCA regarding multivariate characters. According to combining abilities two principal components accounted for 62% of variability in the GCA and SCA of entries. Ordination in biplot was based on discrimination of entries by multivariate GCA and SCA for measured characters. Traits with higher weight in principal components could discriminate entries effectively to exert

multivariate selection. Combining ability of data, seed number (SN), oil yield (OY) and seed yield (SY) had the highest weight in principal component 1, so this component could discriminate entries according to the traits. It can be seen that R23 x CMS78 with highest SCA for SY located further along in the positive direction of its vector (Fig. 1). Two other hybrids with high SCA located near the vector but with a different distance from the vector. The closer one (R21 x CMS52) has a higher SCA. On the other hand,

Table 6. Mean values of agronomic traits in the crosses

Restorer	CMS	Growth Period	Plant Height	Head Diameter	1000 Seed weight	Seed Number /head	Seed yield	Oil Content	Oil Yield
R19	CMS78	106.00	179.63	19.10	70.38	547.26	2,578.35	43.84	1,136.02
R19	CMS52	104.50	192.48	18.05	70.13	710.49	3,241.68	45.37	1,459.63
R19	CMS148	110.00	172.80	18.48	70.50	733.06	3,480.02	43.48	1,503.25
R19	CMS356	106.75	170.88	17.48	75.63	430.36	2,150.01	42.97	915.35
R21	CMS78	103.50	191.25	17.80	59.25	726.07	2,851.68	47.62	1,372.05
R21	CMS52	105.75	194.03	21.78	65.25	965.61	4,200.02	44.33	1,862.48
R21	CMS148	106.25	187.65	17.30	68.75	827.29	3,700.02	44.74	1,663.71
R21	CMS356	109.00	199.13	19.80	82.38	396.79	2,175.01	42.70	928.24
R23	CMS78	106.50	181.48	18.45	77.63	794.72	4,105.02	47.80	1,961.08
R23	CMS52	106.75	200.63	17.15	74.75	594.69	2,920.02	47.68	1,397.32
R23	CMS148	106.75	179.58	18.05	74.38	640.10	3,188.35	46.80	1,502.43
R23	CMS356	117.00	186.40	18.98	84.00	482.57	2,585.01	43.07	1,116.19
R26	CMS78	105.00	181.98	19.05	70.25	579.93	2,678.35	47.59	1,276.24
R26	CMS52	103.75	183.13	19.20	70.63	756.54	3,558.35	47.15	1,674.47
R26	CMS148	105.00	167.33	18.30	67.75	761.51	3,416.68	45.48	1,539.42
R26	CMS356	107.00	176.90	19.38	84.25	401.20	2,365.01	41.69	991.57
R50	CMS78	107.00	196.65	17.30	72.75	699.02	3,365.02	44.21	1,486.29
R50	CMS52	103.50	173.70	19.95	70.50	787.11	3,683.35	43.57	1,608.07
R50	CMS148	102.75	179.13	20.30	67.13	717.11	3,208.35	44.32	1,414.19
R50	CMS356	107.75	196.90	17.33	82.50	543.13	3,083.35	41.39	1,287.25
R56	CMS78	108.25	192.48	17.73	69.00	716.80	3,268.35	47.96	1,571.39
R56	CMS52	108.25	187.15	19.55	70.13	645.72	3,011.68	46.64	1,402.79
R56	CMS148	107.00	179.08	17.15	66.63	665.91	2,976.68	48.44	1,434.26
R56	CMS356	107.75	190.98	18.53	70.13	625.89	3,021.68	45.74	1,397.14
LSD5%		3.53	17.81	3.23	7.72	195.7	819.5	3.74	414.4

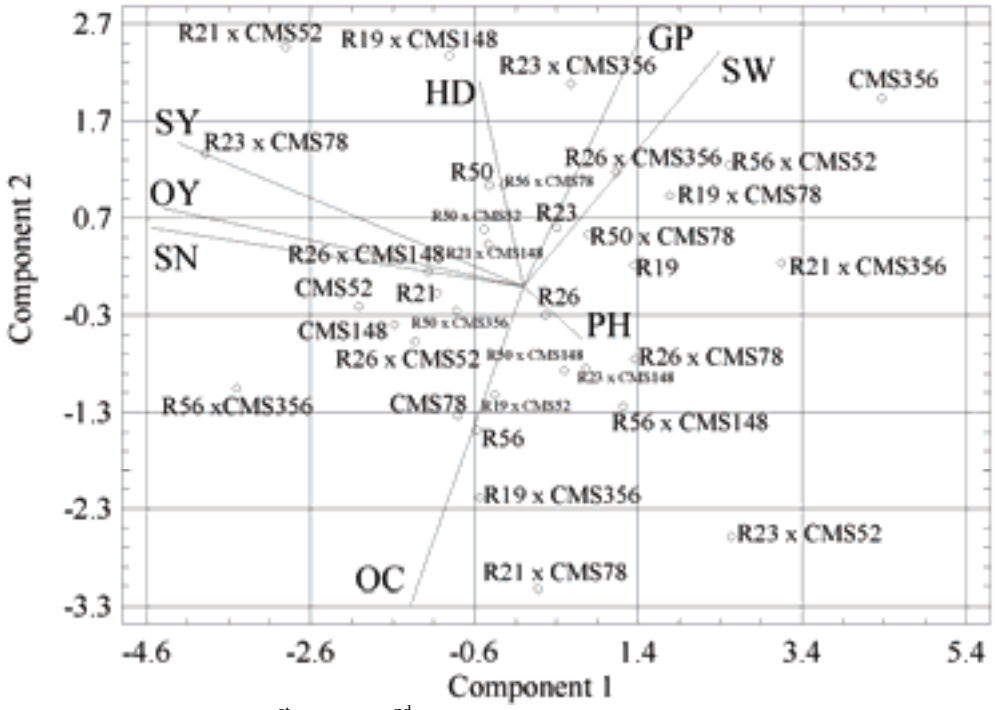

Fig. 1. Biplot of the 1st and the 2nd principal component for general and specific combining abilities.

the lowest SCA belongs to R23 x CMS52, and, logically, it is located further along in the inverse direction of its vector followed by the next high SCA hybrids R21 x CMS356 and R21 x CMS78. This statement is in accordance with de la Vega et al. (1997) who reported the discrimination of genotypes and environment based on their interactions. Acute angles for SY, OY and SN indicated positive associations between them. 1000 seed weight (SW), growth period (GP) and head diameter (HD) is closely associated in this respect. So selection should be made according to one of these traits, accompanied by selection according to the associated traits too, and this would allow making multivariate selection on breeding materials. We found that oil content (OC) has not been associated with SY and OY and that there is a strong negative association for SW with GP and HD because of the obtuse angle for their vectors. Among parental lines, CMS356 had the highest GCA for SW and was located in the same direction of its vector with a distance further away from the origin. R56 and CMS78 had higher SCA for OC and were located in the same direction of their vector. Genotypes with values close to the mean of entries were located near the origin. This was the situation for most parental lines.

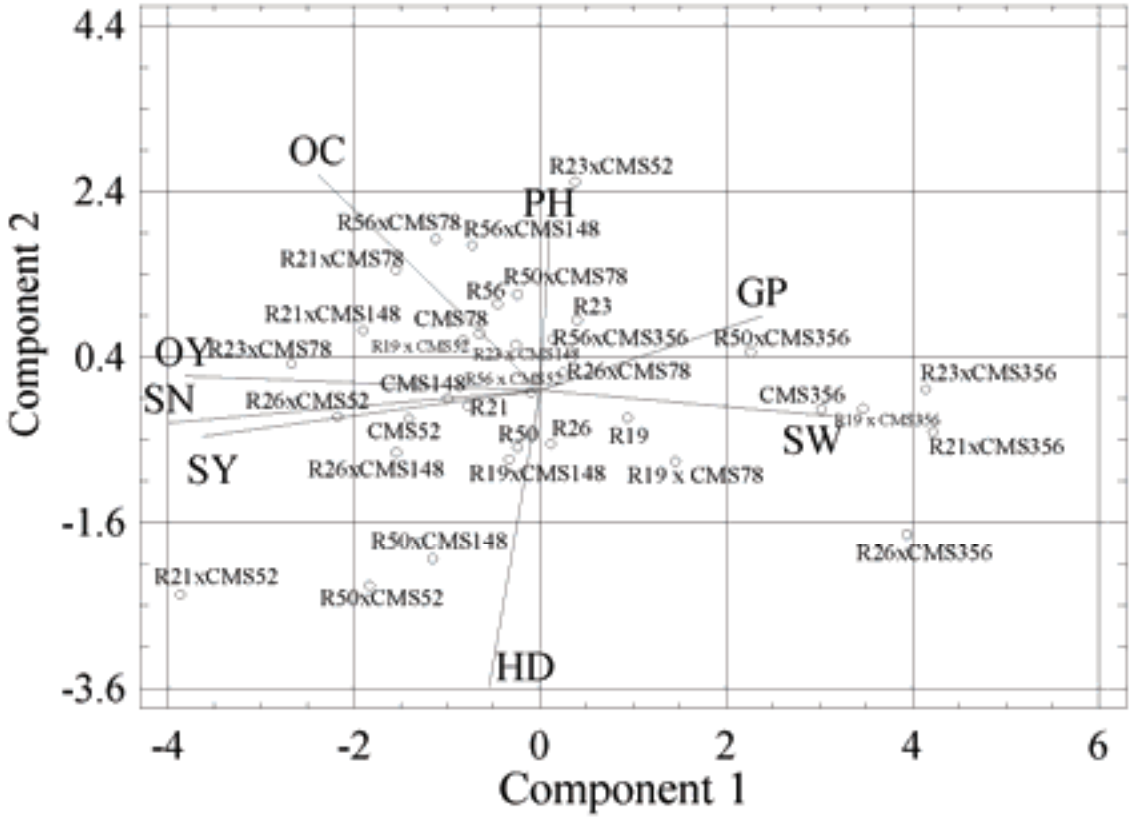

Fig. 2. Biplot of the 1st and the 2nd principal component for agronomic means

Traits with low weight in principal components could not discriminate entries efficiently .This is true for R56 x CMS148 due to its SCA for plant height (PH). Considering agronomic means of SN, OY and SY, these had the highest negative weight in principal component 1, whereas in principal component 2 positive weights of OC and PH and negative weight of HD were higher. A negative association for SN and SW is understood from biplot in Fig. 2. R21 x CMS52 and R23 x CMS78 were located further along in the positive direction of SY vector because of their high seed yields. Hybrids with a low seed yield located on the inverse side of biplot ordination of entries might be influenced by the presence of multivariate effects. For example, ordination of R26 x CMS52 with a lower seed yield than R21 x CMS 148 is not in agreement with the statements, and, in fact, it resulted from the effect of the GP. So, multivariate reactions could cause problems in the ordination of entries, which are felt by breeders

considering multivariate selection, but PCA biplots generate equilibrium ordination due to different traits which could be used for precise selections. In this experiment, if it is desired to select an early mature hybrid with a higher oil yield and oil content using Fig. 2, R23 x CMS78 would be a good choice. Association and discrimination behavior of biplot in Fig. 2 is the same as that mentioned for Fig. 1 but the ordination of entries is slightly different. Except R21 x CMS356, all other combinations of R21 are on the left of the biplot in the same direction as that of their parents, except CMS356. It can be seen that R21 has a stronger positive effect on related hybrids than CMS356, and that agronomic means could discriminate genotypes more effectively than SCA data. Also, CMS 356 has a strong increasing effect on its crosses with all 6 restorer lines considering their SW and a decreasing effect on their SY, OY, SN and OC. R56 x CMS356 with higher SCA for SY has a SY close to the mean of entries and SCA was not able to show its real performance. So these biplots provide more useful information to the breeder, PCA based on agronomic data alone could be used for determining the real performance of entries considering multivariate traits.

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New sunflower hybrids tolerant of Tribenuron-Methyl

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ABSTRACT

Discovery of a tribenuron-methyl resistant wild *Helianthus annuus* L. population (ANN-KAN) created an opportunity for expansion of a sunflower herbicide resistance breeding program. The aim of this work was the creation of sunflower hybrids resistant to tribenuron-methyl. Creation of tribenuron-methyl resistant hybrids would enable the use of a wider palette of herbicides for sunflower, more efficient chemical control of *Cirsium arvense* and more economically profitable post-emergence control of some annual broad-lea weeds in sunflower. Original populations SURES-1 and SURES-2 are homozygous for resistance to tribenuron-methyl. F_1 generations produced from the crossings are completely resistant to tribenuron-methyl, pointing to the dominant way of inheritance of this trait. Studies on the exact number of genes controlling the resistance are in progress. Tribenuron-methyl resistance was transferred from original populations into a number of mother and restorer inbred lines of cultivated sunflower. These inbred lines could enable creation of a number of hybrids resistant to tribenuron-methyl. Hybrids NS-H-2017-SU, NS-H-2018-SU and NS-H-2019-SU are resistant to doubled application dose of tribenuron-methyl. Agronomical characteristics of these hybrids are on the level with the leading conventional sunflower hybrids.

Key words: hybrid – sunflower – tolerance – tribenuron-methyl.

INTRODUCTION

The main aim of plant breeding is to develop new varieties and hybrids to meet the needs of people and domestic animals. Due to the rapid growth of the human population, loss of arable land, global climate change, and water supply problems, the production of sufficient amounts of food will be a challenge in the future. The increase of yields of cultivated plants requires not only the development of new, more productive genotypes but the advancement of growing technology as well. Plant breeding for tolerance to herbicides covers both of these aspects.

The development of plants with herbicide tolerance has been made possible by the latest insights into the mechanism and target place of herbicide action at the molecular level and by the development of new biotechnology methods. In the 1990s, a number of crop genotypes resistant to herbicides have been developed as a result (Table 1). Although it is theoretically possible to develop a plant tolerant of any kind of herbicide, only combinations of major economically important crops and herbicides possessing favorable characteristics (glyphopsate, glufosinate ammonium, sulfonylurea, imidazolinones, etc.) have found an actual commercial application (Malidza et al., 1999).

Table 1. Year of first registration of herbicide tolerant crops (Malidza et al., 1999).

Year	Company	Crop
1992	Cyanamid	IMI, IR, IT Maize
1992	Du Pont	STS Soybeans
1995	Calgene	BXN Cotton
1995	AgrEvo	Liberty Link Canola
1996	Monsanto	Roundup Ready Soybeans
1996	Monsanto	Roundup Ready Canola
1997	Monsanto	Roundup Ready Cotton
1997	AgrEvo	Liberty Link Maize
1997	AgrEvo	Liberty Link Soybeans
1999	Monsanto	Roundup Ready Maize

The initial stages of plant breeding for herbicide resistance did not include any work on sunflower. Crop species for which herbicide-tolerant genotypes had been developed began to be grown more widely thanks primarily to the improved economy of their production. A result of this was a decrease of area in sunflower in South and North America, where the new technologies had been accepted without any legal limitations. Additionally, weed killing herbicides are developed less rapidly in sunflower than in the rest of field crops. Weeds cause significant yield losses in sunflower due to a lack of effective herbicides for

the suppression of broadleaf weeds and use after crop emergence. The currently existing chemical measures are ineffective against large-seeded broadleaf weeds, while the present soil herbicides are often not effective enough in the suppression of small-seeds weed species, especially in years with rainfall deficits occurring after herbicide application (Malidza et al., 2004). All this prompted sunflower researchers to begin working on the crop's tolerance to herbicides. The first major breakthrough came when Al-Khatib et al. (1998) found a population of wild *Helianthus annuus* L. (ANN-PUR) originating from Rossville, Kansas (USA) that was resistant to imidazolinone-based herbicides. Once the genetics of the resistance were studied and understood (Miller and Al-Khatib, 2000; Jocić et al., 2001), this population was used to develop the first sunflower hybrids tolerant of imidazolinone herbicides. These were developed in the USA in 2003 and Serbia and Turkey in 2004 (Jocić et al., 2004).

The discovery in Kansas, USA of a wild *Helianthus annuus* L. (ANN-KAN) population (Al-Khatib et al., 1999) resistant to a sulfonylurea herbicide (tribenuron-methyl) opened up the possibility of expanding the scope of sunflower breeding for tolerance to herbicides. The present study was aimed at the development of sunflower hybrids possessing tolerance of tribenuron-methyl. The introduction of such hybrids provides multiple benefits, including a broadened range of available herbicides in sunflower, more effective control of Canada thistle (*Cirsium arvense*), and greater cost-efficiency in the suppression of some annual broadleaf weeds after sunflower emergence (Zollinger, 2003; Malidza et al., 2006).

MATERIALS AND METHODS

The herbicide Granstar 75 WG was used in the study in two doses, the normal, recommended one (30 g/ha) and twice that (60 g/ha). In the latter years of the program, another herbicide was also used in the study to test the tolerance of the newly developed hybrids. This was Express 50-SX (500 g/kg tribenuron-methyl), a new and improved tribenuron-methyl-based herbicide manufactured by Du Pont. Express 50-SX was applied at 45 g/ha (standard dose) and 90 g/ha (double dose).

The sources of genes for tolerance to tribenuron-methyl were the populations SURES-1 and SURES-2. SURES-1 is a population of B lines obtained from the cross HA 424/3HA 406 // HA 89/ ANN-KAN, while SURES-2 is a population of restorer lines originating from the cross RHA377/3 RHA 392 // RHA 376/ ANN-KAN (Miller and Al-Khatib, 2004). Of cultivated sunflower genotypes, we used the self-pollinated B lines HA-26, VL-A-8 and HA-48 for crosses with SURES-1 and the restorer lines RHA-583, RHA-SES and RHA-N-49 for crossing with SURES-2.

The tolerance of SURES-1 and SURES-2 towards tribenuron-methyl was tested in the greenhouse during September through December 2000. In parallel with this, initial crosses were made between the two populations and the self-pollinated lines chosen for the study. During the 2001 growing season, the tolerance of the resultant F_1 generations was tested under field conditions using the double dose of tribenuron-methyl. After determining the mode of inheritance, pedigree selection was employed, with each inbred generation being treated with the double dose of Granstar 75-WG (60 g/ha). The most tolerant plants from the most tolerant progenies were selected for further breeding work. Treatment with herbicides was performed at the stage of 2-6 leaves using the knapsack sprayer Solo, 350 l/ha of water and a pressure of 2 bars. Twenty days after the treatment, phytotoxicity was assessed visually on a scale of 0 to 100% (0% - no symptoms, 100% - complete plant necrosis). Thanks to the use of a greenhouse, three inbred generations were obtained per year, which enabled us to develop the first experimental hybrids as early as 2004 and to test the general (GCA) and specific (SCA) combining abilities of the newly developed restorer lines. The testing was done using line x tester method (Singh and Choudhary, 1976). The comparative trial was carried out on a well-prepared chernozem soil at the Rimski Sancevi Experiment Field of the Institute of Field and Vegetable Crops using a randomized block design with three replications. The planting dates were optimal, intensive cultural practice was implemented during the growing season, and harvesting was done manually. The best hybrid combinations were selected and tested for tolerance to tribenuron-methyl and performance characteristics in a network of small-plot trials in 2005.

RESULTS AND DISCUSSION

Tribenuron-methyl is a herbicide that inhibits the acetolactate synthase enzyme (ALS), which is responsible for the synthesis of the amino acids valin, leucine and isoleucine. It is also one of the oldest sulfonylurea herbicides in existence (Ferguson et al., 1985) and has been among the most important herbicides in small grains for the past two decades. In Serbia, it is used in wheat crops and is the active ingredient of the Granstar 75-WG formulation (75% tribenuron-methyl) (Mitic, 2004). According to

Kolkman et al. (2004), the SURES-1 and SURES-2 populations have been found to contain the Pro197 mutation. This mutation is one of the most common mutations found in crop species tolerant of herbicides inhibiting ALS. It provides several-fold tolerance towards such herbicides compared with the susceptible genotypes. During the 2001 growing season, progenies of the source populations were found to possess full tolerance to tribenuron-methyl, meaning these populations are fully homozygous for this trait. Full susceptibility of the conventional inbred lines was confirmed as well. The F_1 generations exhibited full tolerance along with slight chlorosis, but there was absolutely no lagging behind in growth of any sort relative to the control treatment, which indicates the dominant mode of inheritance of tolerance to Granstar 75-WG. Determining the genetic basis of herbicide tolerance is a very sensitive kind of research. The first requirement is to use the double dose of the active ingredient. Environmental factors have a great influence on the expression of herbicide tolerance, as does the genetic basis of the lines receiving the tolerance genes. Because the donor populations possess many traits characteristic of the source population of wild *Helianthus annuus*, the determination of the genetics of the tolerance requires prior development of inbred lines tolerant of tribenuron-methyl. Pedigree selection was used to develop 52 inbred lines from crosses between SURES-2 and the restorer lines RHA-583, RHA-SES and RHA-N-49 as well as 46 female inbreds obtained by crossing SURES-1 and the lines Ha-26, VL-A-8 and Ha-48. All these self-pollinated lines are tolerant of the double dose of tribenuron-methyl, since the herbicide was applied at the 2-6-leaves stage in each generation during their development. Besides the herbicide tolerance, the newly developed selfed lines also have other favorable agronomic characteristics (most importantly tolerance to *Phomopsis helianthi*), as these were selected for these characteristics as well during the selection process.

The development of these lines also enabled the development of the first hybrids tolerant of tribenuron-methyl. The GCA and SCA of the new lines were tested and then the experimental hybrids were developed in 2004. All the hybrids were tested for performance characteristics and resistance to the common diseases and treated each year with the double dose of tribenuron-methyl. Based on the results, three of the hybrids were chosen for commercial production.

Due to the large volume of this research program, the present paper shows only the results for the newly developed SU hybrids NS-H-2017-SU, NS-H-2018-SU and NS-H-2019-SU. Table 2 shows the results produced by the three hybrids in two years of testing. The main requirement these hybrids must meet is to have a sufficient level of tolerance to tribenuron-methyl. What this means in concrete terms is that they have to be able to withstand the double dose of the standard, recommended dose of the active ingredient per unit area without showing any signs of phytotoxicity or any significant losses of yield or yield components. The results achieved by our hybrids have shown that they have a sufficient level of tolerance, as there were no statistically significant yield losses or reductions in the other studied traits in the treatment with the double dose of tribenuron-methyl relative to the treatment in which no herbicide was used (Table 2). Additionally, there were no visible signs of phytoxicity either. The only thing observed was that there was some slight chlorosis seven days after the treatment, but these symptoms disappeared completely after two weeks. The second important condition the new hybrids have to fulfil is to have good performance characteristics in addition to tolerance to tribenuron-methyl. Thus, they have to have a high yield potential, a high oil content, and resistance to the common diseases so as to be able to compete with the standard sunflower hybrids used in commercial production. The check hybrids in our trials were NS-H-111, the leading sunflower hybrid in Serbia, and NS-H-43, which is a hybrid that domestic sunflower growers are well familiar with, as it has been present in Serbian sunflower production for a considerable number of years already. The results of the trials have shown that the new SU hybrids are completely on a par with the standard ones in terms of performance. The performance of NS-H-2017-SU and NS-H-2019-SU completely matched that of the class-leading NS-H-111 in terms of seed yield, oil content and oil yield, while NS-H-2018-SU performed as well as NS-H-43 despite being two weeks earlier in terms of maturation (Table 2).

Our results indicate that the new SU hybrids NS-H-2017-SU, NS-H-2018-SU and NS-H-2019-SU will find their niche in the domestic sunflower market very soon. This has been confirmed by their results in the official variety trials of the Serbian Variety Commission and their subsequent registration in the Serbian Variety List.

The source populations SURES-1 and SURES-2 are homozygously tolerant of tribenuron-methyl. The F_1 generations produced in the program are completely tolerant of tribenuron-methyl, indicating the presence of the dominant mode of inheritance. Studies to determine the exact number of genes controlling this resistance are in progress. Resistance to tribenuron-methyl has been transferred from the source populations to a number of female and self-pollinated sunflower lines. This makes it possible to develop a larger number of hybrids tolerant of tribenuron-methyl. The hybrids NS-H-2017-SU, NS-H-2018-SU and

NS-H-2019-SU are tolerant of twice the recommended dose of tribenuron-methyl per hectare and are also as good as the leading sunflower hybrids in the domestic market in terms of agronomic performance.

Table 2. Mean values of several traits in tribenuron-tolerant sunflower hybrids

Hybrid	Treatment	Plant height (cm)	Maturity (days)	Seed yield (kg/ha)	Oil content (%)	Oil yield (kg/ha)
NS-H-2017	Untreated	176.43	122.5	4 265.33	46.36	1 976.43
	Tribenuron-methyl (45 g/ha)	178.29	123	4 230.57	46.25	1 956.20
NS-H-2018	Untreated	161.40	110.4	3 702.26	47.18	1 747.45
	Tribenuron-methyl (45 g/ha)	162.30	109.9	3 806.34	48.53	1 847.28
NS-H-2019	Untreated	189.55	127.8	3 926.57	48.30	1 896.53
	Tribenuron-methyl (45 g/ha)	188.75	127.5	4 157.52	49.25	2 046.11
NS-H-43	Untreated		129	3 938.04	46.49	1 830.35
NS-H-111	Untreated		123	4 258.12	48.53	2 066.36
			LSD	476.93	4.27	260.65

ACKNOWLEDGEMENTS

This work was supported by Ministry of Science of Republic of Serbia.

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Genetic improvement of oil quality in sunflower mutants under water stressed conditions

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ABSTRACT

The objective of the present research was to study 30 mutants, induced by gamma irradiation among a population of M6 sunflower mutant lines and to identify molecular markers associated with different seed quality traits. The experiments were performed in two environments (greenhouse and field) under well-watered and water-stressed conditions. Experiments consisted of three blocks, and each block was split into two main plots (well-watered and water-stressed). The seed quality traits studied were: protein content (PC), oil content (OC), palmitic acid (PA), stearic acid (SA), oleic acid (OA) and linoleic acid (LA). In both environments and both conditions, a large genetic variation was observed between mutant lines and some mutants presented higher values for seed quality traits in comparison to original line. Two mutants M6-862-1NI and M6-826-2 showed the most important values for OC and OA in comparison with the original line AS613 under all conditions. The results revealed the efficiency of gamma-irradiation for inducing genetic variation in sunflower for seed quality traits. Multiple regression analyses showed that some AFLP markers are associated with several traits. The most important were E40M59-5 and E33M49-5 markers, with more than 30% of phenotypic variance for OC and PA under water stressed condition. There were several markers associated with quality traits in both well-watered and water-stressed conditions. Some other markers were specific for only one trait or a given water treatment. The markers which were associated with different traits could be used for marker-assisted selection.

Key words: AFLP – gamma irradiation – genetic variation sulfonylurea – oil quality sulfonylurea – water stress – sunflower.

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the four major annual world oilseed crops which is cultivated for edible oil. In commonly grown sunflower cultivars, the oil contains a high proportion (about 90%) of unsaturated fatty acids; oleic (C18:1) and linoleic (C18:2) acids. The remaining 10% corresponds to the saturated fatty acids palmitic (C16:0) and stearic (C18:0) acids (Garcés et al. 1989).

Mutagenesis has been successfully used for developing variation in the fatty acid profile of sunflower and mutants with an altered fatty acid content have been developed, such as high oleic acid mutant Pervenets (Soldatov, 1976), mutants 275HP and CAS-5 with high palmitic acid content (Ivanov, 1988; Osorio et al., 1995), CAS-12 with high palmitic and oleic acids (Fernandez-Martinez et al., 1997), mutants CAS-8, CAS-4 and CAS-3 with medium to high stearic acid (Osorio et al., 1995) and CAS-14 with very high stearic acid (Fernández-Moya et al., 2002). Using molecular markers in selecting genotypes with desirable traits through marker-assisted selection has been proved to be effective in plants. Identification of markers associated with important traits in a group of genotypes through multiple regression analysis offers an alternative means and has been adopted in several plant species. As an example, Vijayan et al. (2006) identified several ISSR markers associated with yield traits in mulberry. The aim of this study was to identify the interesting mutants for oil quality and to detect the molecular markers associated with oil quality traits.

MATERIALS AND METHODS

A population of gamma rays mutant lines (M6) coming from AS613 genotype was developed in our laboratory (Sarrafi et al. 2000). Among this population, 30 mutants showed morphological differences in comparison with 'AS613', which were used in our experiments.

The quantitative traits were evaluated in two environments: greenhouse under controlled conditions and field. Each experiment was conducted with two conditions: well watered and water stressed. Both experiments consisted of three blocks, and each block was split into two main plots (well- watered and water stressed). Protein content (PC), oil content (OC), palmitic acids (PA), stearic acid (SA), oleic acid (OA) and linoleic acid (LA) were measured by FOSS NIR System 6500 (Foss Analytical, Denmark).

Genomic DNA of AS613 and mutant lines were isolated from two-week old seedlings according to the method of extraction and purification presented by Fulton et al. (1995). Different *MseI* / *EcoRI* primer combinations were used for AFLP genotyping. The AFLP procedure was conducted as described by Al-Chaarani et al. (2004). AFLP bands were scored from the gel as presence (1) or absence (0).

The variability among the mutants for the studied traits was tested through ANOVA. A simple correlation between the quantitative traits was calculated. The association between AFLP markers and quantitative traits was obtained through stepwise multiple regression analysis, where each quantitative trait was considered as a dependent variable while the AFLP marker was treated as an independent variable (Vijayan et al. 2006).

RESULTS

Analysis of variance showed significant variability among mutant lines for all seed quality traits in two environments under well-watered and water-stressed conditions. The effect of water-stress was significant for PC, OC and SA in the greenhouse as well as for SA in the field (data are not presented).

Table 1. Seed quality traits and genetic gain (GG)[1] of sunflower mutants in two experiments: greenhouse (1) and field (2) under well-watered (WW) and water-stressed (WS) conditions.

Mutants	Exp.	PC[2]		OC		PA		SA		OA		LA	
		ww	ws	ww	ws	ww	ws	ww	ws	ww	ws	ww	ws
AS613	1	19.2	18.6	45.7	45.3	5.7	5.3	3.3	3.3	33.3	38.8	59.6	53.9
	2	22.4	21.4	37.6	38.9	5.5	6.0	5.8	4.2	34.2	29.4	53.2	60.3
M6-826-2	1	24.6	24.1	43.6	47.0	5.0	4.7	4.5	4.6	61.0	72.2	29.9	20.5
	2	23.4	19.7	34.1	41.8	5.0	4.8	8.2	9.4	43.2	48.0	42.4	37.7
M6-133-2	1	24.3	23.1	39.9	43.3	5.4	5.1	3.7	4.2	42.1	46.1	49.6	46.0
	2	27.3	22.1	33.7	33.8	7.2	7.2	5.4	5.5	22.7	21.9	64.8	68.1
M6-375-1	1	21.7	20.0	39.0	39.1	5.1	4.9	3.9	3.8	41.5	41.4	50.1	50.1
	2	24.8	23.7	30.3	35.0	5.3	5.1	5.7	5.4	33.2	37.9	55.0	51.0
M6-862-1NI	1	16.4	16.5	54.8	54.3	6.3	5.9	1.8	1.8	36.5	35.8	61.3	53.4
	2	20.8	18.5	43.3	50.1	5.7	5.2	4.0	3.8	19.9	23.1	55.6	52.6
M6-186-1	1	20.8	18.6	44.2	47.8	6.2	6.8	2.7	2.8	28.5	18.5	62.9	72.4
	2	23.4	16.9	37.3	37.9	7.1	7.5	5.0	5.0	23.4	19.9	64.8	67.5
M6-653	1	21.8	21.8	45.2	45.3	5.1	4.9	3.8	4.3	43.4	44.5	48.5	46.8
	2	24.9	20.4	33.8	36.2	9.5	6.3	3.9	5.1	26.7	30.3	58.3	58.2
M6-641-2	1	20.2	21.8	40.8	43.4	5.3	5.6	3.7	4.0	41.9	36.9	49.6	54.6
	2	20.7	16.4	35.2	38.2	5.8	7.4	3.8	3.2	26.7	17.4	63.6	71.9
GG	1	5.1*	5.5^{ns}	9.1*	9.0*	0.6*	1.5*	1.2*	1.3*	27.7*	33.4*	3.3^{ns}	18.5*
	2	4.9*	2.3^{ns}	5.7*	11.2*	4.0*	1.5*	2.4*	5.2*	9.0*	18.6*	11.6*	11.6*

[1]GG: Genetic gain calculated as the differences between the best mutant and original line (*AS613*). Values are presented for the original line AS613 and for 7 selected mutants which present the highest values.

[2]PC, Protein Content; OC, Oil Content; PA, Palmitic Acid content; SA, Stearic Acid content; OA, Oleic Acid content; LA, Linoleic Acid content.

*: significant at 0.05 level, ns: non significant.

Negative significant correlation was observed between OC and PC, OA and LA, PA and SA and SA with LA, whereas correlation between SA and OA was positive (data are not presented). Some mutants presented high values for more than one trait (M6-186-1, M6-862-1NI, and M6-826-2) and some others just for one trait (Table 1). Some mutants presented significant differences with the original line AS613 for several traits. Genetic gain (GG) calculated as the difference between the best mutant and original line was significant for all the studied traits except for PC under water-stressed condition in all environments and for LA under well-watered condition in greenhouse (Table 1).

Table 2. Main markers associated with the seed quality traits in sunflower mutants.

			Well-watered						Water-stressed		
Trait	Exp.	Marker	P	R^2 (%)	M1- M2	Trait	Exp.	Marker	p	R^2 (%)	M1- M2
OC	1	***E40M59-5***	*	15.2	-5.51	OC	1	***E40M59-5***	***	35.3	-8
OC	2	***E40M59-5***	**	24.4	-7.1	OC	2	***E40M59-5***	*	18.7	-5.74
PC	1	*E33M50-17*	**	29.8	-2.91	PC	1	*E33M50-17*	**	21.7	-2.97
PC	1	*E37M50-14*	*	17.1	-2.2	PC	1	*E31M48-4*	*	16.7	2.11
PC	2	***E40M59-5***	*	14.8	3.38	PC	1	***E40M59-5***	*	13.2	3.72
PA	1	*E37M50-14*	*	19.41	0.42	PC	1	*E37M50-6*	**	19.8	-2.57
PA	1	*E31M50-1*	**	22.2	-0.38	PC	2	***E40M59-5***	*	17.3	2.96
SA	1	***E40M59-5***	**	22.8	1.02	PC	2	*E31M50-1*	*	16.4	1.58
SA	1	*E31M50-1*	**	23.7	0.54	PA	1	*E37M50-14*	*	16.2	0.34
SA	2	*E37M50-14*	**	22.8	-1.41	PA	1	***E40M59-5***	*	13.7	-0.50
SA	2	*E33M50-17*	**	23.2	-1.42	PA	2	*E37M50-6*	*	20.7	-0.27
SA	2	*E37M48-8*	*	18	-1.54	PA	2	*E33M49-5*	**	33.2	1
SA	2	*E31M50-1*	**	23.4	1.16	SA	1	***E40M59-5***	**	22.5	1.2
OA	1	*E37M50-14*	**	21.9	-9.52	SA	2	*E37M50-14*	**	23	-1.47
OA	1	*E37M48-8*	**	23	-11.64	SA	2	*E37M50-6*	**	21.3	-1.26
OA	1	*E33M50-17*	**	22	-9.55	SA	2	*E37M48-8*	*	18.3	-1.54
LA	1	*E37M50-14*	**	23	9.75	OA	1	*E37M50-6*	**	18	-6.37
LA	1	*E37M48-8*	**	23.6	11.46	OA	1	*E37M50-14*	*	16.6	-6.75
LA	1	*E33M50-17*	**	22.9	9.71	OA	2	*E37M50-6*	**	29.4	-8.43
LA	2	*E37M48-8*	*	14.2	6.88	OA	1	*E33M59-12*	**	19.7	6.66
LA	2	*E40M59-1*	**	24.8	-6.29	OA	2	*E37M48-8*	**	21.1	-9.9
						LA	1	*E37M50-6*	**	22.2	7.27
						LA	1	*E33M59-12*	**	19.9	-6.95
						LA	2	*E37M48-8*	*	19.7	9.87
						LA	2	*E37M50-6*	**	30.4	8.84

PC, Protein Content; OC, Oil Content; PA, Palmitic Acid content; SA, Stearic Acid content; OA, Oleic Acid content; LA, Linoleic Acid content . 1; greenhouse, 2; field. M1-M2: difference between two marker classes as revealed by analysis of variance of trait by marker genotype

In total 34 and 31 AFLP markers associated with the quantitative traits were identified in greenhouse and field, respectively (Table 2). More than 61% of the detected markers were identical in two experimental environments. Some markers were associated with different traits (for example, E33M50-17, associated with PC, SA, OA and LA) and some others were common across water treatments such as E40 M59-5 associated with OC under well-watered and water-stressed conditions (Table 2).

The results showed that E40M59-5 and E33M49-5 markers are the most important ones in water–stress conditions with more than 30% of phenotypic variance for OC and PA.

DISCUSSION

Significant genetic variation observed among mutant lines for the studied traits revealed the efficiency of gamma-irradiation for inducing genetic variation in sunflower for seed quality traits. Some mutant lines presented advantages over AS613 for different traits. Two mutant lines M6-862-1NI and M6-826-2

showed important values of OC and OA, respectively, in comparison with the original line AS613 in two environments under both conditions (well-watered and water-stressed). These mutant lines could be used in breeding programmes to improve seed oil content under water stress growth conditions. On the other hand, the mutant line M-862-1NI showed the maximum value for PA only under well-watered condition and a low value under water-stressed condition. This mutant is a sensitive genotype under water stress. The significant negative correlation between PC and OC in our mutant lines was observed also by Mahmood et al. (2006) in *Brassica juncea*.

Our results show that some AFLP markers are associated with several traits and some others are specific for only one trait or a given water treatment (Table 2). The phenotypic variance explained by each marker (R^2) was important, ranging from 13.7 to 35.3%. E40M59-5 marker was associated with OC in two environments under both conditions (well-watered and water-stressed). The latter marker is the most important marker in this study as it is associated with some other traits (PC, PA and SA) in well-watered and water-stressed conditions. Thus E40M59-5 could be used in marker assisted selection programmes in sunflower. Also E31M50-1 marker was common between PA and SA under well-watered condition in greenhouse. Overlapping QTLs for PA and SA were reported by Burke et al. (2005). Several common markers for OA and LA were found in each environment. This can be explained by correlation between OA and LA as well as by a specific gene of Δ12-desaturase which converts oleic acid into linoleic acid in grains and modifies fatty acids composition, as reported in sunflower and soybean (Garcés et al., 1989; Heppard et al., 1996). E37M48-8 and E37M50-6 were also common in two environments for OA and LA. E37M50-6 was a specific marker for water-stress while E37M48-8 was non-specific for well-watered and water-stressed conditions. Markers associated with different traits in both water treatments could be used for marker-assisted selection in both environments. Other markers, which are specific for one water treatment but associated with different traits or specific for a trait, could be useful for a given water treatment.

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Estimation of genetic diversity of sunflower single cross hybrids using principal component analysis

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ABSTRACT

In order to study genetic diversity of sunflower single cross hybrids through using principal component analysis, a North Carolina Design I experiment was conducted in a randomized complete block design in three replications at Agricultural and Natural Research Station of Khoy. In 2005, six fertility restorer male lines were crossed with 18 cytoplasmic male sterile (CMS) female lines. Each male line was crossed with three different female lines to make two sets with nine hybrids in each set. In 2006, Single cross F_1 hybrids were planted for studying genetic diversity. Data were collected from agronomic traits including: flowering initiation, seed filling period, maturity, plant height, head diameter, 1000 seed weight, seed numbers per head, harvest index, oil content, seed yield and oil yield. Survey results showed that 3 first components explained variability of all the data (77.20 %). Traits including seed numbers per head, harvest index, oil content, seed yield and oil yield were the main parts of first component while second component was affected mainly by flowering initiation, maturity and plant height. Seed filling period, head diameter, seed yield and oil yield were the important parts of third component. In all, the first component with a large amount of total variance and high correlation with traits including seed yield, oil yield, seed numbers per head, oil content and harvest index could be appropriate and useful for grouping and selecting superior single cross hybrids.

Key words: genetic diversity sulfonylurea – hybrid sulfonylurea – principal components analysis – sunflower.

INTRODUCTION

In recent years, many sunflower single cross and three-way-cross hybrids have been produced in Iran, and a selection of the best of them in regional preliminary experiments is very important task. Multivariate statistical methods that can create relationships between cultivar traits, can help to group cultivars and make it easy to select them on the basis of biplot and triplot diagrams. The principal components analysis method, through the summarizing of preliminary correlated varieties in the form of independent and limited components, provides the possibility of genotype grouping in the 2D or 3D space (Moghaddam et al., 1994). Some researchers, in order to accelerate the genotype selection for aspects of traits like seed yield have used principal components analysis, a method which is also useful for the reduction of the selection cost and in the preliminary stage of cultivar selection (Spranaaij and Bos, 1993). Kroonenberg et al. (1995) also used this method, with three-way-cluster analysis, for the separation of genotypes in limited bunches for their manipulation and Cheres and Knapp (1998) have used the efficient grouping of this method for the evaluation of genetic diversity in sunflower germplasm and determination of ancestral relationship. De La Vega et al. (2001) have used principal components analysis for the determination of interaction among different sunflower cultivars with cultural media and for the consideration of indirect selection possibility of yield in these media and Ghaffari (2003) used this methodologies for rapid screening of 121 sunflower cultivars and hybrids, so that logical cultivar grouping through the influence from agronomic traits could be used as an efficient factor in superior and early mature hybrid selection. Finally, Zeinalzadeh-Tabrizi and Ghaffari (2005) have used this method for the survey of genetic diversity of sunflower genotypes These authors employed in their experiment, commercial cultivars such as Azargol, Record, Armavirski and Hysun33 well-distinguished from three-way-cross hybrids through the high seed and oil yield by first component. In biplot and triplot diagrams, designed on the basis of facts obtained from principal component analysis, trait influence on the genotype grouping in different vector forms and the position of every genotype on the basis of selected component type are shown.

Length of any vector shows the weight of that vector in the creation of distinguished groups and is correlated with the amount of component for the related traits.

Through the designing of a vertical line from a genotype location to trait vector, genotypes can be compared. In effect, the further the distance of conjunction of the line with the origin, the more the diversion of genotype yield from others (Chapman et al., 1997). Any angle among the vectors in these diagrams shows their correlation (Kroonenberg, 1997). With this kind of relationship among the agronomic traits and related vectors in formed diagrams, is possible to group experimental genotypes logically. We can use this method as a way for surveying genetic diversity of evaluated materials and also for the selection of superior genotypes in preliminary experiments.

The aim of this study was the use of principal component analysis for the obtention of biplot and triplot diagrams, that can be used for surveying genetic diversity and selection of superior sunflower single cross hybrids under study, , and for the determination of relationships between traits.

MATERIALS AND METHODS

This experiment was conducted at Agricultural and Natural Resources Research Station of Khoy, Iran (44° 58´ N, 38° 33´ E) during 2004 and 2005. The minimum, average and maximum annual temperature of this station are, respectively, -30, 12.5 and 42°C and the average annual rainfall is 292.6 mm. Plant material used in this experiment were sunflower single cross hybrids obtained from crosses of 6 male restorer lines (R line) with 18 female cytoplasmic male sterile (CMS or A) lines which have been produced at Agricultural and Natural Resources Research Station of Khoy in 2003. In each set, were included 3 R lines crossed with different 3 CMS lines and their developed 9 hybrids. Hybrids containing common male parent were counted as half-sib family. Mating had been done as a nested design in North Carolina Design I plan, as such, CMS lines have been nested inside restorer lines. F_1 hybrids obtained from crosses were planted for the principal components analysis and to survey genetic diversity in 2005.

Surveyed traits were: flowering initiation, seed filling period, maturity, plant height, head diameter, 1000 seed weight, seed number per head, harvest index, oil content, seed yield and oil yield. Correlation among the varieties was done through the use of SPSS software and the principal components analysis employing Statgraphics, biplot and triplot diagrams being designed. On the basis of the characteristics of every hybrid, and the direction and angle of the related vectors, the position of 18 hybrids in biplot and triplot diagrams have been clarified, and, on that basis, the range of the existing diversity and methods of grouping obtained have been considered.

Hybrids characteristics are shown in charts below.

First Set		
Hybrid	**R line**	**A line**
A	R_{43}	CMS_{28}
B		CMS_{128}
C		CMS_{346}
D	R_{27}	CMS_{330}
E		CMS_{78}
F		CMS_{328}
G	R_{34}	CMS_{336}
H		CMS_{52}
I		CMS_{148}

Second Set		
Hybrid	**R line**	**A line**
J	R_{56}	CMS_{344}
K		CMS_{260}
L		CMS_{32}
M	R_{25}	CMS_{222}
N		CMS_{96}
O		CMS_{356}
P	R_{32}	CMS_{356}
Q		CMS_{196}
R		CMS_{376}

RESULTS AND DISCUSSION

Equation of every 3 first components has been shown in Table 2. For example, equation of first component is:

Z1= -0.05 FI-0.32 SFP-0.23 MA+0.06 PH+0.16 HD-0.35 SW+0.41 SN+0.32 HI+0.35 OC+0.36 SY+0.40 OY

Table 1. Variance of components in principal component analysis method

Component number	Eigen value	Variance percentage	Cumulative percentage
1	5.10	46.40	46.40
2	2.01	18.32	64.73
3	1.37	12.47	77.20
4	0.99	9.03	86.24
5	0.74	6.72	92.96
6	0.38	3.49	96.46
7	0.20	1.88	98.35
8	0.13	1.19	99.54
9	0.03	0.32	99.86
10	0.01	0.12	99.99
11	0.00	0.00	100

Table 2. Structure of first 3 components for agronomic traits

Traits	Component 1	Component 2	Component 3
Flowering Initiation (FI)	-0.05	0.57	0.09
Seed Filling Period (SFP)	-0.32	-0.01	0.32
Maturity (MA)	-0.23	0.50	0.29
Plant Height (PH)	0.06	0.36	-0.06
Head Diameter (HD)	0.16	-0.44	0.49
1000 Seed Weight (SW)	-0.35	0.03	0.23
Seed Number per Head (SN)	0.41	0.06	0.17
Harvest Index (HI)	0.32	0.00	-0.37
Oil Content (OC)	0.35	0.28	-0.16
Seed Yield (SY)	0.37	0.07	0.46
Oil Yield (OY)	0.40	0.14	0.31

Through carrying out the principal components analysis and grouping the genotypes on the basis of the quantity of two first components, it was clarified that genotypes, on the basis of trait weight in every component, obtain a special position in correlation with the agronomic trait vector and are scattered according to the correlation of considered traits with components and according to the quantity of trait under study (Figs. 2 and 3).

This kind of genotype scattering in provided vectors can afford at least the possibility of fast rejection or selection of main parts of genotypes and this could be useful in preliminary evaluations. Because genetic materials used each experiment are different, genotype orientation around the related vectors of agronomic traits will depend on the correlations obtained in every experiment and trait weight in the formation of every component. Because of that, the method of selection in every experiment will be different from the other. The use of this method is not limited to sunflower crop, and like other multivariate methods, it can be used in other products.

In this study, on the basis of general waypoint we have reached the conclusion that the first component obtained from principal components analysis with 46.40 percent of the total variance and high correlations with traits like seed numbers per head, harvest index, oil content, seed yield and oil yield, can be used in an efficient way in the fast selection and screening of genetic materials in the initial stages. Genotypes which are in the right half of the biplot diagram and around the vector related to the seed yield, besides having a high seed yield, have a high oil yield, oil content and number of seed per head. Selection on the basis of these genotypes with a short maturity period is beneficial to the effort to achieve the most important aims of production of sunflower hybrids seed programs.

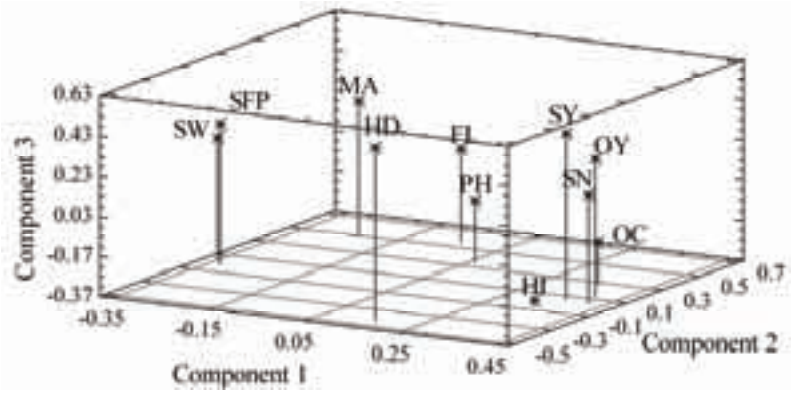

Fig. 1. Diagram of first three component weights for agronomic traits on sunflower single cross hybrids. Abbreviations are given in table 2.

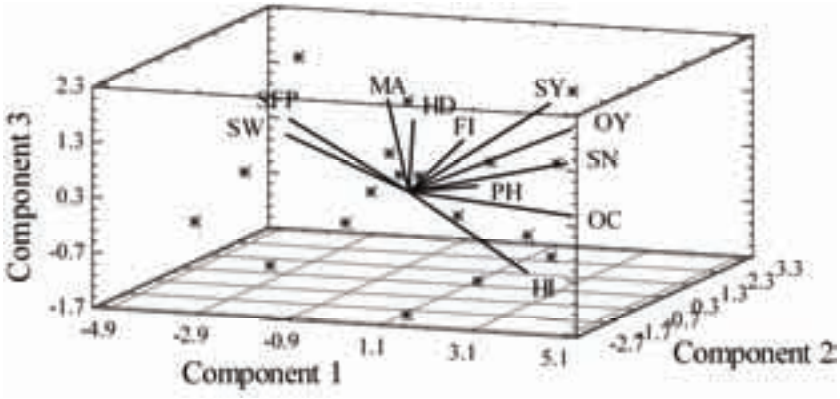

Fig. 2. 3D diagram for position of sunflower genotypes and trait vectors in principalcomponent analysis method. Abbreviations are given in table 2.

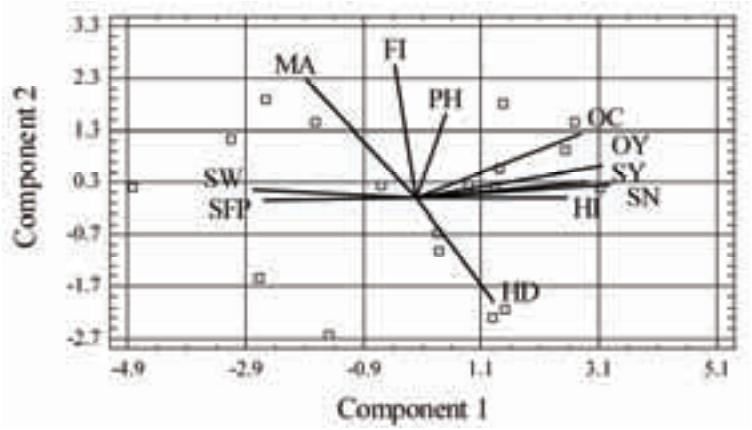

Fig. 3. Biplot for position of sunflower genotypes and trait vectors in principal components analysis method. Abbreviations are given in Table 2.

Table 3. Correlation among agronomic traits of sunflower single cross hybrids and components of principal component analysis

Traits	Flowering Initiation	Seed Filling Period	Days to Maturity	Plant Height	Head Diameter	1000 Seed Weight	No. of seeds per Head	Harvest Index	Oil Content	Seed Yield	Oil Yield
FI	1										
SFP	-0.115^{ns}	1									
MA	0.648**	0.600**	1								
PH	0.133^{ns}	-0.146^{ns}	0.113^{ns}	1							
HD	-0.379^{ns}	-0.142^{ns}	-0.479*	-0.200^{ns}	1						
SW	0.142^{ns}	0.579*	0.439^{ns}	0.143^{ns}	-0.064^{ns}	1					
SN	-0.043^{ns}	-0.548*	-0.314^{ns}	0.079^{ns}	0.346^{ns}	-0.811**	1				
HI	-0.073^{ns}	-0.641**	-0.490*	0.121^{ns}	0.083^{ns}	-0.572*	0.583*	1			
OC	0.073^{ns}	-0.523*	-0.187^{ns}	0.339^{ns}	-0.002^{ns}	-0.651**	0.717**	0.614**	1		
SY	0.034^{ns}	-0.417^{ns}	-0.200^{ns}	0.142^{ns}	0.529*	-0.449^{ns}	0.863**	0.388^{ns}	0.558*	1	
OY	0.066^{ns}	-0.504*	-0.221^{ns}	0.205^{ns}	0.388^{ns}	-0.561*	0.903**	0.493*	0.750**	0.965**	1
Component 1	-0.107^{ns}	-0.735**	-0.530**	0.140^{ns}	0.376^{ns}	-0.788**	0.932**	0.727**	0.790**	0.820**	0.896**
Component 2	0.804**	-0.016^{ns}	0.713**	0.509**	-0.623**	0.050^{ns}	0.082^{ns}	0.005^{ns}	0.395^{ns}	0.095^{ns}	0.199^{ns}
Component 3	0.106^{ns}	0.381^{ns}	0.344^{ns}	-0.077^{ns}	0.580*	0.275^{ns}	0.198^{ns}	-0.431^{ns}	-0.189^{ns}	0.535*	0.364^{ns}

ns, * and **: Non significant, significant at 5 % and 1 %, respectively.

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Relationship between genetic distance and heterosis based on quantitative traits and SSR markers in sunflower

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ABSTRACT

The objective of this study was to determine the relationship between SSR based genetic distance (GD) of new NS sunflower inbred lines for most important agronomic traits and heterosis. Twenty three sunflower inbred lines (twenty restorer lines and three female lines used as testers) were selected based on their diverse genetic background for plant height, head diameter, thousand seed mass, oil content, seed yield per plant and oil yield per plant. Significant heterosis was observed in hybrid combinations for all examined traits except oil content. Genetic distance between pairs of tested sunflower inbred lines ranged from 0.13 to 0.8. There was no significant positive correlation between genetic distance and mid- and better-parent heterosis, specific combining ability and mean value in any of the examined traits for all 60 hybrids. A highly significant negative correlation was found between GD and mean oil percentage ($r=-0.33$ $p<0.01$). However significant correlations between GD and better-parent heterosis for thousand seed weight were found for hybrids of the tester line HA-19 ($r=0.43$ $p<0.05$) and between GD and mid-parent heterosis for plant height for hybrids of the tester line HA-26 ($r=0.47$ $p<0.05$). Although GD was generally a poor predictor of heterosis, better results are obtained if hybrid combinations for each tester and each trait are analyzed separately.

Key words: correlations sulfonylurea – genetic distance sulfonylurea – heterosis sulfonylurea – hybrid performance – sunflower.

INTRODUCTION

Identification of parental combinations that produce hybrids of superior yield is the most important step in the breeding program of sunflower (*Helianthus annuus* L.). However, developing hybrids is a costly and long term process, as it is necessary to cross a lot of inbred lines and evaluate hybrids in field trials. Therefore, only a limited number of hybrids among all possible crosses can be tested. Utilisation of genetic distance for predicting hybrid heterosis has been of great interest to breeders. The efficiency of hybrid breeding programs could be increased if the inbred lines *per se* could be screened and the superior crosses predicted before field evaluation (Melchinger et al., 1990).

Studies of genetic diversity in relation to hybrid performance have been undertaken in several crops. Investigations in corn, *Zea mays* L. have shown that the genetic diversity of parents was significantly correlated with hybrid performance and that yield heterosis could be predicted using molecular markers (Smith et al., 1990; Betran et al., 2003; Rief et al.; 2003; Schrag et al., 2006). Conversely, weak correlations have been reported between genetic distance and hybrid performance and heterosis in oilseed rape, *Brassica napus* L. (Diers et al., 1996), pepper, *Capsicum annuum* L. (Geleta et al., 2004), faba bean, *Vicia faba* L. (Zeid et al., 2004), and alfalfa, *Medicago sativa* L. (Riday et al., 2003).

Different sunflower gene pools have been studied for their genetic diversity with different marker systems (Tersac et al., 1993; Gentzbittel et al., 1994; Berry et al., 1994; Zhang, 1995; Hongtrakul, 1997, Cheres and Knapp, 1998; Yu et al., 2002; Tang and Knapp, 2003; Pankovic et al., 2004; Solodenko et al., 2005). However, the literature data on the predication of sunflower heterosis and hybrid performance by marker based genetic distance of the parental lines is scarce (Tersac et al., 1994; Cheres et al., 2000). Cheres et al. (2000) used AFLP markers and found a significant correlation between GD and seed yield, but genetic distance was generally a poor predictor of hybrid performance. The objective of this study was to determine the association between SSR based genetic distance of new NS sunflower inbred lines for most important agronomic traits and heterosis.

MATERIALS AND METHODS

Twenty three sunflower inbred lines (20 restorer lines and three female lines used as testers) were selected based on their diverse genetic background for examined agronomic traits. The selected restorer lines (labeled R-1 through R-20) are new inbred lines developed in the breeding program of the Oil Crops Department, of the Institute of Field and Vegetable Crops, in Novi Sad, Serbia. Female lines used as testers (HA-48, HA-26 and HA-19) are commercial lines with good combining abilities.

Female lines were crossed with restorer lines to produce all possible combinations of F_1 hybrids using the line x tester method (Singh and Choudhary, 1976). Seeds of the 60 F_1 hybrids produced and their parents were sown in a breeding nursery of the Oil Crops Department, of the Institute of Field and Vegetable Crops. The experimental design was a randomized block system with four replications.

Plant height (PH), head diameter (HD), thousand seed weight (TSW), oil content (OC), seed yield per plant (SY) and oil yield per plant (OY) were used for quantitative characterization of 23 parental lines and their 60 F_1 hybrids. Plant height and head diameter were measured at the end of flowering. Seed yield was measured by harvesting the middle row of each plot by hand. Seed samples from each plot were analyzed for oil content by nuclear magnetic resonance.

Analysis of variance and specific combining abilities (SCA) for quantitative traits were performed using the line x tester method (Singh and Choudhary, 1976). Heterosis was determined as follows:

Mid-parent heterosis (MPH) (%) = $((F_1\text{-MP})/MP)*100$

Better-parent heterosis (BPH) (%) = $((F_1\text{-BP})/BP)*100$

where, F_1 is the F_1 performance, MP = $(P_1+P_2)/2$ in which P_1 and P_2 are the performances of inbred parents and BP is the betterparent value (Geleta et al., 2004). Significance of heterosis was determined by the t-test (Kraljevic-Balalic et al., 1991).

Genomic DNA of 23 parental lines was extracted following the modified method of Dellaporta et al. (1983). The 15 SSR sunflower primers used in the study were: ORS 1, ORS 5, ORS 7, ORS 8, ORS 10, ORS 12, ORS 14, ORS 16, ORS 31, ORS 37, ORS 47, ORS 66, ORS 78, ORS 509 and ORS 595 (Tang et al., 2002). The selected primers have previously revealed DNA polymorphism of sunflower NS breeding material (Pankovic et al., 2004; Terzic et al., 2006). Fragments were separated using 2% agarose and 6% denaturing polyacrylamide gels. DNA polymorphism between two inbred lines was estimated by comparison of amplified fragments. Jaccard coefficient (J) of similarity was calculated according to Staub et al. (2000). Genetic distances (GD) among the 23 parental lines were estimated according to Spooner et al. (1996) as GD = 1-J.

Values of genetic distance as measured by SSR markers were correlated with MPH and BPH to estimate their relationship. Correlations were done for F_1 combination from each tester line separately and all tester lines.

RESULTS AND DISCUSSION

Parental lines and 60 F_1 hybrids were evaluated in field trials for plant height, head diameter, thousand seed weight, oil content, seed yield per plant and oil yield per plant. There was a great variation among inbred lines and hybrids, respectively (Table 1). The mean values of the hybrids were significantly higher than the parental lines for plant height, head diameter, thousand seed mass, seed and oil yield per plant.

Table 1. Mean values, standard error of the means and coeficient of variation (V) for the sunflower parental lines and their F_1 hybrids

Trait	Female line		F_1 hybrid		Restorer	
	Mean	V	Mean	V	Mean	V
Plant height (cm)	157.77±0.87	20.10	201.88±0.45	45.19	141.48±0.36	51.43
Head diameter (cm)	18.69±0.01	19.47	22.48±0.02	36.98	14.21±0.01	66.04
Tousand seed weight (g)	50.66±0.21	9.49	54.33±0.07	8.62	34.49±0.25	22.07
Oil content (%)	46.77±0.10	6.11	47.36±0.09	5.42	47.90±0.13	6.12
Seed yield (g per plant)	35.38±0.65	10.15	57.05±0.58	14.60	12.24±0.20	38.69
Oil yield (g per plant)	16.46±0.33	4.66	26.99±0.26	14.83	5.91±0.05	42.46

The heterotic effect was observed in all examined traits, except oil content (Table 2). The mean values of hybrids were between parental means for oil content and both parental lines were selected for high oil quantity. The highest effect of heterosis (MPH) was observed for oil yield per plant (143.77%) followed by seed yield per plant (142.04%).

Table 2. Mean values and range of heterosis (%) for six quantitative traits of the 60 F_1 sunflower hybrids (PH=plant height, HD=head diameter, TSW=thousand seed weight, OC=oil content, SY=seed yield per plant and OY=oil yield per plant)

Heterosis		PH	HD	TSW	OC	SY	OY
MPH							
	Mean	35.36**	37.17**	21.37**	0.06	142.04**	143.77**
	Range	15.32-66.86	17.24-66.56	0.20-65.22	-7.12-9.72	60.17-249.44	55.77-247-24
BPH							
	Mean	21.28**	19.00**	3.45*	-0.46	62.04**	64.10**
	Range	-4.01-42.70	0.74-47.81	-18.27-34.85	-9.98-6.98	29.14-130.92	34.72-125.32

**significant at P=0.05 , *significant at P=0.01

Analysis of fifteen SSR markers detected 44 alleles, with an average polymorphism PIC= 45.3%. The number of alleles per locus ranged between 2 and 5, with a mean of 2.93. Genetic distance between pairs of tested sunflower inbred lines ranged from 0.13 (HA-19 vs. HA-48 and R-12 vs. R-18) to 0.8 (HA-19 vs. R-18) (data not presented).

The relationship between genetic diversity based on SSR markers of all inbred lines and their hybrid performance depended on the trait examined. Correlation coefficients between GD and parental means, SCA and heterosis were not significant for the most examined traits (Table 3). The only significant correlation was a negative one, between GD and mean oil content ($r=-0.33$ $p<0.01$). For plant height, correlation between GD and heterosis was positive but not significant (r=0.232 and 0.172). Similar results were obtained for thousand seed weight (0.226 and 0.245).

Table 3. Correlation between genetic distance (GD) and mid- (MPH) and better-parent heterosis (BPH), specific combining ability (SCA) and mean values (MV) for each trait in sunflower hybrids (PH=plant height, HD=head diameter, TSW=thousand seed weight, OC=oil content, SY=seed yield per plant and OY=oil yield per plant).

	PH	HD	TSW	SY	OC	OY
GD vs. MPH	0.232	0.096	0.226	-0.213	-	-0.202
GD vs. BPH	0.172	0.101	0.245	-0.067	-	-0.071
GD vs. SCA	0.020	0.099	0.090	-0.159	-0.154	-0.178
GD vs. MV	-0.115	-0.102	0.071	0.021	-0.330**	-0.103

$r_{(0,05)}$=0,25, $r_{(0,01)}$=0,325

Correlation between genetic distance and heterosis was not significant for most of the examined traits. The poor correlation might be due to several causes. SSR markers used in this study were chosen solely for their high PIC values. Charcosset et al. (1991) and Bernardo et al. (1992) suggested that genetic distance cannot accurately predict hybrid performance unless the DNA markers used in the analysis were linked to the genes affecting the trait. Therefore, the 60 F_1 hybrids were divided into three groups according to the parental tester line and correlation of the GD with hybrid performance, and heterosis within the groups was examined for all six traits. Only significant correlations were found between GD and better-parent heterosis for thousand seed mass for hybrids with the tester line HA-19 ($r=0.43$ $p<0.05$) and between GD and mid-parent heterosis for plant height for hybrids with the tester line HA-26 ($r=0.47$ $p<0.05$) (Fig. 1). In these two cases hybrid heterosis increased linearly with increased GD between parental lines. However, the correlations obtained were too low to be of any predictive value.

Tersac et al. (1994) described relationships between heterosis and enzymatic polymorphism of 39 sunflower populations. The correlation coefficients for all enzyme systems were too low to be used as predictors of the general combining ability, but when enzyme systems were analyzed separately, four of them turned out to be useful markers for breeding purposes. Cheres et al. (2000) have used 360 AFLP markers and found that although genetic distances were significantly correlated with hybrid seed yield and percent of heterosis for seed yield (r=0.79 and 0.76), hybrid performance varied greatly among hybrids of inbreds with similar genetic distance (GD). Zeid et al. (2004) pointed out that the lack of association between heterosis and genetic dissimilarities for inter group hybrids might be explained by absence of crosses between related parents i.e. by the absence of variation for parental relatedness: all crosses have unrelated parents.

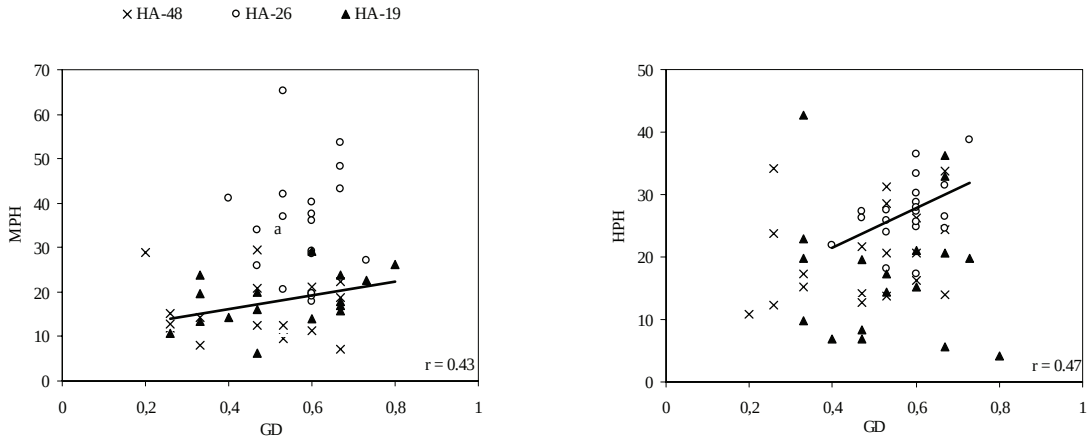

Fig. 1. Plots of genetic distance vs. mid-(MPH) and better-parent heterosis (BPH) for plant thousand seed weight (left) and plant height (right) of sunflower hybrid combinations ($r_{(0,05)}$=0.42, $r_{(0,01)}$=0.54).

The results of this study confirm that GD generally correlates poorly with heterosis and specific combining abilities. Previous studies in various crop species such as corn, pepper, alfalfa, wheat, and rapeseed also showed low correlations of GD with heterosis (Melchinger et al., 1990; Diers et al., 1996; Geleta et al., 2004; Zeid et al., 2004; Riday et al., 2003). Although genetic distance is a poor predictor of hybrid performance, our results indicate that better results are obtained if hybrid combinations for each tester and each trait are analyzed separately. Our further field trials for identification of sunflower heterotic performance will be planned on prior information on genetic distance of inbreds, obtained by more molecular markers, involving the ones associated with QTLs for examined traits.

ACKNOWLEDGEMENTS

This work was supported by Ministry of Science of Republic of Serbia.

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The Pervenets mutation in sunflower knocks out the wild microsomal oleate desaturase gene and leads to high oleic acid content in the seed oil

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ABSTRACT

Mutations in the seed oil pathways are of interest to release cultivars with modified fatty acid composition without transgenic methods. In sunflower, Pervenets mutation has been widely used to release sunflower cultivars with about 90% of oleic acid content in the seed oil without the exact mechanism of the mutation being understood. Here, we report new features of the Pervenets mutation organization and function as we have revealed the part of the microsomal oleate desaturase which is duplicated and we point out the expression of siRNA homologous to the oleate desaturase cDNA in the developing embryo at the stage when the oleic acid is synthesized. This new fact infers that the oleate desaturase underwent post transcriptional gene silencing (PTGS), which explains all the difficulties faced by breeders in stabilizing the oleic acid content in cultivars whatever the environmental conditions.

Key words: gene silencing – mutation – oleic acid content – PTGS – seed oil – siRNA

INTRODUCTION

Sunflower oil is naturally rich in linoleic acid (55-70%), and, consequently, poor in oleic acid (20-25%). Classic varieties are qualified as low oleic (LO). Until the 1970s, mutagenesis programs were conducted in order to produce varieties with an increased oleic acid content (OAC) compared to the classic (LO) varieties. The Pervenets sunflower population was obtained by chemical mutagenesis. It displayed OAC in seed oil around 65% (Soldatov, 1976). New varieties with OAC higher than 83% (HO for high oleic) varieties were then derived from the Pervenets population through breeding programs. This fatty acid composition modification of oil is located specifically in embryo tissues (Garcés et al., 1989). Due to the increased health interest of oleic acid and the similar agronomic performance of the HO compared to the classic varieties, HO varieties are now widely used in the world covering about 1.2 million ha (Collectif, 2004). In these varieties, the microsomal oleate-desaturase (MOD)-mRNA accumulation is reduced compared to the classic genotypes, leading to a decrease in MOD activity in the seeds during lipid reserve elaboration steps (Garcés and Mancha, 1989; 1991; Kabbaj et al., 1996a,b,c; Hongtrakul et al., 1998b). Using a candidate gene approach in diversity analysis, linkage disequilibrium was reported between the Pervenets mutation and an HO-specific MOD allele. Genetic studies performed on F_2 and recombinant inbred lines (RI Lines) populations revealed that this linkage disequilibrium is due to a closely genetic linkage between the Pervenets mutation and the HO-specific MOD allele (Hongtrakul et al., 1998b; Lacombe et al., 2001; Lacombe and Bervillé, 2001). However, these approaches could not determine whether the HO-specific MOD allele carries or is genetically linked to the Pervenets mutation. Consequently, the nature of the mutation is still unknown. Recently, Schuppert et al. (2006) studied the HO-specific MOD allele using PCR based approaches to identified molecular markers of the Pervenets mutation. However, no PCR codominant markers were found between wild type and mutant oilseed inbred lines due to a lack of DNA polymorphism in the region tested.

Recently, Lacombe et al. (2001), in studying RI lines progenies, showed that half the families carrying the Pervenets duplication were not as high oleic as expected. They therefore suggested that a genetic factor masked the Pervenets mutation effect and that it segregated in the progenies. The factor as a suppressor was called olesup. Crosses between a RI line carrying an efficient *olesup* allele and an HO line led to a low oleic F_1 progeny, whereas crosses between a RI line without an efficient *olesup* and a high oleic line led to a high oleic F_1 progeny (Lacombe and Bervillé unpublished; Y. Demurin, pers com).

Post transcriptional gene silencing (PTGS) is a way to regulate gene expression when expressed sequences are repeated. It is not widely spread (Baulcombe, 2004) and it is associated with 21 to 25 bp RNA molecules homologous to the gene under knock out (Hamilton and Baulcombe, 1999; Della-Vedova et al., 2005). Their presence points to the PTGS mechanism.

In this work, we present the organization of the Pervenets specific MOD allele in two different parts. The first part is present in both Pervenets and classic genotypes and carries a classic MOD gene (MOD-Cs). The second section is specific in Pervenets genotypes and carries a duplication of the MOD gene (MOD-Per). We also showed that the Pervenets mutation acts in *trans* to induce MOD mRNA under- or no-accumulation. We propose that the Pervenets mutation corresponds to MOD duplication and induces gene silencing on the normal MOD gene. Moreover, this work allowed us to identify co-dominant SSR in the intron of the MOD gene and other PCR markers corresponding to the Pervenets mutation. Such molecular markers may represent advantageous and useful tools in breeding programs. Finally, we characterized siRNA in Pervenets embryos that are absent in classic sunflower and it sustains PTGS. Moreover, this mechanism is known to be suppressed, and, consequently, it questions whether OAC variation in high oleic hybrids is due to the environment or to a genetic suppressor as we revealed *olesup* alleles.

MATERIALS AND METHODS

Plant Material

The RI lines segregating population was produced as described by Lacombe et al. (2001). The classic line 83HR4 (INRA) as the female parent was crossed with the HO line RHA345 (USDA-Fargo). One F_1 plant was self-fertilized to produce the F_2 progenies composed of 390 plants. Further generations were obtained by selfing ten individuals from each progeny, but only one producing many seeds was kept for the next generation. Because of inbreeding depression and self incompatibility, the F_6 generation was composed of 174 lines. Five seeds of the F_6 generation were analysed and put in Jiffy pots and then transferred to the field, but only one (plant 2 of each line, or, if lacking, plant 3) was retained for inheritance analysis. Half a cotyledon from each seed was analyzed for oil composition before germination, and each plant was further genotyped with the MOD cDNA as a probe (RFLP) and with different PCR primer pairs.

For mRNA and siRNA accumulation studies in immature seeds, self progenies and controlled crosses were obtained under a protective paper bag set up a few days before flowering. Each plant from the LO and HO lines was numbered and studied separately for all steps. Crosses were performed by transferring pollen from the male to the female under a paper bag to prevent illicit fertilization.

Total RNA was extracted using TriReagent (Sigma) according to the manufacturer's instructions from immature seeds at 10 to 15 days after pollination. For OD mRNA accumulation studies, northern blots were performed according to Sambrook et al. (1989) and northern blots were probed with 32P random priming labelled OD-cDNA (U91341). For siRNA detection, northern blots were made as described previously (Herr et al., 2005). T7-OD riboprobe was generated by T7 transcription (Promega) of a 1176 bp fragment that was amplified with Forward OD primer carrying a T7 extension (5'ataatacgactcactatagggtcgctaacccgttcgttctc3') and a reverse OD primer (5'tctaaaacacacccaacacg3')

RESULTS

The MOD-HO allele carries the common part of the MOD-LO allele on a sequenced fragment of 13.5kb. It carries the common 5.5kb *Eco*RI fragment, as predicted (Fig. 1). One single intron of 1684nt was detected between nt 83 and 1767 in the putative 5' untranslated region, 29nt before the ATG. A 16nt repeats of a $^{5'}$ATT$^{3'}$ SSR motif was revealed in the intron between nt 784 and 832. A *Hind*III site is present in the intron. It enables a 2.1kb *Hind*III fragment carrying only 83nt similar to the MOD sequence. This fragment was never revealed in RFLP profiles of HO or classic genotypes probably due to the small size of the MOD sequence on this fragment. No other *Hind*III site was detected in the rest of the putative gene or in the 4.3kb sequenced on its 3' side. This suggests that the HO-specific insertion is beyond this sequenced region on the 3'side on the common fragment.

A primer pair was selected to amplify the SSR locus located in the intron (Table 1). Polymorphism of the putative MOD was evaluated and three different alleles with 14, 15 and 17 TTA were found. These three alleles occurred in both the HO and classic genotypes showing that no linkage disequilibrium exists between the Pervenets mutation and a specific SSR allele despite the tight genetic linkage between the two loci. This suggests that natural variation at the SSR locus has occurred since the Pervenets mutation event.

To isolate parts of the HO-specific fragment, long PCR experiments were performed on DNA from the RHA345 HO and the 83HR4 classic lines using the F_c-a primer in combination with primers designed for the whole MOD cDNA sequence in both orientations (F1 to F8 and R1 to R8, Table 1). PCR products

were detected with the F_c-a primer in combination with R1, R2, R3, R4, R5, R6 and R7 whereas for the classic line no PCR product was detected with any of the primer sets.

The 4kb F_c-a_R7 PCR fragment from the HO line was cloned and sequenced. The organisation of this fragment is, from 5' to 3': 1) - 1219nt overlapping with the previously sequenced MOD region as expected according to the F_c primer position; 2) - 1357nt without any similarity to database sequences or to previously sequenced regions; 3) - 1487nt identical to the putative MOD gene between nt1447 and nt2421 corresponding to 239nt of intron and the following 1248nt of the exon 2. The 5' extremity of the putative gene was not detected. This MOD sequence and the putative gene had the same orientation. No *Eco*RI or *Hind*III site was detected in the F_c-a_R7 fragment. This agrees with the physical map of the OD-HO allele established from RFLP profiles (Fig. 1).

Table 1. List of primer pairs used in pairwise combination and size of fragments.

F_R	5'-F_ 3'	5'-R_ 3'	Expected_observed
1_8	accctaagcctctgtcgctc	tctaaaacacacccaacacg	most of cDNA
8-4	agatgatgaagggaaaggag	gccatagcaacacgataaag	3.974 kb across insertion
Fc-a_7	caaaccaccacccactaac	ggttctgggtctgggtctggtt	902 bp
Fc-b_1	gagaagagggaggtgtgaag	agcggttatggtgaggtcag	880 bp
Fc-b_1	gagaagagggaggtgtgaag	acaaagcccacagtgtcgtc	1247 bp
Fc-b_4	agaagagggaggtgtgaag	gccatagcaacacgataaag	1608 bp
1f_1r -	ttggagttcggtttatttat	ttagtaaacgagcctgaac	240 Ssr-ha-diapc MOD*RHA345*
1f_1r -	ttggagttcggtttatttat	ttagtaaacgagcctgaac	237 Ssr-ha-diapc-MOD*83HR4*

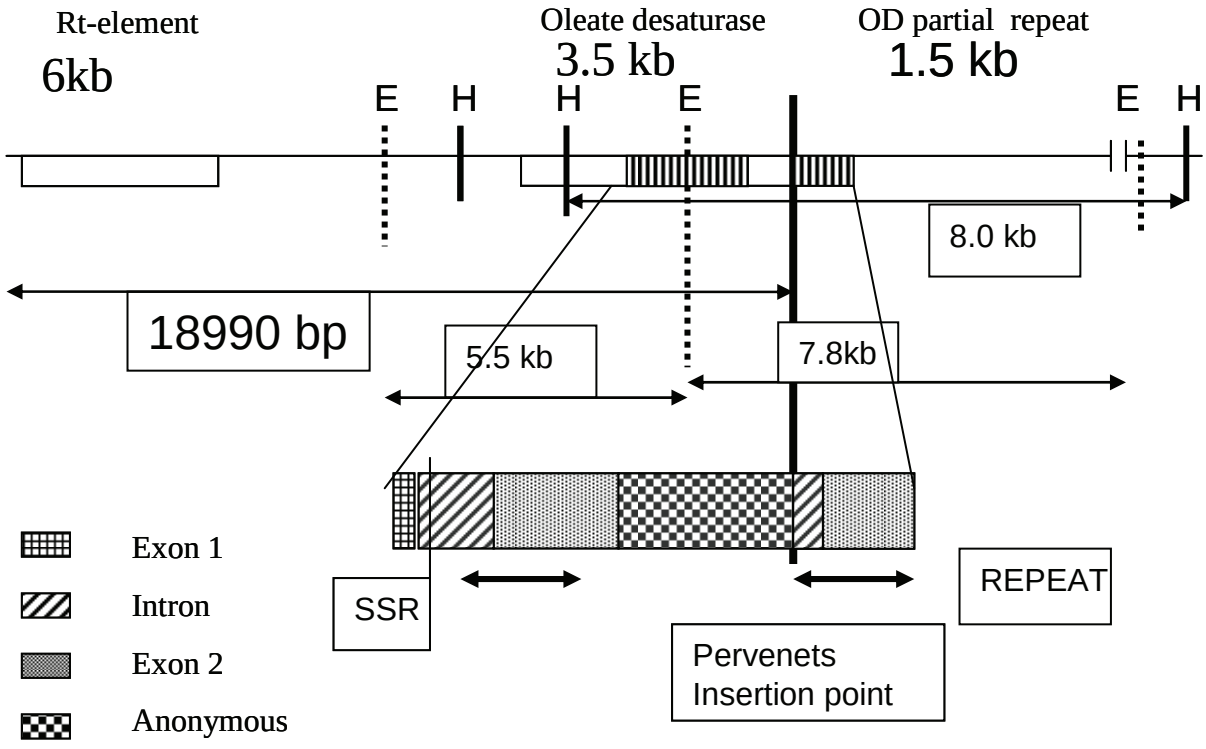

Fig. 1. A. Organisation of a microsomal MOD region in classic sunflower. Some features for regulation of the expression according to Lacombe and Bervillé (2001) and Bervillé et al. (2004). **B:** Organisation of the Pervenets mutation. Physical maps of the OD-Per region carrying an MOD gene and an extra fragment of 7.8kb carrying MOD-like sequences. E: *Eco*RI, H: *Hind*III site.

PCR amplification products of the expected size for all the HO genotypes tested were obtained with F_c-b designed on the F_c-a _R7 fragment in order to amplify an 880, 902, 1247, or a 1608bp fragment, in combination with R1, R2 or R4, respectively. These primer sets are suitable for the routine PCR technique according to the size of amplification products. They led to no amplification for the classic genotypes. An F2_R2 primer set used as a PCR control generated amplification at the expected size for

all the HO and classic genotypes. Consequently, the absence of PCR amplifications in classic genotypes with F_c-b / R1, R2 or R4 was not due to PCR failure, but, indeed, to the absence of these PCR fragments. Therefore, linkage disequilibrium exists between the Pervenets mutation and these HO-specific PCR fragments generated by F_c-b_R1, _R2 or _R4 primer sets.

The Pervenets mutation leads to an absence of, or a weak, MOD-mRNA accumulation in embryos (Fig. 2). No hybridisation signal was detected in HO embryos resulting from selfing or crosses. Moreover, results obtained in the hybrid embryos reveal that the mutation is dominant.

We selected primer pairs to amplify a 240pb PCR fragment carrying the microsatellite repeat in the MOD intron to map with Pervenets mutation. The use of these primers revealed polymorphism between the RHA345 HO line (17 TTA repeats) and the 83HR4 classic line (16 TTA repeats). For 174 RI lines studied, eighty-two display the 16-TTA allele and ninety-two RI lines carried the 17-TTA allele that fits the 1:1 ratio for the two SSR alleles (χ^2 P>0.1).

In the F_6 RI lines population, we then compared the segregation of the SSR polymorphism with the 7.8kb *Eco*RI Pervenets-specific fragment from the RHA345 line and with the PCR-specific 872bp fragment. This is in agreement with a tight genetic linkage between the Pervenets mutation and OD-HO allele (Table 2). PCR tests were set up to detect the Pervenets insertion. PCR assays included positive and negative controls Pervenets mutation segregation studies in RI lines

The positive control was provided by the 880bp PCR fragment amplified across the insertion point. The negative control was as previously the F2_R2 MOD primer pair wise combination. In 174 F_6 RI lines, eighty-two RI lines carry the MOD-SSR allele of the classic parent, and ninety-two lines carry the MOD-SSR from the HO parent. Seventy-eight RI lines displayed the HO-specific 7.8kb *Eco*RI and fourteen RI lines carried a shortened insertion (Table 2).

Table 2. Number of RI lines in each HO or LO class according to the MOD-Cs or MOD-Per alleles at the MOD- locus. Six plants were heterozygous and excluded from further analysis; Sup0, non expressed form assumed for the suppressor of silencing; *Supole*, expressed form of the suppressor of silencing; *excluded from further analysis; Sup0, non expressed form of the suppressor

174 RI lines	LO (OAC<50%)	HO (OAC>50%)
5.5kb *Eco*RI 8kb *Hind*III	125	35
SSR 237	82	0
SSR 240	43 (6*)	35
*Hind*III 13kb		
PCR OD-Per 872bp	0	88
Sup	Sup0 (ND)	*Supole* 43

SiRNA were revealed specifically in RHA345 lines in ten-days-old embryos, whereas they were absent in the classic line 83HR4 (Fig. 2).

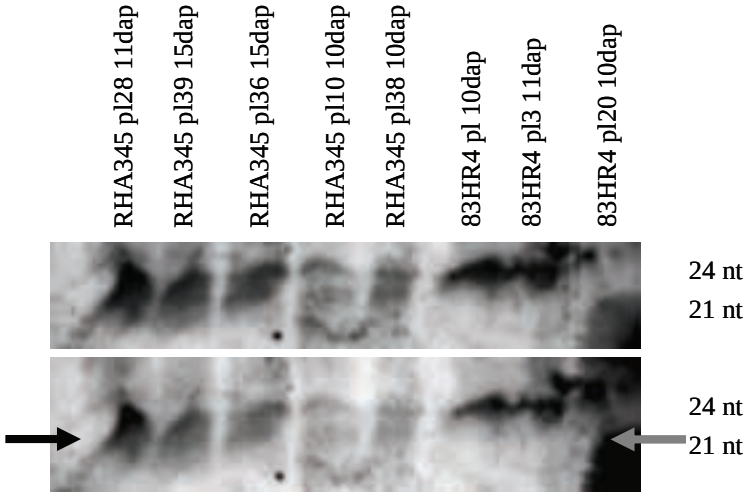

Fig. 2. Hybridization pattern with oleate desaturase as a riboprobe of total RNA fraction from immature (10 DAP) embryos harvested on different plants of lines RHA345 and 83HR4 showing (black arrow) and not showing (grey arrow) SiRNA at 21nt size, respectively.

DISCUSSION

Here we present evidence that the Pervenets mutation leading to the HO phenotype does not directly modify the MOD gene sequence but corresponds to MOD duplications. For other plant species, the mutations leading to an increase in OAC that have been characterised so far, directly affect MOD genes (Jung et al., 2000a,b; Patel et al., 2004; Okuley et al., 1994). The common MOD region is the only one detected in classic genotypes but other oleate desaturase do exist according to Martinez-Rivas et al. (2003). However, we showed that MOD-mRNA did not accumulate in segregating HO seeds whereas it accumulated in classic seeds confirming results already reported (Kabbaj et al., 1996a,b,c; Hongtrakul et al., 1998a,b). Moreover, the Pervenets mutation acts in *trans* to prevent MOD mRNA accumulation. A mutation in the MOD gene could not explain this dominance behaviour. Considering this result and the fact that the Pervenets mutation is associated with MOD duplications, we propose that the duplication induces a gene-silencing on the normal MOD gene leading to mRNA under accumulation.

Detection of MOD siRNA in the Pervenets mutant confirms the gene silencing mechanism. In Eukaryotes, gene silencing is a process that affects gene expression through sequence specific interactions. It involves 21nt and 24nt small interfering RNA (siRNA) produced from double strand RNA resulting from transcription of antisense or hairpin RNA and can act as dominant or semi dominant (Baulcombe, 2004). Strategies based on gene silencing against MOD genes have already been reported in crops to obtain transgenic plants with increased OAC. High OAC soybean and rapeseed were obtained through antisense and co-suppression mediated down-regulation of MOD (Kinney, 1996; Stoutjesdijk et al., 2000). Cotton transformed with an MOD inverted repeat construct showed high OAC (Liu et al., 2002). For the Pervenets mutant, the duplicated fragment is partially sequenced. Thus, we cannot predict whether antisense or hairpin RNAs is involved in the process. In sunflower, the Pervenets mutation associated with a gene silencing against the MOD gene may represent a new example of non-transgenic induced gene silencing in plants.

Molecular markers linked to the Pervenets mutation would represent advantageous and useful tools in breeding programs for rapid and early screening of genotypes carrying the mutation. PCR-based molecular markers linked to the Pervenets mutation were first co-dominant molecular markers.

The combination of these PCR-based molecular markers with the ones developed on the mutation itself would allow the determination of the homozygous or heterozygous status of the Pervenets mutation locus. Schuppert et al. (2006) have identified the same region and developed similar PCR tools for marker-assisted selection. However, some features did not fit our results. We cannot identify a 4.2kb *Eco*RI fragment on any autoradiogram. Probably, this fragment results from sequence concatenation of PCR products. Because several oleate desaturase genes do exist in sunflower (Martinez-Rivas et al., 2001), PCR may have produced a fragment from other MOD genes generating some confusion in the resulting sequence. We have also recognized some difficulties by primer extension upwards the insertion site. Apparently, the fragment obtained did not belong to the Pervenets locus since we obtained the same fragments for classic and Pervenets sunflower.

We provide evidence in the RI lines families that the Pervenets insertion can still be rearranged. All features for those RI lines converged, proving that the insertion shortened. Our results suggest that some RI lines may have lost duplicated MOD sequences. In fact, all these lines display low OAC in the seed oil, but the olesup suppressor in segregation may also be the cause of the LO phenotype. Genetic analyses are planned on these progenies to verify whether it may be due to the suppressor activity or to the loss of the duplication, which cancels silencing mechanisms.

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Vegetation period and hybrid sunflower productivity in breeding for earliness

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ABSTRACT

Development of a new cultivar combining short vegetation period with high productivity is a rather challenging problem. The aim of our work was to find out connections between duration of the vegetation period as a whole and its separate parts and seed yield, oil content and oil yield in sunflower hybrids. The study was done in the Hybrid Sunflower Breeding Department of All-Russia Oil Crops Research Institute (VNIIMK) in Krasnodar, Russia. Released, prospective and experimental sunflower hybrids of VNIIMK were used as a material. It was concluded that increasing the vegetation period itself does not lead to a high productivity. In each groups of earliness there were hybrids with high and low productivity. It was concluded also that seed yield is mainly determined by duration of the period from emergence to flowering (VE-R5.1), and oil content is mainly determined by duration of the period from beginning of flowering to maturity (R5.1-R8). Oil yield, being an integrated trait, it is determined by the whole vegetation period (VE-R8). As part of our study, a set of new hybrid combinations was produced and tested. One of them is recommended for release in the near future. Hybrid combination Kubanskiy 86 x VK 789 (tentative name is Aurora) is a three-way cross that throughout a 3-year trial showed better results than all tested open pollinated varieties and hybrids with a similar vegetation period.

Key words: breeding – earliness – sunflower – vegetation period.

INTRODUCTION

Development of a new cultivar combining short vegetation period with high productivity is a rather challenging problem. Many researchers report correlations between yield and duration of the vegetation period for different crops, including sunflower (Putt, 1943; Kovacik and Skaloud, 1972; Stoenescu, 1985; Merrien, 1992). At the same time, the possibility of a successful combination of these traits in one hybrid is not completely denied (Pustovoit, 1939). The aim of our work was to develop new ultra-early inbred lines and hybrids and also to find out relationships between duration of the vegetation period as a whole and its separate parts, and seed yield, oil content and oil yield in sunflower hybrids.

MATERIALS AND METHODS

The study was carried out during 2004-2006 in the Hybrid sunflower breeding department of All-Russia Oil Crops Research Institute (VNIIMK) in Krasnodar, Russia. Released, prospective and experimental sunflower hybrids of VNIIMK breeding were used as a material. The number of hybrids varied from 112 to 120 from year to year. In addition to the hybrids two open pollinated (OP) varieties (SUR and Enisey) were used as checks, because VNIIMK had not had such ultra-early hybrids before. The method described by Shneiter and Miller (1981) was used for phenological observations. The main registered periods were VE-R1, VE-R5.1, R5.1-R8, R1-R5.1 and VE-R8. Among the agronomy characteristics only the most important ones were used: seed yield per hectare, oil content in the absolutely dry seed and oil yield per hectare. Experimental design was randomized blocks with three replications. Each replication had four rows and two central rows were analyzed only to exclude the border effect. Oil content was evaluated by NMR-analyzer.

RESULTS AND DISCUSSION

During the three years, correlation coefficients were calculated between duration of the main parts of vegetation period and the most important yield characters. The number of hybrid combinations studied varied from a minimum of 112 in 2006 to a maximum of 120 in 2004. The correlation coefficients obtained are presented in Table 1. As is clear from the table, no significant and (or) stable relationships for all the years were found.

Table 1. Correlations between duration of the main parts of vegetation period and yield characters in sunflower hybrids (Krasnodar, 2004-2006).

Character	Year	Duration of the parts of the vegetation period				
		V-E – R 1	V-E – R 5.1	R 5.1 – R 8	R1 – R 5. 1	V-E – R 8
Seed yield, t/ha	2004	- 0.11	0.12	0.05	0.12	0.17
	2005	0.11	0.09	0.00	0.01	0.08
	2006	-0.15	-0.11	0.34**	-0.04	0.12
Oil content, %	2004	0.11	-0.03	-0.05	-0.11	-0.07
	2005	-0.04	-0.04	0.12	-0.03	0.05
	2006	-0.35**	-0.53**	0.20*	-0.52**	-0.38**
Oil yield, t/ha	2004	-0.05	0.08	0.03	0.05	0.11
	2005	0.07	0.05	0.07	0.01	0.10
	2006	-0.26**	-0.28**	0.36**	-0.22*	-0.03

**P = 0,01; *P = 0,05

Correlation analysis were also calculated after dividing the data to include only high-yielding hybrids from each group of earliness. The number of studied hybrid combinations was 20 during the whole period. In this case we obtained results showing tight correlations between the studied traits (Table 2).

Table 2. Correlations between duration of the main parts of vegetation period and yield characters in high-yielding sunflower hybrids of different groups of earliness (Krasnodar, 2004-2006).

Character	Year	Durations of the parts of the vegetation period				
		V-E – R 1	V-E – R 5.1	R 5.1 – R 8	R1 – R 5. 1	V-E – R 8
Seed yield, t/ha	2004	0.21	0.59**	0.64**	0.37	0.88**
	2005	0.56*	0.66**	0.27	0.35	0.77**
	2006	0.50*	0.59**	0.27	0.54*	0.69**
Oil content, %	2004	0.14	-0.31	0.27	-0.51*	-0.01
	2005	-0.16	-0.36	0.42	-0.34	-0.04
	2006	-0.36	-0.60**	0.19	-0.67**	-0.48*
Oil yield, t/ha	2004	0.27	0.41	0.75**	0.11	0.83**
	2005	0.44*	0.37	0.48*	0.08	0.65**
	2006	0.33	0.29	0.41	0.20	0.49*

**P = 0,01; *P = 0,05

From these results it was concluded that increasing the vegetation period itself does not lead to high productivity. In each group of earliness there were hybrids with high and low productivity. It was concluded also that seed yield is mainly determined by the duration of the period from emergence to flowering (VE-R5.1), and oil content is mainly determined by the duration of the period from beginning of flowering to maturity (R5.1-R8). Oil yield, being an integrated trait, is determined by the whole vegetation period (VE-R8).

As a part of our study a set of new hybrid combinations was produced and tested. One of them is recommended for release in the near future (Table 3). Hybrid combination Kubanskiy 86 x VK 789 (tentative name is Aurora) is a three-way cross that during the whole 3-year trial showed better results than all tested OP varieties and hybrids with a similar vegetation period.

Table 3. Characteristics of the new ultra-early sunflower hybrid (Krasnodar, 2006)

Hybrid or OP variety	Period VE-R8, days	Seed yield		Oil content, %	Oil yield	
		t/ha	± to check		t/ha	± to check
Enisey (Chek OP variety)	76	3,09	-	42,2	1,17	-
SUR (OP variety)	78	3,19	+0,10	47,8	1,37	+0,20
Kubanskiy 86 x VK 789	74	3,41	+0,32	45,9	1,41	+0,24
$LSD_{0,5}$	-	-	0,20	-	-	0,09

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Homo- and heterozygous longitudinal gradient of oleic acid content in sunflower seeds

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ABSTRACT

The results of the research on a seed oleic acid gradient present both in inbred lines and hybrids of a high oleic genotype with suppressor-carrying normal lines of sunflower are presented. Seven inbred lines were used: high oleic VK508, VK876; increased oleic LG27; low oleic RIL100, LG28, RHA416 and K824. The oil obtained from cotyledons of the line LG27 had a higher content of oleic acid than that obtained from gemmule. The difference was about 12.7%. Other lines did not show any significant discrepancy. The phenomenon could be called environmental homozygous increase of oleic acid content. A wide range of distribution of heterozygous *Olol* F_1 seeds on oleic acid classes in the crosses of VK876×LG28 and VK876×K824 was observed. One half of the seeds belonged to the high oleic class (76 to 91%) and the other half varied in an intermediate class (46 to 76%). This abnormal variation of the F_1 seeds is believed to be due to the genetic suppressor of *Ol* mutation from normal lines. The oleic acid content of gemmule and cotyledon was the same for the F_1 seeds of the mutant class. On the contrary, a significant difference in oleic acid content was observed for the F_1 seeds of the intermediate class. In this case, the cotyledon had a lower content of oleic acid than that of gemmule with a difference of about 11.6%. This longitudinal seed gradient could be called an epigenetic heterozygous decrease in oleic acid content. The environmental homozygous change in oleic acid content is in the opposite direction to the epigenetic heterozygous variation. The portion of mosaic F_1 seeds was 23%. Only one type of mosaic heterozygous embryo was found, i.e. mutant gemmule and intermediate cotyledon.

Key words: heterozygote – mutation – oleic acid – seed gradient

INTRODUCTION

The sunflower achene (fruit) consists of a seed (kernel) and pericarp (hull). The seed includes a seed coat, endosperm and embryo (Seiler, 1997). The embryo is mostly made up of two cotyledons and a gemmule (seed tip), which consists of rootlet, hypocotyl and budlet. All parts of the embryo contain oil-rich cells with a maximum content of reserve lipids in cotyledons of about 65% (Dyakov and Perestova, 1975; Popov and Dyakov, 1975).

The spatial longitudinal difference between the embryo tip and the cotyledons of a sunflower seed in the oleic acid content was described for the first time for both the high oleic and normal homozygous genotypes. The oleic acid content changed from 87.5 for the seed tip to 91.3 mol% for cotyledons of a high oleic genotype and from 44.4 to 56.9 % for the seeds of normal inbred line. The increase in oleic acid percentage was 3.8 and 12.5, respectively (Garcés et al., 1989). Another type of spatial difference was associated with the decrease in oleic acid content from 82.1 to 77.5 % for inner seed cotyledon and cotyledon emerged out of the hull for a high oleic genotype (Garcés and Mancha, 1989).

The content of oleic acid was found to be higher, about 5% (that of linoleic acid being lower), at the distal end of the cotyledon comparatively to the seed tip for the seeds of normal line CAS-6. This phenomenon could be explained with a gradient of oxygen throughout the seed due to the oxygen impermeability of the seed coat resulting in its diffusion mainly through the contact between the seed and the capitulum. Obviously, the oxygen could be a limiting environmental factor of oleate desaturation. A contrarily longitudinal difference was observed for the high stearic mutant lines CAS-14. The percentage of oleic acid was about twice times lower at the distal end of the cotyledon (reduction from 39.3 to 16.8 %) due to the increase of stearic acid content (Fernández-Moya et al., 2003).

All of the above-mentioned cases of the seed longitudinal gradient of oleic acid content were observed for the homozygous genotypes of sunflower. The spatial heterogeneity of a heterozygous F_1 seed of the oleic acid content was described for the first time in the crosses of a high oleic mutant with normal inbred lines HA89 and VK678. About 35% of individual F_1 seeds were mosaic with only one type of "high oleic gemmule – normal cotyledon". All of them belonged to the intermediate phenotypic class of whole seeds from 45 to 65% of oleic acid content (Demurin and Škorić, 1996).

This paper shows the results of the research on the seed oleic acid gradient in the crosses of a high oleic genotype with suppressor-carrying normal lines of sunflower. This was done in the development of hypothesis of incomplete penetrance of the *Ol* mutation.

MATERIALS AND METHODS

Seven inbred lines of sunflower were used: high oleic VK508 (*Ol*), VK876 (*Ol*); increased oleic LG27; low oleic RIL100, LG28, RHA416 and K824.

The plants were grown and self-pollinated in a field plot of VNIIMK, Krasnodar in summer 2004. The crosses were made with hand-emasculation. Each individual seed (embryo) of the inbred line or F_1 was cut with the razor into two parts, gemmule and cotyledons, which were analyzed separately. The oleic acid content of a whole seed was calculated with the weight ratio of 0.1 gemmule and 0.9 cotyledons.

Fatty acid composition of the oil from the seed parts was determined by gas chromatography of methyl esters.

RESULTS AND DISCUSSION

The oil obtained from cotyledons of the line LG27 had a higher content of oleic acid than that obtained from gemmule. This difference was about 12.7% (Table 1). Neither high oleic lines VK508, VK876 nor low oleic lines RIL100, LG28, RHA416, K824 showed any significant discrepancy. This type of spatial gradient along an embryo in the oleic acid content seems to be caused by an environmental lowering of oxygen concentration. The phenomenon could be called environmental homozygous increase in oleic acid content. These results agree with earlier research (Garcés et al., 1989; Fernández-Moya et al., 2003).

Table 1. Average oleic acid content in the oil of seeds and seed parts of inbred lines

Line	Oleic acid content, %				LSD_{05}
	embryo	gemmule	cotyledon	Δ^1	
VK508	92.3	92.2	92.3	0.1	0.4
VK876	89.2	88.8	89.2	0.4	1.9
LG27	72.0	60.6	73.3	12.7	2.3[2]
RIL100	36.3	35.8	36.3	0.5	9.1
LG28	28.4	27.9	28.5	0.6	5.1
RHA416	27.3	24.8	27.6	2.8	3.7

[1] Difference between cotyledon and gemmule (five seeds per line)
[2] $p < 0.05$

A wide range of distribution of heterozygous *Olol* F_1 seeds on oleic acid classes in the crosses of VK876×LG28 and VK876×K824 was observed (Fig. 1). One half of the seeds belonged to the high oleic class from 76 to 91% and the other half varied within the intermediate class from 46 to 76%. This abnormal variation of the F_1 seeds is believed to be due to the genetic suppressor of *Ol* mutation of normal lines LG28 and K824. As a result of the incomplete penetrance of a dominant mutation *Ol* in a heterozygote can be detected.

The oleic acid content of gemmule and cotyledon was the same for the F_1 seeds of the mutant class with the embryo mean of 87.6% (Table 2). Differences between the cotyledon and the gemmule of the individual seeds of the mutant class varied accidentally. On the contrary, a significant directional difference in oleic acid content was observed for the F_1 seeds of the intermediate class with the embryo mean of 60.1%. In this case, the cotyledon had a lower content of oleic acid than that of the gemmule with a difference of about 11.6%. This spatial seed gradient could be called an epigenetic heterozygous decrease in oleic acid content. A heterozygous *Olol* embryo under the action of the suppressor seems to possess a reversion to the normal phenotype during mitotic division of the cotyledon cells and seed development. It should be noted that the environmental homozygous change in oleic acid content is in the opposite direction to the epigenetic heterozygous variation.

Table 2. Average (range) oleic acid content in the oil of F_1 seeds and seed parts.

Phenotypic class of whole seed	Number of seeds	Oleic acid content, %				LSD_{05}
		embryo	gemmule	cotyledon	Δ^1	
Mutant, high oleic	20	87.6 (77.3-91.4)	87.5 (75.6-90.7)	87.6 (77.3-91.5)	0.1	2.4
Intermediate	20	60.1 (45.8-75.6)	70.5 (51.4-87.3)	58.9 (44.7-75.3)	-11.6	6.6[2]

[1] Difference between cotyledon and gemmule
[2] $p < 0.05$

The data on the phenotypic coincidence in oleic acid content between gemmule and cotyledon of the F_1 seeds are evidence of the above assumption (Table 3). The portion of mosaic F_1 seeds was 23% (9/40). It is very important to stress that only one type of mosaic heterozygous embryo was found, i.e. mutant gemmule and intermediate cotyledon. These results agree with our previous observation (Demurin and Škorić, 1996).

Table 3. Phenotypic coincidence in oleic acid content between gemmule and cotyledon of F_1 seeds.

Cross	Number of F_1 seeds				Portion of mosaic seeds
	G_m/C_m	G_m/C_{int}	G_{int}/C_m	G_{int}/C_{int}	
VK876×LG28	13	4	0	3	0.20
VK876×K824	7	5	0	8	0.25
total	20	9	0	11	0.23

G – gemmule, C – cotyledon, m – mutant (high oleic), int – intermediate oleic class

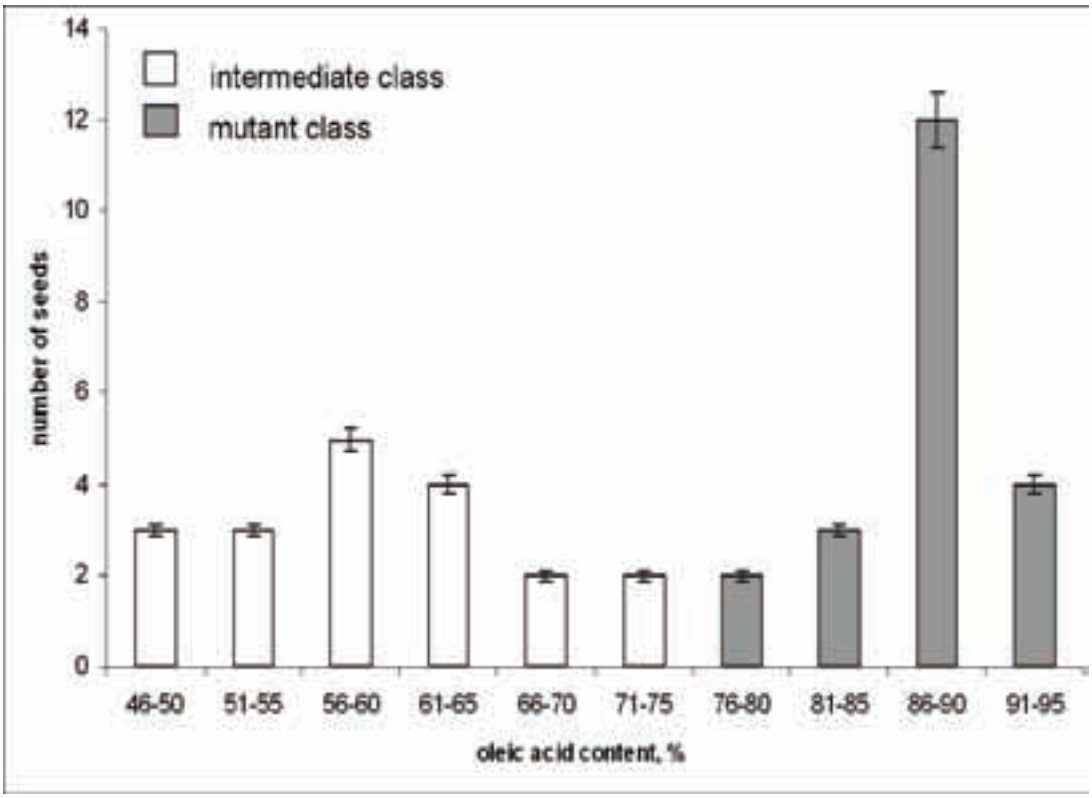

Fig. 1. Distribution of heterozygous *Olol* F_1 seeds on oleic acid classes in the crosses of VK876×LG28 and VK876×K824, n=40

In conclusion, the phenomenon of the longitudinal gradient of oleic acid content in the sunflower seeds has to be taken into account when the half-seed technique is used.

ACKNOWLEDGEMENTS

This work was supported by a grant № 06-04-97603 from the Russian Foundation of Basic Research (RFFI) and Administration of Krasnodar region, Russia.

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White rot resistance, seed weight and seed oil content in sunflower test-crosses

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ABSTRACT

In Balcarce, we wanted to obtain sunflower restorer R inbred-lines capable of transmitting to their off-spring a suitable level of partial resistance to white rot, without being inferior for other agronomic attributes. A series of test-crosses obtained by crossing new R inbred-lines with a tester were evaluated. Preliminary results indicated that some R inbred-lines had favourable effects concerning resistance for at least one phase of the white rot development in capitula, as well as seed weight and seed oil content. The level of disease resistance was independent of both agronomic characters, measured on healthy plants. During the cultivar development it should be possible to obtain sunflower hybrids with moderate level of resistance to white rot combined with high seed weight and oil content.

Key Words: biological cost – breeding – disease resistance – *Sclerotinia* – seed oil content – seed weight

INTRODUCTION

White rot symptoms caused by *Sclerotinia sclerotiorum* infections on capitula reduce seed yield and oil content in sunflower cultivars in relation to disease intensity (Gulya et al., 1997). Therefore, it is necessary to develop sunflower hybrids with a suitable level of disease resistance and adapted to be grown in environments where there is a risk of white rot.

In sunflower, white rot disease can be considered as being composed of different phases of a process beginning with the infection during flowering, followed by mycelium invasion in the parenchyma tissues during the grain-filling, and ending with sclerotia formation at maturity. Partial resistance of genotypes can be evaluated by means of indicators related to each of these phases (Castaño, 2007). For example, for the disease incidence and the relative incubation period (Vear and Tourvieille, 1984), the beginning of the disease development (penetration - initial mycelium growth) was measured. With disease severity (Russi et al., 2004) as well as daily white rot progress (Castaño and Giussani, 2006) the severity of intermediate and final phases (mycelium invasion and sclerotia formation) of the white rot development can be estimated

At Balcarce we developed a series of restorer R inbred-lines after inbreeding and selection from old and modern sunflower cultivars. Because this germplasm is potentially interesting for seed weight and oil content, it became necessary to evaluate the capacity of the R inbred-lines to transmit both white rot resistance, and the agronomic characters to their off-spring in order to define which of the lines should usefully be continued in the sunflower breeding program.

In this study, a series of R inbred-lines were evaluated, through their test-cross behaviour, for their reaction to the different phases of white rot development, as well as for the seed weight and seed oil content produced by plants without symptoms.

MATERIALS AND METHODS

Sunflower genotypes

46 test-cross hybrids, with sufficient seed, were produced by pollinating the male-sterile inbred-lines CMS GU and/or CMS GB with 37 restorer R inbred-lines selected from Argentine, French and Italian commercial hybrids, and with the line PSC8 of well known performance to white rot (Castaño et al., 1993). The lines GU (Serre et al., 2004) and GB (F.Vear, pers. com.) were bred by INRA, France, and showed a high level of white rot symptoms quite rapidly after infection in France.

Two sunflower cultivars, PARAÍSO 20 and ACA 884, were used as checks for disease incidence since they showed moderate resistance to this variable in more than 70% of trials carried out in the last decade at Balcarce. In addition, the cultivar VDH 487 was utilized as check for the seed weight and seed oil because of its good performance during the 2005/06 and 2006/07 seasons in the southern sunflower growing area in Argentina (Quillehauquy et al., 2007).

Experiment
The test-crosses and the three sunflower cultivars were sown in the field following a randomized complete block design with two replicates. Plots had at least 15 plants. The lines GU and GB, in the male fertile versions (B), were grown beside this experiment.

Inoculation and disease variables measured
The inoculation protocol of Vear and Tourvieille (1984) was used. The floral surface of 12 plants/plot in the R5.3 sunflower stage (Schneiter and Miller, 1981) (= F3.2, Cetiom, 1992), was sprayed once with an aqueous suspension containing about 25000 ascospores. Inoculated inflorescences were covered with Kraft paper bags until the end of the experiment. Twice weekly irrigations of approximately 5 mm each were made with sprinklers until the sunflower maturity stage.

Capitula were felt twice a week from 15 days after inoculation until first white rot symptoms appeared. Then, each capitulum was examined every 7 days until the end of the experiment. At each observation, the date and the proportion of the diseased capitulum area were scored. The following variables were quantified: 1) disease incidence (%), at the M4 stage, 2) relative incubation period, 3) disease severity, at 40 days after inoculation (40 dai), R8-9 stages (= M2), 4) maximum disease severity reached at the M4 stage and, 5) daily white rot progression (%). An average was calculated per plot for the last four white rot variables.

Seed weight and Seed oil content
The capitula of uninoculated plants (at least three per plot) were covered with netting bags. At maturity, capitula were harvested and seeds weighed. Means per plant and per plot were calculated Seed-oil percentage was determined by nuclear magnetic resonance (NMR) and a mean per plot estimated.

Statistical analyses
Analyses of variance using two criteria of classification (genotypes and blocks) were made, by means of the GLM (SS type III) procedure of SAS. The LSD values were calculated and, in addition, a correlation coefficient between white rot resistance and seed weight and seed oil content obtained. All the analyses were based on Reza-Hoshmand (1998).

RESULTS AND DISCUSSION

Means of each variable measured are shown in Table 1. In 5 of the 7 variables, the coefficient of variability was over 30%. These relatively high values could be related to the insufficient environmental humidity during the experiment despite the irrigations carried out.

White rot variables
General disease incidence reached 23.8% and the range between extreme incidence values was 63.3%. The checks PARAÍSO 20 y ACA 884 had 13.6% and 28.1% of diseased plants, respectively. The disease incidence for the line GU (B) was 40.6% and for GB (B) 34.2% (data not shown). In checks, the disease incidence was 5% (ACA 884) and 12% (PARAÍSO 20) lower than estimated mean values from 10 experiments made previously at Balcarce (Quillehauquy et al., 2007). Concerning the line GU (B), the relative number of diseased capitula was 59.3% lower than the average value reported by Serre et al. (2004) after 13 trials made in France. In relation to the bibliography, the lower mean values of checks as well as in the line GU (B) could be related to inadequate environmental humidity during disease development. Analysis of variance detected significant (α=0.01) differences between genotypes.

Table 1. Average responses of sunflower test-crosses and commercial hybrids to *S .sclerotiorum* inoculations and seed weight and seed oil in plants without white rot symptoms.

VARIABLES[1] ➔	INC (%)	RIP	SEV-40 (%)	SEV-MX (%)	WRP (% / d)	SW (g/cap)	SO (%)
GENOTYPES							
Test-cross							
GB x R1	* 25.0	& 1.38	* 8.3	45.0	1.6	10.0	& 46.8
GB x R2	* 11.8	1.12	* 8.6	* 32.5	1.0	9.4	& 50.6
GB x R3	55.6	0.72	81.7	88.0	4.5	15.0	& 49.7
GB x R4	36.4	& 1.31	* 18.0	44.6	2.2	12.2	& 50.7
GB x R5	* 20.9	1.07	* 51.7	90.0	4.7	11.9	& 49.9
GB x R6	33.1	& 1.18	57.4	88.1	9.2	& 24.5	& 49.7
GB x R7	* 17.4	0.93	87.1	100.0	9.1	14.8	& 50.6
GB x R8	* 0.0	& 1.52	* 0.0	* 0.0	# 0.4	15.6	42.1
GB x R9	* 17.0	0.85	65.3	85.0	5.4	13.5	& 46.0
GB x R10	* 7.1	0.63	100.0	100.0	5.7	& 18.2	& 46.3
GB x R11	63.3	0.87	67.6	83.3	5.9	& 29.3	& 51.3
GB x R12	31.4	0.88	* 27.8	56.7	2.9	16.3	& 48.5
GB x R13	* 23.1	0.84	* 46.6	58.8	3.7	9.6	& 46.6
GB x R14	48.3	0.85	67.4	83.9	6.2	& 17.7	& 51.2
GB x R15	* 19.4	0.91	* 37.1	61.3	3.0	& 18.6	& 48.5
GB x R16	36.4	& 1.20	* 29.9	48.8	2.5	& 26.4	43.3
GU x R6	* 7.7	0.95	* 25.6	62.5	3.8	& 21.2	& 46.4
GU x R7	42.9	1.00	* 6.8	* 25.0	0.8	& 22.0	33.5
GU x R8	* 28.6	0.73	71.9	100.0	6.1	4.5	37.3
GU x R9	* 16.7	0.99	* 40.8	62.5	3.3	9.4	& 49.4
GU x R11	* 10.0	0.81	66.3	100.0	5.8	10.9	& 50.6
GU x R12	* 20.8	0.96	65.0	68.8	6.9	11.4	42.2
GU x R15	* 10.0	0.78	* 15.0	* 15.0	0.8	14.0	& 50.4
GU x R16	* 9.1	0.95	* 3.3	* 22.5	0.5	11.5	& 49.1
GU x R17	* 22.6	0.99	* 5.3	* 40.0	1.3	6.0	45.2
GU x R18	* 4.5	0.81	100.0	100.0	4.8	8.3	44.1
GU x R19	* 26.6	0.98	* 48.8	64.6	3.5	6.8	& 49.7
GU x R20	* 30.2	0.86	63.5	73.8	8.6	10.0	& 48.6
GU x R21	29.9	& 1.31	* 0.0	* 20.6	0.9	9.1	45.1
GU x R22	* 25.4	0.85	* 42.4	59.4	2.3	9.7	& 48.4
GU x R23	36.0	1.02	54.9	69.1	5.8	14.4	& 51.0
GU x R24	* 16.1	1.01	* 22.2	73.3	6.2	& 22.7	& 52.0
GU x R25	59.2	0.76	93.4	100.0	6.4	11.4	& 49.8
GU x R26	* 16.7	0.91	* 9.7	53.3	1.8	13.5	& 48.2
GU x R27	* 18.2	0.63	55.8	87.5	3.0	1.2	& 51.2
GU x R28	* 19.8	1.04	61.0	80.0	7.5	14.9	& 50.0
GU x R29	* 11.5	& 1.26	* 0.0	* 27.5	0.5	13.5	& 52.5
GU x R30	* 15.3	0.85	* 35.0	50.0	2.1	9.7	& 51.7
GU x R31	* 23.0	0.94	* 34.0	52.2	8.2	12.1	& 47.5
GU x R32	27.9	1.00	* 34.1	50.3	3.6	11.5	& 50.9
GU x R33	* 15.0	1.06	* 23.9	86.7	7.6	5.3	& 48.4
GU x R34	* 19.9	1.09	* 25.0	56.3	6.6	7.7	& 49.9
GU x R35	46.2	& 1.14	* 28.3	80.0	2.9	& 18.9	& 47.7
GU x R36	33.3	0.92	* 23.8	61.3	4.2	16.8	& 50.4
GU x R37	* 0.0	& 1.52	* 0.0	* 0.0	# 0.4	& 22.8	& 47.9
GU x R38	* 16.7	1.01	* 1.9	* 38.8	3.6	14.6	& 49.2
Cultivars							
ACA 884	* 28.1	1.08	* 28.6	48.8	2.7	13.4	42.8
PARAÍSO 20	* 13.6	& 1.16	* 3.6	97.5	13.3	12.4	& 47.1
VDH 487	* 18.2	0.99	* 7.6	* 23.3	0.6	12.6	& 52.4
General mean	23.8	1.0	38.4	61.9	4.2	13.7	48.0
CV (%)	66	17.1	63.8	31.5	70.6	41.4	6.5
$LSD_{0.05}$	30	0.39	54	43	---	11.8	6.6

[1]INC= Incidence; RIP= Relative Incubation Period; SEV-40= Severity at 40 days after inoculation; SEV-MX= Maximum Severity; WRP= White Rot Progress; SW= Seed Weight; SO= Seed Oil.
*Value equal to the minimum one in the variable, since the $LSD_{0.05}$ test; [&]Value equal to the maximum one in the variable, according to the $LSD_{0.05}$ test; [#] Rounded value.

The LSD value of 30% indicated that 30 test-crosses as well as the three cultivars were not significantly different from the GBxR8 and GUxR37 test-crosses, without symptoms in this experiment. In these 32 test-crosses the disease incidence was lower than in the lines GU and GB. Therefore, the R inbred-lines used must have contributed to reducing disease incidence in these test-crosses in relation to the testers. The inbred-lines R8, R9, and R15, were crossed to both testers (CMS GU and CMS GB), and their hybrids had similar disease incidence values to those of both check cultivars.

The relative incubation period of the GBxR8 and GUxR37 test-crosses, which showed no symptoms, was estimated according to Castaño et al. (1993). The calculated value (1.52) was 10% greater than the highest one (1.38) shown by GBxR1 in this experiment. General mean was 1.00 and range 0.89. Analysis of variance detected significant (α=0.02) effects of genotypes. The LSD value (0.39) detected 7 test-crosses and the cultivar PARAÍSO 20 with similar relative incubation period values to the maxima calculated for GBxR8 and GUxR37.

The general mean of the disease severity at 40 days after inoculation had a value of 38.4% and the range was 100%. Analysis of variance showed significant (α=0.01) differences between genotypes. The LSD value (54%) determined that 26 test-cross and all three cultivars had similar severity 40 dai values to the four following test-crosses: GBxR8, GUxR37, GUxR21, GUxR29, with the minimum (0%).

Maximum disease severity had a general mean value of 61.9% and a range of 100%. Analysis of variance indicated that genotype responses differed significantly (α=0,001). LSD value was 43%, determining that 8 test-crosses and the cultivar VDH 487 showed similar maximum disease severities as both GBxR8 and GUxR37 test-crosses without symptoms.

The daily white rot progress of two non diseased test-crosses was estimated in the same way as for the relative incubation period. The calculated value was 0.45, 10% less compared with the lowest value (0.5) observed for GUxR29 and GUxR16 in this experiment. Mean of daily progression of symptoms was 4.2% and the range was 12.8%. Unlike previous four white rot variables, the analysis of variance did not detect any differences between genotype responses (α=0.12). In spite of the high range of white rot progress values, the absence of different genotype responses could be related to the coefficient of variation of 70.6% showed by this variable, the highest one in the experiment.

Test-crosses obtained from the line CMS GU with the restorers R21, R29, and R37, as well as CMS GBxR8, showed favourable responses for white rot variables. These R inbred-lines contributed to increasing the level of resistance of all phases of the disease development in the test-crosses evaluated. Excepting R3, R20, R25, and R23, the inbred-lines showed advantageous effects for at least one phase of white rot development. The inbred-line R11 however showed a good performance with respect to disease incidence when it was crossed to CMS GU, but was very susceptible when it was crossed with CMS GB since this test-cross reached the maximum incidence value (63. 3%).

Seed weight and seed oil content

Mean value for seed weight was 13.7 g/cap and the range was 28.1 g/cap. The check VDH 487 had 12.6 g/cap, this value being lower than the average (52 g/cap) shown by these cultivars in 8 trials of the National Network of Sunflower in Argentina during 2006/07 (Quillehauquy et al., 2007). According to Hall et al. (1985), Ravishankar et al. (1991), and Nel et al. (2000) the absence of adequate environmental humidity would have decreased both the seed size and seed weight in this cultivar, but also in the other genotypes grown in this experiment. Analysis of variance showed significant (α=0.02) differences between genotypes. The LSD value of 11.8 g/cap showed that 10 test crosses had statistically similar seed weight to GBxR11, which was the best (29.3 g/cap). Three test crosses: GBxR11, GBxR16, GBxR6, showed significantly more seed weight than the check VDH 487.

General mean of seed oil content was 48% and the range was 19%. The fertile inbred lines GU and GB showed 48% and 44%, respectively (data not shown). The cultivar VDH 487 had a value of 52.4%, which was higher than the mean value (48%) shown by the same cultivar in 19 trials in the National Network of Sunflower in Argentina (Quillehauquy et al., 2007). According to Steer et al. (1988), the fact the seed size and seed weight were altered by the lack of adequate humidity could be related to the maintenance of seed oil content in this cultivar and other genotypes in this experiment. Analysis of variance detected significant (α=0.01) differences between genotypes. The LSD value was 6.5% and it determined that 37 test-crosses as well as cultivars VDH 487 and PARAÍSO 20 had similar seed-oil percentage as the maximum (52.5%) shown by GUxR29. All these test-crosses, excepting GUxR6, GUxR31, GUxR35, and GUxR37, showed higher seed-oil values than the fertile inbred-lines GU and GB, whose male-sterile version were the female in the test-crosses. This effect suggests a favourable contribution of R inbred-lines in the seed oil content in these test crosses.

Relationship between white rot resistance and seed-weight and oil content of healthy plants

The association between genotype responses to *S. sclerotiorum* inoculations and the agronomic attributes measured in plants without symptoms was calculated and the correlation coefficients are shown in Table 2.

Table 2. Correlation coefficients, linear (r) and rank (r_s, in italics), between white rot variables and seed weight and seed oil content of plants without symptoms

	Incidence	Relative incubation period	SEV-40dai	SEV- MX
Seed-Weight	0.26	0.21	-0.04	-0.08
Seed-Oil	*0.04*	0.04	*0.09*	*0.06*

Coefficients of linear correlation varied between r= 0.26 and r= -0.08, while those of rank correlation oscillate between r_s= 0.09 and r_s= -0.04. None of the coefficients were statistically different from zero (n=49, p> 0.05). In this experiment, the level of disease resistance in genotypes could be considered as being independent of the seed weight and seed oil content measured on healthy plants.

The apparent absence of effects of *S. sclerotiorum* resistance on the agronomic traits measured would suggest that the level of white rot resistance does not have any substantial cost, from a biological point of view, for the sunflower genotypes when the disease is absent.

This characteristic is not exclusive of the sunflower-*Sclerotinia* interaction, since the bibliography reports similar results with other crops and pathogens. For example, Miles et al. (1980) and St. Martin et al. (1994) found that in environments without disease the seed yield of genotypes with a higher level of resistance was not reduced compared with that shown by less resistant genotypes in maize-*Heminthosporium turcicum* and soybean-*Phytophtora infestans* pathosystems, respectively.

Development of cultivars with high levels of resistance implies selecting genotypes with the best disease performance. These genotypes must be adapted for use in environments with disease risk. Then, if the disease appeared, resistant cultivars will have a better agronomic behaviour than susceptible or non-adapted ones (Brown, 2002).

In this sense, Creus et al. (2007) showed that resistant sunflower isohybrids to *Verticillium dahliae* yielded 30% more than the homologous susceptible ones, when Verticillium wilt was present. In the available bibliography, there does not appear to have been any similar work using white rot disease, but it can be assumed that genotypes with a higher level of resistance yield more than susceptible ones when white rot occurs.

Sunflower breeders consider many factors, such as white rot resistance for example, when deciding whether or not to release a hybrid. The fact that the resistance to *Sclerotinia* infection on capitula does not condition the seed weight and seed-oil content in non diseased test-crosses would allow the possibility of combining adequate levels of white rot resistance with high seed weight and seed-oil content in the same genotype. If these selected cultivars were used, the agronomic stability of sunflower crop could be improved given that the seed weight and seed oil content oscillations between environments with and without white rot appearance could be diminished.

CONCLUSIONS

Because the results were obtained from only one experiment they must be considered as being preliminary ones. A further trial will allow an estimation of their repeatability. In this first experiment it could be concluded that:

1- Genotypes were diversified for all white rot variables, except daily white rot progression.
2- 72% of test-crosses (33/46) had similar disease incidence values as the checks ACA 884 and PARAÍSO 20. In these test-crosses, the R inbred-lines contributed to reduction of disease incidence compared with testers.
3- There were some R inbred-lines with favourable resistance contribution for at least one phase of the disease development.
4- There was variability in seed weight and seed oil content in genotypes not inoculated with *S. sclerotiorum*.
5- Test-crosses produced by the inbred-lines R6, R11, and R16 with CMS GB, had higher seed weight than the check VDH 487. Those obtained after crossing the inbred-lines R6, R31, R35, and R37 with CMS GU, had similar oil contents to those of the check but higher than the tester.
6- The level of white rot resistance did not restrict the seed weight and seed oil content when the disease was absent in the genotypes evaluated

ACKNOWLEDGEMENTS

We would like to thank Dr. Félicity A. Vear for her critical reading of the manuscript. Miss Mª: A. Giussani is a student of the "Programa de Posgrado en Ciencias Agrarias" at the UIB. The UNMdP and INTA supported this investigation.

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Gene effects and combining abilities of sunflower morphophysiological traits

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ABSTRACT

The development of sunflower hybrids with high genetic potentials for seed and oil yields requires information on the GCA and SCA for agronomically important traits in the F_1 generation. In this study were used seven new divergent cms inbred lines (A) lines, three Rf restorers utilized as testers, and 21 F_1 hybrids developed at the Institute of Field and Vegetable Crops in Novi Sad. Significant differences were found between the A lines, Rf testers and their F_1 hybrids for petiole length (PL), total leaf number per plant (TLN), total leaf area per plant (TLA), seed yield per plant (SY), seed oil content (OC), and oil yield per plant (OY). Analysis of variance of the combining abilities revealed highly significant differences for GCA and SCA. The nonadditive component of genetic variance (dominance and epistasis) contributed more to total genetic variance, as evidenced by the GCA/SCA ratio in the F_1 generation being below the value of one for all the traits studied. The greatest average contribution to the expression of all of the traits was found in the female A lines, while the contributions of the Rf testers and line x tester interaction were less significant. Evaluation of the combining abilities of the line NS-GS-5 suggests that this line could justifiably be used as a parent in breeding programs for increasing sunflower seed and oil yields. Analysis of SCA for OY per plant identified the following hybrids as promising: NS-GS-5xRHA-R-PL-2/1, NS-GS-6xRHA-N-49, NS-GS-2xRHA-N-49, NS-GS-1xRUS-RF-OL-168.

Key words: GCA – *H. annuus* – interspecific hybridization – SCA.

INTRODUCTION

Sunflower is the main crop for producing vegetable oil in Serbia. NS sunflower hybrids are developed primarily for Serbian growing conditions while taking into account the limiting factors present in the domestic sunflower production. They are the dominant brand on the Serbian sunflower market and are also grown on over two million hectares in various markets abroad (Miklič et al., 2007).

Plant height, head size, form, and position on the stem, and leaf number, size, duration, and distribution on the plant all play important roles in defining optimum plant architecture for a sunflower hybrid (Škorić et al., 1989; 2002). Breeding for morphophysiological yield components and the creation of a new sunflower ideotype require an increased use of wild *Helianthus* species in breeding programs. One of the surest ways to increase the genetic variability of sunflower is by the use of interspecific hybridization (Škorić et al., 2007). The main precondition for designing the model of a hybrid is to obtain parental lines possessing desirable genes so as to be able to pair up parent lines that produce superior F_1 progeny over the existing hybrids for the largest number of agronomic traits.

Petiole length (PL) has caught the attention of breeders because of the possibility of changing existing sunflower plant architecture. The shortening of the sunflower petiole so that it is closer to the stem results in the faster conveyance of photoassimilates from the leaf blade to the sinks as well as in an increased plant number per unit area (Marinković et al., 2003).

Along with leaf size, total leaf number per plant (TLN) determines the total leaf area for accumulation, which makes it necessary to know the genetics of this trait (Marinković et al., 2003). Total leaf area (TLA) depends on the position of the leaf, leaf area profile, plant development, and genotype (Panković et al., 1991).

One of the main directions of sunflower breeding both in Serbia and elsewhere is the development of hybrids with high genetic potential for seed yield and altered plant architecture capable of adapting to the conditions of the specific area in which they are being grown. Seed oil content (OC) depends on the genotype, soil and climate conditions, and the level of cultural practice used. OC is greatly influenced by the origin of the material and the year of study (Hladni et al., 2006). It ranges from 38.1 to 49.2% depending on the location (Škorić et al., 1996) and from 36.0 to 54.4% according to year (Škorić and Marinković, 1990).

Oil yield (OY) is the main indicator of any sunflower hybrid's productivity (Škorić et al., 2005) and is dependent on SY and OC. Nonadditive gene action in OY inheritance has been reported by Škorić et al. (2000), Laureti and Del Gatto (2001), Ortis (2005) and Gvozdenović (2006).

The objective of this study was to investigate GCA effects in new divergent inbred lines obtained by interspecific hybridization, SCA effects of the F_1 hybrids, gene effects, components of genetic variance, average percentage contributions of the lines, testers and their interactions to the expression of the six morphophysiological traits studied (PL, TLN, TLA, SY, OC, OY).

MATERIALS AND METHODS

Seven new divergent cms inbred lines (A) lines, three Rf restorers utilized as testers, and 21 F_1 hybrids developed at the Institute of Field and Vegetable Crops in Novi Sad were used in this study. The female inbreds (NS-GS-1, NS-GS-2, NS-GS-3, NS-GS-4, NS-GS-5, NS-GS-6, NS-GS-7) had been developed by interspecific hybridization. The male restorer lines (RHA-R-PL-2/1, RHA-N-49, RUS-RF-OL-168) with good combining abilities were used as testers in the form of fertility restorers. The F_1 hybrids had been developed by crossing each tester with each female inbred line. The trial was carried out at the Institute's Experiment Field at Rimski Šančevi.,There were three replications, and the experiment was designed according to the line x tester method. The lines and hybrids were planted manually at an optimum time on a well-prepared soil. The plots consisted of four rows with 12 plants in each. The row-to-row spacing was 70 cm and the plants were spaced at 30 cm intervals within the rows. Each trait was analyzed on a sample consisting of 30 plants (10 per replicate) taken from the middle rows in each block. PL (cm) was determined in the field at budding by measuring the 12th leaf. TLN and TLA (expressed as cm^2) were determined in the laboratory at flowering by counting the total number of leaves (dry and green) and using a device for measuring leaf area LI-300- Licor, respectively. SY was determined by measuring total seed quantity in each individual open pollinated plant using a scale with an accuracy of 0.01 g. OC was determined on an NMR at the chemical laboratory of the Institute's Oil Crops Department.

Mean values were calculated according to Hadživuković (1991). The mean values of the inbred lines and F_1 hybrids were used to calculate the values of the combining abilities and assess the gene effects for morphophysiological traits using the line x tester method (Singh and Choudhary, 1976).

RESULTS AND DISCUSSION

Significant differences were observed among the A lines, Rf testers and their F_1 hybrids for all the traits studied, indicating the presence of genetic differences among the genotypes concerned .

The mean values of the inbred lines and F_1 hybrids were used to calculate the combining abilities in order to estimate gene effects for all the morphophysiological traits under study. Analysis of the combining abilities showed that the A lines and Rf testers differed in their GCA.

Highly significant positive GCA values for PL were found in the female inbreds NS-GS-3, NS-GS-4, and NS-GS-5 and the male inbred RHA-N-49, so these lines can be regarded as good general combiners for this trait. Highly significant negative GCA effects and the lowest PL means were recorded in the female inbred lines NS-GS-6 and NS-GS-7. These lines, along with the male inbred RHA-R-PL-2/1, can be considered poor general combiners for PL. In the case of SCA, the hybrid combinations NS-GS-4xRHA-R-PL-2/1(4x8), NS-GS-7xRHA-R-PL-2/1(7x8), NS-GS-5xRHA-N-49(5x9), and NS-GS-4xRUS-RF-OL-168(4x10) had highly significant positive PL values in the F_1 generation, while NS-GS-2xRHA-R-PL-2/1(2x8), NS-GS-3xRHA-N-49(3x9) and NS-GS-6xRHA-N-49(6x9) had highly significant negative ones. The nonadditive component of genetic variance played the main role in the imheritance of PL, as shown by analysis of combining abilities and analysis of genetic variance components. This was further confirmed by the GCA/SCA ratio for PL in the F_1 generation, which was below the value of one (0.43) (Table 1). These findings are not in agreement with those of Marinković (1993) and Hladni et al. (2002), who reported a greater contribution of additive genes to the expression of PL.

The female inbreds NS-GS-1 and NS-GS-3 and the male tester RUS-RF-OL-168 had highly significant positive GCA values for TLN and can hence be considered good general combiners for this trait. The female inbreds NS-GS-6 and NS-GS-7 and the male inbred RHA-R-PL-2/1 had highly significant negative GCA effects and are therefore poor general combiners for TLN. The hybrids NS-GS-6xRHA-R-PL-2/1(6x8), NS-GS-1xRHA-N-49(1x9), and NS-GS-4xRUS-RF-OL-168(4x10) had highly significant SCA values for TLN. The main role in the inheritance of TLN was that of the nonadditive component of genetic variance, as confirmed by the GCA/SCA ratio being below one (0.40) in the F_1

generation (Table 1). Additive gene action in the inheritance of TLN has been reported by Hladni et al. (2000), whereas Nedeljković et al. (1992), Kumar et al. (1998), and Naik et al. (1999) found nonadditive gene action in the inheritance of this trait.

The most highly significant positive GCA effects for TLA were found in the female inbreds NS-GS-4, NS-GS-5 and NS-GS-7, while the lowest negative ones were recorded in NS-GS-1 and NS-GS-2. Among the Rf testers, RHA-R-PL-2/1 had the most pronounced positive GCA effect for TLA, while RUS-RF-OL-168 had the most pronounced negative one. The hybrids NS-GS-1xRHA-R-PL-2/1(1x8), NS-GS-5xRHA-R-PL-2/1(5x8), NS-GS-3xRHA-N-49(3x9), NS-GS-7xRHA-N-49(7x9), NS-GS-6xRUS-RF-OL-168(6x10), and NS-GS-7xRUS-RF-OL-168(7x10) had highly significant positive values of SCA for TLA, whereas NS-GS-7xRHA-R-PL-2/1(7x8), NS-GS-5xRHA-N-49(5x9) and NS-GS-5xRUS-RF-OL-168(5x10) had highly significant negative ones. In the inheritance of TLA, the dominant component of genetic variance predominated, as confirmed by a GCA/SCA ratio of less than one (0.07) (Table 1). These findings are in agreement with those of Kovačik and Škaloud (1990), Joksimović et al. (1997), Bath et al. (2000) and Hladni et al. (2003).

Highly significant positive GCA values for SY were found in the female inbreds NS-GS-4 and NS-GS-5, while the lines NS-GS-1 and NS-GS-2 had highly significant negative ones. The largest highly significant positive value of SCA for SY was found in the hybrid NS-GS-5xRHA-R-PL-2/1(5x8). The nonadditive component of genetic variance was more influential in the inheritance of SY, as confirmed by the GCA/SCA ratio being below one in the F_1 generation (0.08) (Table 1). Studies of the mode of inheritance of SY in sunflower have produced varying results. In agreement with the present findings, greater contribution of nonadditive genetic variance in the inheritance of this trait has been reported by Škorić et al. (2000), Hladni et al. (2002), and Gvozdenović (2006). Marinković et al. (2000) and Shekar et al. (2000), on the other hand, have reported greater contribution of additive genetic variance. Qingyu et al. (2002) has found equal contributions of the additive and nonadditive components of genetic variance in SY inheritance.

Highly significant positive values of GCA for OC were found in NS-GS-1 and NS-GS-2, while NS-GS-4 and NS-GS-5 had highly significant negative ones. The largest highly significant positive SCA value for OC was recorded in the hybrid NS-GS-6xRHA-R-PL-2/1(6x8), while the highest negative one was observed in NS-GS-5xRHA-R-PL-2/1(5x8). As evidenced by the GCA/SCA ratio being smaller than one (0.33), tab. 1, genes with nonadditive effects had more influence in the inheritance of OC, which is in agreement with the results of Marinković (1993) and Škorić et al. (2000).

The female inbred NS-GS-5 had the largest highly significant positive effect for OY and was deemed the best general combiner for the trait. Highly significant negative OY values were found in NS-GS-1, NS-GS-2, NS-GS-6, and NS-GS-7. The male inbred RHA-N-49 was the best general combiner for OY, as it had the largest positive GCA effect for this trait. The hybrid NS-GS-5xRHA-R-PL-2/1(5x8), which had the best SCA for OY, had been developed by crossing one parent with good GCA with one with poor GCA for the trait. Highly significant average values of SCA for OY were recorded in the hybrids NS-GS-1xRUS-RF-OL-168(1x10), NS-GS-6xRHA-N-49(6x9), and NS-GS-2xRHA-N-49(2x9). The nonadditive component of genetic variance was more significant in the inheritance OY, since the GCA/SCA ratio was below the value of one (0.07) (Table 1). These results are in agreement with Škorić et al. (2000) and Gvozdenović (2006).

The greatest average contribution to the expression of PL (49.9%), TLN (75.6%), TLA (57.1%), SY (61.5%), OC (77.3%), and OY (56.3%) was found in the female A lines. The contributions of the Rf testers and line x tester interaction were less significant (Table 2).

The differences in the findings of different authors referenced in the present paper can be attributed to the divergence of the material used in their studies. Identification of inbred lines with high positive GCA values is of great importance for the development of new productive sunflower hybrids. None of the inbred lines from the present study (either male or female) had a highly significant positive or negative effect for all six traits studied.

One of the goals of sunflower breeding is to decrease PL, so any line with a negative GCA value for this trait is considered desirable in a breeding program. In the present study, the lines NS-GS-6 and NS-GS-7 had the lowest highly significant negative values of PL and were rated the best general combiners for this trait. These findings are in agreement with those of Marinković (1982), who argues that in studying a particular trait advantage should be given to the line that is the best combiner for that particular trait regardless of whether the value is positive or negative, which depends on the direction in which selection for that trait goes.

Table 1. Values of GCA inbred lines and SCA hybrids for sunflower morphophysiological traits

No.	Parents and hybrids	PL	TLN	TLA	SY	OC	OY
	GCA values						
1	NS-GS-1	-0.921	0.659	-0.062	-10.053	3.56**	-1.221
2	NS-GS-2	-1.076	-0.486	-0.052	-12.439	2.38**	-3.468
3	NS-GS-3	2.096**	2.881**	-0.024	-0.072	0.15	0.466
4	NS-GS-4	1.435**	0.170	0.700**	3.521**	-2.067	-0.272
5	NS-GS-5	1.302**	0.014	0.157**	31.356**	-3.10	10.769**
6	NS-GS-6	-1.493	-2.297	-0.035	-9.021	-0.64	-4.700
7	NS-GS-7	-1.343	-0.941	0.113**	-3.292	-0.30	-1.574
8	RHA-R-PL-2/1	-1.589	-0.573	0.022**	0.273	-0.82	-1.070
9	RHA-N-49	1.511**	-0.259	-0.007	2.219**	0.49	1.758
10	RUS-RF-OL-168	0.078	0.832	-0.015	-2.492	0.33	-0.688
LSD (1-7) 5%		0.31	0.29	0.016	1.826	0.86	1.232
1%		0.46	0.43	0.024	2.739	1.29	1.848
LSD (8-10) 5%		0.20	0.19	0.010	1.196	0.56	0.806
1%		0.30	0.75	0.015	1.794	0.85	1.209
SCA values							
1	1x8	-0.506	-0.949	0.080**	-10.01	-0.60	-4.891
2	2x8	-1.300	-0.138	0.004	-5.01	0.31	-1.661
3	3x8	0.094	0.329	-0.039	-9.75	1.10**	-3.317
4	4x8	1.506**	-0.794	0.023	7.94**	-1.07	2.800*
5	5x8	-0.378	0.295	0.181**	31.88**	-1.95	11.188**
6	6x8	0.467*	1.173**	-0.095	-11.64	1.73**	-3.433
7	7x8	0.117**	0.084	-0.154	-3.41	0.52	-0.686
8	1x9	0.278	1.037**	-0.029	0.22	0.84	0.482
9	2x9	0.667*	0.548*	-0.036	7.75**	-0.02	3.592**
10	3x9	-0.706	-0.552	0.061**	4.60*	0.26	2.384*
11	4x9	-0.511	-0.175	-0.002	-10.80	0.91	-4.222
12	5x9	0.856**	-0.252	-0.120	-15.98	1.16**	-5.276
13	6x9	-0.600	-0.308	0.034*	12.10**	-1.53	3.816**
14	7x9	0.017	-0.297	0.092**	2.10	-1.63	-0.777
15	1x10	0.228	-0.087	-0.051	9.78**	-0.24	4.410**
16	2x10	0.633*	-0.410	0.031*	-2.74	-0.29	-1.932
17	3x10	0.611*	0.224	-0.022	5.15**	-1.32	0.933
18	4x10	0.994**	0.968**	-0.021	2.86	0.16	1.422
19	5x10	-0.478	-0.043	-0.061	-15.90	0.79*	-5.912
20	6x10	0.133	-0.865	0.061**	-0.46	-0.20	-0.384
21	7x10	-0.133	0.213	0.062**	1.31	1.12	1.463
LSD 5%		0.52	0.50	0.028	3.16	0.564	2,134
1%		0.78	0.75	0.042	4.74	0.846	3.201
GCA		0,30	0.21	66739.94	16.03	0.41	1,93
SCA		0,68	0,52	977433.14	201.15	1.23	26,02
GCA/SCA		**0.43**	**0.40**	**0.07**	**0.08**	**0.33**	**0.07**

PL – petiole length
TLN – total leaf number per plant
TLA – total leaf area per plant
SY – seed yield per plant
OC – seed oil content
OY – oil yield per plant

Table 2. Average percentage contributions of the female lines, testers and their interactions to the expression of sunflower morphophysiological traits.

Average contribution	PL	TLN	TLA	SY	OC	OY
	%	%	%	%	%	%
Female line	**49.89**	**75.63**	**57.06**	**61.51**	**77.29**	**56.33**
Tester line	39.51	12.65	1.83	1.20	5.65	4.01
Line x tester	10.61	11.71	41.12	37.29	17.06	39.66

If the goal is to change sunflower plant architecture, then the NS-GS-7 genotype with its highly significant negative GCA value for PL and highly significant positive GCA value for TLA is desirable in breeding programs. The line NS-GS-5 had highly significant positive effects for four traits (PL, TLA, SY, OY), while the line NS-GS-4 had these effects for three of the characters (PL, TLA, SY).

Based on the GCA values, selection was made of the lines with the best GCA for PL and TLN (NS-GS-3), TLA (NS-GS-4), SY and OY (NS-GS-5), and OC (NS-GS-1). Combining all the positive traits in a single hybrid combination is very difficult. The hybrid NS-GS-5xRHA-R-PL-2/1(5x8) had the best SCA for SY and OY and highest average values of those traits. Highly significant positive values of SY and OY were found in the hybrids NS-GS-6xRHA-N-49(6x9), NS-GS-2xRHA-N-49(2x9), and NS-GS-1xRUS-RF-OL-168(1x10).

The assessment of the combining abilities of the female line NS-GS-5 indicates that this line could justifiably be used as a parent in breeding for increased oil yield in sunflower. The hybrids NS-GS-5xRHA-R-PL-2/1(5x8), NS-GS-1xRUS-RF-OL-168(1x10), NS-GS-6xRHA-N-49(6x9), and NS-GS-2xRHA-N-49(2x9), obtained by interspecific hybridization, have been identified in the present study as promising because of their high SCA for OY, and, as such, should be subjected to further testing. The findings of this study can be used in the development of new sunflower hybrids with high oil yields based on interspecific hybridization.

CONCLUSIONS

Significant differences in the mean values of all the traits studied were observed among the inbred lines (females and testers) and the F_1 hybrids.

Analysis of the combining abilities showed that the A lines and Rf testers differed in their GCA and SCA for all the traits studied. The nonadditive component of genetic variance played the main role in the inheritance of all the traits, as evidenced by the GCA/SCA ratio being below the value of one. The greatest average contribution to the expression of all the traits was found in the female A lines, while the contributions of the Rf testers and line x tester interaction were less significant.

Evaluation of the combining abilities of the line NS-GS-5 suggests that this line could justifiably be used as a parent in breeding programs for increasing sunflower oil yields. The hybrids NS-GS-5xRHA-R-PL-2/1(5x8), NS-GS-1xRUS-RF-OL-168(1x10), NS-GS-6xRHA-N-49(6x9), and NS-GS-2xRHA-N-49(2x9), obtained by interspecific hybridization, have been identified as promising due to their high SCA for OY, and, as such, should be subjected to further tests in the future.

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Ichraq: Première variété de tournesol d'automne au Maroc

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RESUME

Traditionnellement, le tournesol est semé au printemps dans différentes régions du Maroc. Dès les années 80 du cycle dernier, un programme d'amélioration du tournesol visant la sélection de matériel génétique qui se sème en automne, tolérant le froid du début de cycle, a été initiée à l'Institut National de la Recherche Agronomique du Maroc (INRA). A partir de la variété population 'Salima', prise comme matériel de départ, une dizaine de cycles d'évaluation, sélection et épuration ont été réalisés. Comme résultat de tout ce travail, la première variété population dite de tournesol d'automne, adaptée aux semis précoces au Maroc, a été développée. Elle a été nommée Ichraq et inscrite au Catalogue Officiel marocain des variétés en 1999. C'est une variété tardive qui tolère le froid du début de cycle et se caractérise par un rendement grain et une teneur en huile élevés. Dans 9 environnements, les valeurs moyennes enregistrées pour ces 2 derniers caractères, respectivement, ont été de 28 q/ha et 44%. En semis conventionnel de printemps, et dans les 9 environnements, la variété parentale 'Salima' a produit un rendement grain de 15 q/ha et une teneur en huile de 43%. La variété 'Ichraq' est recommandée pour les semis d'automne ou d'hiver dans les régions à précipitations annuelles supérieures à 350 mm.

Mots clés: Maroc – semis précoce – tournesol d'automne – variété Ichraq

ABSTRACT

Traditionally, sunflower is sown at spring in different regions of Morocco. Early in the eighties of the last century, a specific sunflower improvement program was launched at the National Institute for Agricultural Research of Morocco (INRA). The objective was to select a genetic material which could be sown at autumn being cold tolerant in the early stages of the crop cycle, using 'Salima', a Moroccan population variety, as the initial germplasm. After several cycles of evaluation, selection and rouging, the first population variety of autumn sunflower, adapted to early sowing in Morocco, has been developed. This variety, named Ichraq, was released in 1999. Ichraq is late maturing, cold tolerant and combines good grain yield and high oil content. Across 9 environments, this variety exhibited a grain yield of 28 q/ha and an oil content of 44%. For 'Salima', the parental population, the obtained values in the same environments, but for a spring conventional sowing, were 15 q/ha and 43%, respectively. Ichraq variety is well adapted to autumn or winter sowing in areas where rainfall is more than 350 mm per year.

Key words: autumn sunflower – early sowing – Ichraq variety – Morocco.

INTRODUCTION

Sous climat méditerranéen, en culture pluviale, le rendement en grains est affecté significativement par les retards au semis (Andrade, 1995; Barros et al., 2004). De même, des variations de rendement plus importantes sont rapportées lors des semis tardifs (Bange et al., 1997). Au Maroc, le tournesol classique, cultivé en printemps a des limites de production sachant qu'il ne bénéficie pas des précipitations automnales et/ou hivernales et qu'il se trouve souvent exposé aux sécheresses et hautes températures de fin de cycle. Ces contraintes coïncident avec les périodes de floraison et de remplissage des graines qui sont critiques pour la détermination de la productivité en grains et de la teneur en huile (Ouattar et al., 1992). Sa culture reste souvent secondaire et est souvent considérée comme culture de rattrapage suite à des sécheresses ou des inondations de début de cycle affectant l'installation des cultures automnales, en particulier les céréales. Une des solutions envisagées pour promouvoir la culture du tournesol au Maroc était sa transformation en culture d'automne dans le but de surmonter les contraintes précitées et d'être, par la suite, une culture principale dans l'assolement de toute la zone qui lui soit favorable. Des études ont montré l'intérêt des semis précoces (en automne ou début d'hiver) dans l'amélioration de la productivité

en grains et la teneur en huile au Maroc (Boujghagh, 1993; Gosset et Vear, 1995; Aboudrare et al., 2000) et en Espagne (Gimeno et al., 1989).

Dès le début des années 80, l'INRA a commencé un programme de sélection de variétés populations dites d'automne. L'objectif de ce programme est de créer des variétés caractérisées par leur tolérance au froid en début de cycle de la culture. Ichraq est la première variété marocaine d'automne ou d'hiver développée par l'INRA et a été inscrite au Catalogue Officiel des variétés en 1999.

MATÉRIELS ET MÉTHODES

Matériel végétal de départ ou germoplasme

Le matériel de départ a été constitué de la variété population Salima, connue aussi sous le nom «Record-Maroc», obtenue en 1972 et inscrite au catalogue officiel en 1990 (Nabloussi et al., 2006). Elle est semi-tardive et elle a toujours été semée conventionnellement au printemps surtout dans la zone Gharb du Maroc.

Méthode de sélection et expérimentation

A partir de 1984 environ, des essais de semis d'automne de Salima ont été entamés dans la station expérimentale de l'INRA située à Annoceur en zone montagneuse, dans la province de Sefrou. L'objectif était de sélectionner un matériel génétique qui tolère le froid du début de cycle. Entre 1984 et 1988, c'était donc une période d'évaluation suivie de sélection. La méthode de sélection qui a été adoptée est la massale. Entre 1990 et 1993, des cycles de sélection et d'épuration supplémentaires ont été conduits afin d'écarter tous les hors-types du matériel préalablement sélectionné. Les essais de rendement concernant la matériel génétique définitivement sélectionné ont été conduits dans 9 environnements différents. Ces 9 environnements correspondent à 3 années d'évaluation au site Annoceur et 2 années d'évaluation aux sites Douyet (Province de Fès), Allal Tazi (Province de Kénitra) et Merchouch (Province de Khémisset). Deux témoins ont été considérés, la variété parentale Salima et la variété Karima, une autre population à cycle biologique plus court (Nabloussi et al., 2006).

RESULTATS ET DISCUSSION

La variété population Salima a montré une grande hétérogénéité pour les principaux critères de sélection, à savoir le rendement grain, la teneur en huile et la durée du cycle. A la fin des années 80, une population codée « INRA-89 » a été développée. Elle a montré une tolérance au froid et un cycle végétatif très long. De même, les essais de rendement ont prouvé sa grande performance et sa supériorité par rapport à d'autres populations sélectionnées dans les mêmes conditions. Néanmoins, il a été observé un certain niveau d'hétérogénéité chez INRA-89 dû à un mélange avec d'autres matériels génétiques. Enfin, en 1994, la population épurée et homogène dérivée de INRA-89 a été mise au point. Les résultats des essais d'évaluation de la valeur agronomique et technologique (VAT) de INRA-89, Salima et Karima sont rapportés dans le Tableau 1.

.

Tableau 1. Evaluation de la population INRA-89 de tournesol en comparaison avec d'autres obtentions (variétés populations) antérieures de l'INRA du Maroc évaluées dans 9 environnements

Variété	RDTG	HTRP	DUCY	DCA	TRH	PMG	PS	Mal. M/S
INRA-89	28.0	1.8	150-190	17.0	44.0	78.9	41.2	+/+
KARIMA	16.0	1.5	120	13.7	41.5	58.0	38.4	+/+
SALIMA	14.7	1.6	110-150	15.0	43.0	47.3	39.1	+/+

RDTG: rendement grain exprimé en quintaux par hectare; HTRP: hauteur moyenne de la plante en mètres; DUCY: durée du cycle exprimée en jours; DCA: diamètre moyen du capitule en centimètres; TRH: teneur en huile exprimé en % de matière sèche; PMG: poids de mille graines en grammes; PS: poids spécifique exprimé en Kilogrammes par Hectolitre; Mal. M/S: maladies M: Mildiou et S: Sclérotinia; +: réaction de susceptibilité.

Les résultats présentés dans le Tableau 1 montrent que INRA-89 est la meilleure population. La productivité de cette population adaptée aux semis d'automne dépasse de loin celle de la variété dont elle est dérivée, Salima, et celle de la variété précoce Karima. Le rendement grain moyen d'Ichraq obtenu dans 9 environnements, en conditions de semis précoces, est presque le double du rendement moyen de Salima, en conditions de semis conventionnels. INRA-89 se caractérise surtout par un poids de mille graines très élevé qui dépasse 78 g et un poids spécifique plus important que ceux des autres populations. Elle possède, par ailleurs, la hauteur de plante la plus élevée et le cycle végétatif le plus long. Cette

longueur du cycle permet à cette variété de bénéficier du maximum des précipitations de la campagne agricole. En plus, elle dépasse toutes les autres populations quant à la teneur en huile qui peut atteindre, dans certaines conditions, 47%. Dans les pires des cas, elle est de l'ordre de 42%. Cependant, et à l'image de toutes les autres variétés, INRA-89 est susceptible aux deux principales maladies du tournesol au Maroc, le mildiou et la sclérotinia.

Sur la base de ces résultats très encourageants, la population INRA-89 a été multipliée et par la suite déposée au Catalogue Officiel des variétés pour inscription. Après trois années d'évaluation agronomique et technologique (VAT) et des essais de distinction, homogénéité et stabilité (DHS) par le service concerné, cette population a été acceptée pour inscription en 1999 en tant que première variété de tournesol d'automne au Maroc. Elle a été dénommée, depuis lors, Ichraq. Les semences de pré-base sont produites et maintenues par l'Institut National de la Recherche Agronomique du Maroc. Expérimentée dans des conditions françaises (Toulouse), Ichraq a exprimé un grand potentiel concernant sa productivité en grains et sa teneur en huile. Ses performances ont été très comparables à celles de variétés hybrides françaises (Roche, 2005).

Dans les zones du Maroc où les précipitations annuelles moyennes dépassent 350 mm, Ichraq peut être semée tôt dès le mois d'octobre ou novembre. Elle est aussi adaptée aux semis précoces de saison au mois de janvier ou début février dans les principales zones de production du tournesol au Maroc, le Gharb, le Loukkos et le Saïs.

REMERCIEMENTS

Les auteurs tiennent à remercier l'ensemble du personnel des domaines expérimentaux de l'INRA (Annoceur, Douyet, Allal Tazi et Merchouch) pour leur contribution à cette obtention.

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Estimation of breeding potential for tocopherols and phytosterols in sunflower

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ABSTRACT

Sunflower (*Helianthus annuus* L.) oil is a good source of tocopherols and phytosterols, interesting bioactive molecules with beneficial health effects. The objective of this work was to determine the possibility of breeding sunflowers with improved oil quality for these traits. Seven B lines (females) and seven R lines (males) were crossed to obtain 49 F_1 hybrids progenies. The F_1 hybrids were then grown for two consecutive years (2005 and 2006) on six locations / year. General combining abilities (GCA) for total tocopherol content were higher than for the total phytosterol content. In both B and R parental lines, there was a positive correlation between the parental lines values and their GCA for total tocopherol content, suggesting additive effects and the possibility of genetic gain for this parameter. This was not the case for total phytosterol content, which displayed important year effects on GCA. The specific combining abilities (SCA) were very important, particularly the negative ones, for the total tocopherol content indicating that some dominance effects could change the predicted hybrid performance. The results of this study indicate the existence of genetic variance for total tocopherol content, but phytosterol content variability is lower that that of tocopherols and subjected to year interactions.

Key words: breeding – GCA– phytosterols – tocopherols – sunflower.

INTRODUCTION

Several studies have shown that tocopherols and sterols can have many positive health effects: they prevent cancer (Bramley et al., 2000), reduce blood cholesterol level (von Bergmann et al., 2005; Patel and Thompson, 2006) and they are effective antioxidants (Niki and Noguchi, 2004). An increasing interest in such active molecules has promoted research in the natural sources of these substances. Tocopherols are lipid antioxidants, vitamin E-active substances, with four isomers (α-, β-, γ-, and δ-tocopherol) with species-dependent proportions. Naturally, sunflower oil has more than 95% of α-tocopherol (Ayerdi Gotor et al., 2006a), the most efficient Vitamin E bioactive tocopherol homologue. In literature, references can be found on sunflower tocopherol mutants with a high content in β-, δ-, or γ-tocopherol (Velasco et al., 2004a,b; Demurin et al., 2007). The total content can vary between 300 to 1873 $mg \cdot kg^{-1}$ of oil (Demurin et al., 1996; Velasco et al., 2002; Nolasco et al., 2006). Phytosterol content in sunflower oil varies from 200 to 700 $mg \cdot 100g^{-1}$ of oil (Vlahakis and Hazebroek, 2000; Ayerdi Gotor et al., 2007) and β-sitosterol is the major form (40-60%).

Genotype as well as environment can influence the total tocopherol content in sunflower oil. Temperature is one of the most influential environmental factors (Velasco et al., 2002; Ayerdi Gotor et al., 2006b; Nolasco et al., 2006). Phytosterols content is less influenced by genotype or by environmental factors (Ayerdi Gotor et al., 2006b; Roche et al., 2006).

In spite of the growing importance of tocopherols and phytosterols as micronutrients and as natural oil stabilizers, few studies have focused on breeding programs for these minor components in oilcrops, especially on sunflower. It has been shown in rapeseed that genetic progress is possible for the oil tocopherol content (Goffman and Becker, 2001a,b). In these studies, tocopherol content and composition inheritances were highly associated with additive gene action.

Breeding for tocopherol and phytosterol contents can increase the market value of sunflower oil by means of health-promoting effects associated with these nutriments. The objective of the present study was to determine the feasibility of breeding for these molecules in sunflower oil.

MATERIALS AND METHODS

Plant material

Seven restorers (males) and seven females (cytoplasmic male sterile) parental lines of sunflower, *Helianthus annuus* L., were selected for their high and low tocopherol and phytosterol content. The F1 hybrids seeds were produced in a 7 X 7 factorial design (NCII). Crosses were made in Chile during winter 2005. Three of the 49 hybrids formed were not viable and produced no seed. The fourteen parental lines were provided by six sunflower breeders: Caussade semences, Maïsadour semences, Monsanto Dekalb SAS, RAGT-R2n, Soltis and Syngenta seeds.

Field trials

The progenies (from grains F_1) were cultivated in the summers of 2005 and 2006 in six different places throughout France (Table 1), with two blocks in each place. Hybrids were randomized in the blocks to limit the effect of interactions between plants. Just before flowering, the buds were covered with microperforated bags to ensure self-pollinated achenes; these bags were taken away at the end of flowering. F_2 achenes from F_1 plants were collected at maturity, the lab samples (for analysis) were made with 5 plants from the same plot.

Table 1. F_2 hybrid growing places in 2005 and 2006.

Breeder company	Place (French department)	Geographical location
Caussade semences	Cayrac (81)	44º6'N 1º28'E
Maïsadour semences	Conan (41)	47º48'N 1º15'E
Monsanto Dekalb SAS	Savenès (82)	43º49' N 1º11'E
RAGT-R2n	Villampuy (28)	48º2'N 1º30'E
Soltis	Mondonville (31)	43º40'N 1º17'E
Syngenta seeds	Saint sauveur (31)	43º45'N 1º24'E

Chemical analysis

Oil extraction

Grains were ground in a sample mill (KnifeTec 1095; Foss Tecator AB, Sweden) for 2 periods of 10 s. Around 15 g of ground seeds were placed in a 33ml cartridge with Fointainebleu sand for extraction in an accelerated solvent extractor apparatus (ASE-200, Dionex, France) with the following extraction conditions: 120ºC, 10 min of static extraction, 95% Hexane (n-hexane Prolabo-Subra, France) and 5% Propanol-2 (HPLC grade, SDS, France) under a pressure of 100 bar. Oil was recovered after solvent evaporation under low pressure with a rotavapor (HS 40 Huber, Bioblock Scientific, Heildolph, Germany). Lipid extracts were weighed and conserved at –18°C to minimize oxidative reactions before analysis.

Tocopherol determination

Complete separation of all native tocopherols was achieved using high-performance liquid chromatography (HPLC) (SpectraPhysics; TSP, USA) (ISO 9936, 1997). A normal-phase LiChrosorb Si60 column was used. The mobile phase was hexane/isopropanol (99.7:0.3 v/v) and the solvent flow was 1 mL/min. One gram of oil sample was diluted in 25 mL of hexane and 20 µL were injected. Tocopherols were identified by comparison of retention times with their respective standards (Tocopherol Kit; ChromaDex, USA). Total tocopherol content was calculated as the sum of α- , β-, γ- and δ-tocopherol contents and expressed in mg kg^{-1}oil.

Phytosterol determination

The analyses of sterol required a saponification with KOH 0.5M and a purification on an aluminium oxide basic (Panreac, Spain) column. The total and the individual sterol contents were analyzed by GC, after silylation with trimethylsylil (TMS) ether derivatives. 1µl of the TMS solutions were injected on a silica capillary column (ZB-5) in a gas chromatograph (Clarus 600, Perkin Elmer, USA) fitted with a flame ionization detector. Sterols were identified by their retention time relative to betulin internal standard. They were quantified using the ratio obtained between betulin (Internal standard, Sigma-Aldrich, France) and sterol standards. Sterols were expressed in mg 100 g^{-1} oil (NF EN ISO 12228, 1999).

RESULTS AND DISCUSSION

Total tocopherol content in the parental lines varied between 548.0 to 1096.4 mg·kg^{-1} oil. Total phytosterol content varied between 260.7 to 455.7 mg 100g^{-1} oil. Mean values of F_2 seeds of the 6 growing places and the two years are in Table 2.

Table 2. Mean values, standard deviation (SD) and range of tocopherol and phytosterol total contents for the 6 locations in 2005 and 2006 of the 46 F1 hybrids

Minor component	2005			2006		
	Mean	SD	Range	Mean	SD	Range
α-tocopherol (mg·kg^{-1} oil)	452.3	52.6	346.9- 570.2	516.5	65.0	390.4-679.6
Total tocopherol (mg·kg^{-1} oil)	469.8	55.8	354.8- 590.0	469.8	76.8	427.7- 733.9
β-sitosterol (mg·100g^{-1} oil)	215.7	14.3	176.4- 241.7	227.5	23.4	183.6- 317.1
Total phytosterol (mg·100g^{-1} oil)	315.7	20.6	254.7-366.4	319.6	27.6	262.2- 398.8

The general combining ability (GCA) for each parent was calculated as the difference between the mean of its half sib offsprings and the mean of its overall hybrids, which was calculated separately for each growing place. A mean GCA was then calculated over the six places. The correlations between parental line values and the corresponding mean GCA of their offsprings are shown Fig. 1. GCA for the total tocopherol content was greater than for total phytosterol content, in accordance with the fact that phytosterol variability is less important than that of tocopherols (Ayerdi Gotor et al., 2006b).

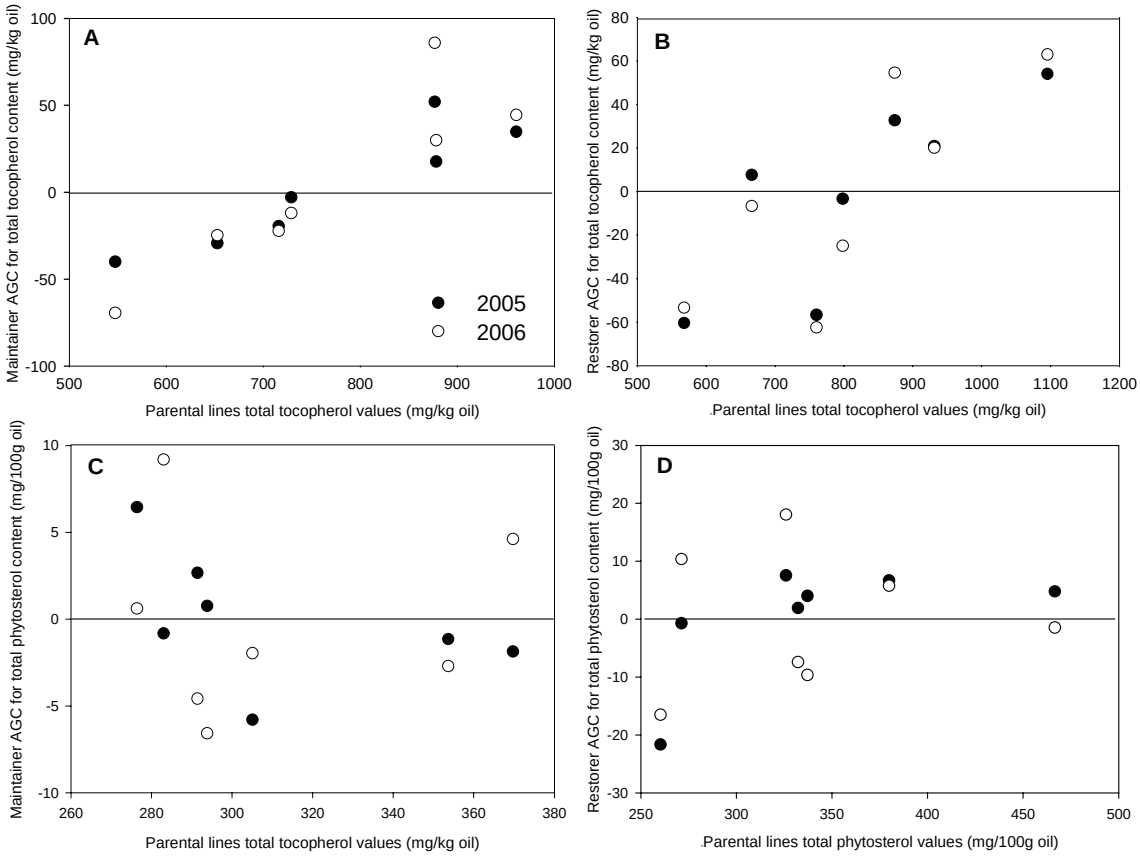

Fig. 1. Correlations between parental values and general combining ability (GCA) in 2005 and 2006. A - B: total tocopherol contents (mg/kg oil), female lines and restorer lines, respectively; C - D: total phytosterol contents (mg/100g oil), female lines and male lines, respectively.

For the total tocopherol content, the GCA and parental values were positively correlated for both female and restorer lines. The tendency was the same in the two years. Such correlations were not observed for the total phytosterol content, which also showed an important year effect.

For each location by year, the specific combining ability (SCA) was calculated as differences between a given hybrid mean and its two parental half sib means deviations from the general mean. Then the means were given by year over the six locations.

Table 3. Range of specific combining ability (SCA) of the 49 hybrids for 2005 and 2006

Year	SCA total tocopherol content (mg/kg oil)		SCA total phytosterol content (mg/100g oil)	
	Maximum	Minimum	Maximum	Minimum
2005	40.25	-52.84	58.94	-29.25
2006	57.18	-117.92	56.49	-48.32

SCA range was larger in 2006 than in 2005 for total tocopherol content. On the contrary, the SCA range showed a lesser year effect for total phytosterol content. Negative SCA for phytosterol and tocopherol content were of a greater amplitude in 2006.

New statistical treatment is currently under development to improve the accuracy of the genetic parameters obtained with these data. This work will soon be completed with heritability information from data of the F3-F4 hybrid seeds from three F2 families selected for their F1 highest GCA (negative or positive, female or restorer) grown during summer, 2007.

These first results suggested that total tocopherol content is influenced by an additive effect showing the possibility of a genetic gain for this parameter. For the total phytosterol content there was a larger year effect on GCA, so genetic gain could be less important. The SCA values indicated that total tocopherol and phytosterol contents could be affected by dominance effects, which could change the predicted hybrid performance. Both families of minor components were subjected to year interactions. These results open up possibilities for breeders to improve sunflower composition by increasing the content of these interesting compounds for human health.

ACKNOWLEDGEMENTS

The authors thank Caussade Semences, Maïsadour Semences, Monsanto Dekalb SAS, RAGT-R2n, Soltis, Syngenta Seeds for field experiments and supply of sunflower plant material. Financial support was provided by ASEDIS-SO, ONIDOL (French oilseeds organization) and Midi-Pyrenees Region. We also thank Cognis-France for their technical support.

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Studies on general and specific combining abilities in sunflower

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ABSTRACT

Understanding the genetic basis and mode of gene(s) action for grain yield and important agronomic traits of sunflower would facilitate the improvement of sunflower production in Iran. The objectives of this study were to determine the mode of gene action, the general combining ability (GCA) and specific combining ability (SCA) for seed yield and important agronomic traits in the F_1 generation of Iranian sunflower hybrids. An experiment with four male sterile testers, four Rf-lines, and their 16 F_1 hybrids were carried out at the Experimental Field of Seed and Plant Improvement Institute (SPII) at Karaj, Iran in 2007. The trials were arranged in a randomized complete block design with two replicates. Results showed that variance due to the GCA of the males was significant for plant height, 1000 seed weight, oil content and vegetation period characteristics. Variance due to the general combining ability of the females was significant for vegetation period, plant height, oil content and seed yield. Highly significant positive or negative effects were recorded for agronomic characters in several hybrid combinations. The GCA/SCA ratio in the F_1 generation was lower than 1, indicating that the non-additive component of genetic variance (dominance and epistasis) made a larger contribution to total genetic variance for seed yield than the additive one. The contribution of the non-additive component of genetic variance in the expression of seed oil content was higher than that of the additive one. Understanding the genetic control of traits in newly released hybrids with good agronomic characteristics would facilitate the efficiency of sunflower breeding programs.

Key words: GCA – gene effect – inheritance – SCA – sunflower.

INTRODUCTION

A sunflower breeding program was started during late 1970's at the Oil Crops Research Department of Seed and Plant improvement Institute (SPII), with the cooperation of the Field and Vegetable Crops Institute at Novi Sad, Serbia. Some sunflower hybrids have been introduced by the Oil Crops Research Department at SPII during the last forty years. Now, we are going to release the new generation of Iranian sunflower hybrids. A large number of researchers have studied the general (GCA) and specific combining abilities (SCA) of agronomic traits, mode of gene action and the inheritance of important traits, as reviewed and summarized by Škorić et al. (2007).

MATERIALS AND METHODS

Plant materials consisted of four new Rf-lines **(**RF81-154/2, RF81-R27/2, RF81-R125/1 and RF81-R131/1) and and four male sterile A-lines (AF81-48, AF81-166, AF81-226 and AF81-222). Hybrid combinations of the A-sterile lines and the Rf testers were produced under cages to obtain genetically pure material.

Comparative trials with four A-sterile lines, four Rf testers, and their 16 F_1 hybrids were carried out at the Experimental Field of the Seed and Plant Improvement Institute (SPII) at Karaj, Iran in 2007. The trials were arranged with a randomized complete block design in two replicates with a net plot of 40 plants. All observations and measurements were made during the growing season of 2007.

Analysis of variance for grain yield of parents and F_1 hybrids was performed according to the analysis of the combining abilities using the line x tester method suggested by Singh and Chaudhary (1976).

RESULTS AND DISCUSSION

Analysis of variance showed that the genotypes differed significantly for all the characters (P<0.01) (Table 1). The mean squares due to parents also differed significantly (P<0.01), indicating a great deal of diversity among them. Highly significant differences (P<0.01) were also observed for the variance components, viz. parents vs. crosses for all characters. These results showed the expression of heterosis for above mentioned characters. The variance due to the GCA of the males was highly significant for growing period, plant height, 1000 seed weight and oil content. Variance due to the GCA of females was highly significant (P<0.01) for vegetation period, plant height and oil content. Lines x testers effects were non significant for all of the characters except for oil content, which was significantly different (P<0.05).

Table 1. Analysis of variance for combining ability on different traits

Sources of variation	df	Mean squares				
		Growing period	Plant height	1000 seed weight	Seed yield	Oil content
Replications	1	3.056ns	91.230ns	117.480 *	0.840ns	0.565 ns
Genotypes	23	159.827**	1151.569**	314.448 **	2.158 **	15.498 **
Parents	7	137.252**	1129.110**	651.422 **	1.978 **	17.314 **
Parents vs. crosses	1	153.495**	8497.568**	1321.837 **	26.131 **	48.741 **
Crosses	15	170.785**	672.317**	90.035 **	0.644*	12.434 **
Lines	3	68.886**	1845.128**	282.759 **	0.608 ns	23.607 **
Testers	3	742.382**	980.135**	76.123 **	0.916 ns	29.511 **
Lines x Testers	9	14.219ns	178.775ns	30.430ns	0.565 ns	3.017 *
Error	23	15.109	200.875	27.207	0.274	1.118

ns,*, ** Nonsignificant, significant at 5% and 1 % level, respectively

Among the A lines (females), the shortest plant height was observed in AF81-166 (131.18 cm) and the tallest in AF81-266 (165.29 cm). Of the Rf testers (males), RF81-27.2 was the shortest (131.50 cm) and RF81-154/2 the tallest one (156.49 cm). Regarding seed yield, the lowest-yielding A-line was AF81-166 with 2.656 t/ha, while the high-yielding one was AF81-48 with 3.255 t/ha. Among the Rf testers, RF81-R27/2 had the lowest and RF81-125/1 the highest seed yield (2.700 and 3.503 t/ha, respectively). The lowest seed oil content among the A lines was found in AF81-226 (39.88%) and the highest in AF81-222 (43.63%). Among the Rf testers, the lowest value was recorded in RF81-27/2 (40.42%) and the highest in RF81- R131/1 (44.11%).

Analysis of the combining abilities for plant height revealed significant differences (P<0.05) between the Rf-lines (males) and A lines (females) with respect to GCA. The most prominent negative effect of the GCA for plant height was found in the Rf-lines RF81-R27/2, while the most prominent positive effect was observed in RF81-154/2. In the A lines, the most prominent negative and positive effects of the GCA for this trait were recorded in AF81-166 and AF81-226, respectively (Table 2).

Table 2. Value and general combining ability effects of the parents on different traits

	Growing period		Plant height		1000 seed weight		Seed yield		Oil content	
	Days	GCA	cm	GCA	gr	GCA	t/ha	GCA	%	GCA
Lines										
A F81-48	106.35	4.320	139.81	-7.902	56.77	-4.505	3.255	0.189	43.31	0.898
A F81-166	101.37	-0.653	131.18	-16.537	57.61	-3.664	2.656	-0.409	42.81	0.406
A F81-226	100.09	-1.941	165.29	17.575	69.77	8.498	3.164	0.099	39.88	-2.527
A F81-222	100.30	-1.725	154.58	6.864	60.95	-0.329	3.187	0.122	43.63	1.223
Testers										
RF81-154/2	106.16	4.135	156.49	8.772	64.37	3.097	3.118	0.053	41.10	-1.305
RF81-R27/2	87.62	-14.408	131.50	-16.214	63.48	2.206	2.700	-0.365	40.42	-1.986
RF81-R125/1	106.47	4.447	151.32	3.604	58.73	-2.550	3.503	0.438	44.11	1.698
RF81- R131/1	107.85	5.825	151.55	3.837	58.52	-2.753	2.940	-0.125	44.00	1.593
SE GCA / lines and testers		1.374		5.011		1.844		0.185		0.374
SE (Gi-Gj) / lines and testers		1.943		7.087		2.608		0.262		0.529

As the number of testers and lines were equal, SE for GCA and (Gi-Gj) were the same for both lines and testers.

Regarding the SCA for plant height, highly significant (P<0.01) positive effects were recorded in several hybrid combinations, most notably in AF81-48 x RF81-125/1 and AF81-222 x RF81-27/2. Highly significant (P<0.01) negative values of the SCA for plant height were also found in several combinations, most notably in AF81-226 x RF81-27/2 and AF81-48 x RF81-125/1. The GCA/SCA ratio for plant height was less than 1, namely 0.328 (Table 4). These results were in agreement with those of Škorić et al. (2000), although not in full agreement with Tiagi (1988), Mihaljevic (1988) and Ortegon et al. (1992), who reported an equal effect for GCA and SCA in plant height. On average, the female parents made the largest contribution (54.89.8%) to the expression of plant height in the F_1 hybrids. The contributions of the Rf-lines and line x tester interactions were less significant (Table 5).

Table 3. Mean value and specific combining ability effects of the crosses on different traits

	Testers	RF81-154/2		RF81-R27/2		RF81-R125/1		RF81- R131/1	
	lines	value	SCA	value	SCA	value	SCA	value	SCA
Growing period (Days)	A F81-48	113	2.40	93	1.04	109	-1.7	110	-1.74
	A F81-166	106	0.10	88	0.75	104	-1.86	108	1.02
	A F81-226	100	-3.98	86	-0.09	105	0.82	109	3.25
	A F81-222	106	1.49	84	-1.71	108	2.74	104	-2.53
	SE for SCA				2.749				
	SE(Sgi-Sgj)				3.887				
Plant height (cm)	AF81-48	139.64	-8.94	125.51	1.91	157.79	14.38	136.31	-7.34
	AF81-166	144.22	4.27	113.01	-1.96	129.95	-4.84	137.54	2.52
	AF81-226	181.64	7.58	138.46	-10.62	169.55	0.66	171.51	2.38
	AF81-222	160.44	-2.91	149.03	10.67	147.99	-10.19	160.86	2.44
	SE for SCA				10.022				
	SE(Sgi-Sgj)				14.173				
1000 seed weight (gr)	AF81-48	59.68	-0.18	59.14	0.17	56.56	2.34	51.70	-2.32
	AF81-166	55.85	-4.86	60.11	0.29	59.63	4.57	54.86	0.00
	AF81-226	75.80	2.92	71.53	-0.45	66.91	-0.32	64.87	-2.15
	AF81-222	66.17	2.12	63.15	-0.01	51.81	-6.59	62.67	4.47
	SE for SCA				3.688				
	SE(Sgi-Sgj)				5.216				
Seed yield (t/ha)	AF81-48	3.334	0.03	2.254	-0.64	4.524	0.83	2.907	-0.22
	AF81-166	2.370	-0.34	2.318	0.03	2.846	-0.25	3.090	0.56
	AF81-226	3.493	0.28	2.695	-0.10	3.422	-0.18	3.045	0.01
	AF81-222	3.275	0.04	3.534	0.71	3.222	-0.40	2.717	-0.34
	SE for SCA				0.370				
	SE(Sgi-Sgj)				0.523				
Oil content (%)	AF81-48	40.83	-1.17	42.12	0.80	45.09	0.09	45.18	1.08
	AF81-166	40.99	-0.52	42.37	1.54	43.48	-1.03	44.42	0.58
	AF81-226	38.37	-0.20	37.14	-0.76	42.57	1.00	41.44	-2.54
	AF81-222	44.22	1.89	40.06	-1.59	45.27	-0.05	44.97	0.88
	SE for SCA				0.748				
	SE(Sgi-Sgj)				1.057				

The mean squares of treatments, parents, parents vs. crosses in the variance analysis for seed yield were highly significant (P<0.01) and for crosses they were significant (P<0.05). Highly significant (P<0.01) positive effects of the GCA for seed yield (t/ha) were observed in the A line AF81-48 and the Rf line RF81-125/1. Highly negative (P<0.01) effects for seed yield were found in lines AF81-166 and RF81-27/2 (Table 2). Results of analysis of the SCA effects of the inbred lines in the F_1 hybrids showed that the highest positive values for seed yield were achieved by the following combinations: AF81-48 x RF81-125/1 and AF81-222 x RF81-27/2 (Table 3). The highest negative values of the SCA for seed yield were recorded in the combination AF81-48 x RF81-27/2. The GCA/SCA ratio for this trait in the F_1 generation was lower than 1 (0.01), indicating that the non additive component of genetic variance (dominance and epistasis) made a larger contribution to total genetic variance for seed yield than the additive one (Table 4). A similar relationship was established in the studies of Škorić et al. (2000), Sindagi et al. (1996) and Farrokhi (2003). The average contribution of the testers, lines and lines x testers was 16.91%, 62.81%, and 20.28%, respectively (Table 5).

The highest positive GCA value for seed oil content was achieved by the lines RF51-125/1 and RF81-131/1. The highest negative values were those of AF81-226 and RF81-27/2 (Table 2). The highest positive SCA values for seed oil content were recorded in AF81-222 x RF81-154/2 and AF81-166 x RF81-27/2, while the highest negative ones were found in the combinations RF81-131/1 x AF81-226 and RF81-27/2 x AF81-228. The GCA/SCA ratio for seed oil content was less than 1, namely 0.2 (Table 4). Also, the other components of genetic variance showed that the contribution of the non additive component of genetic variance in the expression of this trait was higher than that of the additive one. The average contribution of the tester lines to the expression of seed oil content was 28.46% (Table 5). The same results were reported in oil content by Škorić et al. (2000), but the results of Farrokhi (2003) showed additive gene effects.

Table 4. Components of genetic variance for different traits

		VA	VD	VD/VA	GCA	SCA	GCA/SCA
Growing period	F = 0	32.618	6.664	0.204	8.154	6.664	1.224
	F = 1	16.309	13.328	0.817			
Plant height	F = 0	102.821	78.337	0.762	25.705	78.337	0.328
	F = 1	51.411	156.674	3.047			
1000 seed weight	F = 0	12.418	16.827	1.355	3.104	16.827	0.184
	F = 1	6.209	33.654	5.420			
Seed yield	F = 0	0.016	0.428	26.750	0.004	0.428	0.010
	F = 1	0.008	0.857	107.125			
Oil content	F = 0	1.962	2.458	1.253	0.490	2.458	0.200
	F = 1	0.981	4.917	5.012			

The highest positive value of the GCA for vegetation period was found in RF81-131/1 and AF81-48. Significant ($P<0.05$) negative GCA values for this trait were found in RF81-27/2 (Table 2). The combination AF81-226 x RF81-131/1 had the highest positive and AF81-226 x RF81-154/2 the highest negative value of the SCA for this trait (Table 3). The GCA/SCA ratio for vegetation period was higher than 1, meaning that additive gene action played a considerably more important role in the inheritance of vegetation period than non additive gene action (Table 4). The average contributions to the expression of the vegetation period were as follows: tester lines 86.94%, A lines 8.07%, and tester x lines 5% (Table 5).

Table 5. Average contributions (%) of the lines, testers, and their interactions to the expression of different traits

Average Contribution	Growing period	Plant height	1000 seed weight	Seed yield	Oil content
Lines	8.07	54.89	22.84	62.81	18.88
Testers	86.94	29.16	12.93	16.91	28.46
Lines x testers	5.00	15.95	64.23	20.28	52.67

ACKNOWLEDGEMENTS

The financial support from Seed and Plant Improvement is highly appreciated. We would like to thank Dr. Bahram Alizadeh, Dr. Mohammadreza Ahmadi and Dr. Alireza Nabipour for their technical and editorial assistance.

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Heredabilidad de componentes de rendimiento en dos poblaciones de girasol de la EEA Pergamino

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RESUMEN

La expresión de la variabilidad es causada por la variación genética determinada por el genotipo de los individuos de una población y por la variación ambiental que influye en la expresión de un carácter. La heredabilidad indica la proporción de la variabilidad total de origen genético. Se analizó la variabilidad y la heredabilidad en familias S_1 de dos poblaciones de diferente fondo genético: P3 de origen local con genes de girasol silvestre, tardío con un ciclo promedio a floración de 71días, y P6 de origen americano, precoz de 65 días de ciclo a floración y de alto aceite. Durante la campaña 2006/07, se sembraron las progenies S_1 en un diseño de bloques al azar con tres repeticiones. Se analizaron cuatro caracteres: diámetro de capítulo (cm), peso de cien aquenios (g), número de aquenios por capítulo y contenido de aceite (%). La variancia ambiental tuvo la mayor incidencia en número de aquenios por capítulo tanto en P3 como en P6; por otra parte la menor incidencia se obtuvo en peso de 100 aquenios en P3 y en diámetro de capítulo y porcentaje de aceite en P6. Se esperarían buenos resultados en la selección por porcentaje de aceite teniendo en cuenta los altos valores de heredabilidad obtenidos para este carácter. En las poblaciones estudiadas existe variabilidad para ser explorada con altas probabilidades de lograr progresos por la aplicación de métodos de selección.

Palabras clave: componentes de rendimiento - girasol – heredabilidad - variabilidad.

ABSTRACT

Variability is caused by the genetic variation determined by the genotype of the individuals of a population and by the environmental variation that influences the expression of a character. Heritability indicates the proportion of the total variability of genetic origin. The variability and the heritability in S_1 families of two populations from different backgrounds were analyzed: P3 of local origin with genes of wild sunflower and with a cycle of 71 days average to flowering, and P6 of American origin, with a cycle of 65 days to flowering and high oil. During 2006/07, the S_1 families were seeded at random in a block design with three repetitions. Four characters were analyzed: diameter of head (cm), weight of 100 seeds (g), number of seeds per head and oil content (%). The environmental variance had a higher incidence in number of seeds per head in both P3 and P6; on the other hand, a lower incidence was obtained in weight of 100 seeds in P3, and in diameter of chapter and percentage of oil in P6. Considering the high values of heritability obtained for percentage of oil, good results are to be expected in the selection for this trait. In addition, in the studied populations there is a variability which should be explored as there are high probabilities of obtaining some progress by applying selection methods.

Key words: heritability – sunflower – variability – yield components.

INTRODUCCIÓN

El desarrollo de poblaciones de base genética amplia como fuente de extracción de líneas, contribuiría a aumentar la variabilidad genética para la obtención de híbridos comerciales (Harvey, 1977).

El mejoramiento de poblaciones constituye una valiosa herramienta para el aumento de la ganancia genética en caracteres de interés agronómico (Alvarez et al., 1992). El conocimiento de la misma permite aplicar el método de selección adecuado para aumentar la frecuencia de genotipos superiores.

La expresión de la variabilidad es causada por la variación genética determinada por el genotipo de los individuos de una población y por la variación ambiental que influye en la expresión de un carácter. La heredabilidad indica la proporción de la variabilidad total de origen genético (Allard, 1980). Las estimaciones de este y otros parámetros genéticos son herramientas para determinar el mejor método de selección para caracteres específicos (Alvarez et al., 1996).

Ortegón y Escobedo (1995), en un estudio sobre cinco líneas de girasol, encontraron baja variabilidad genética y baja heredabilidad para los caracteres de rendimiento de grano y aceite. Por otra parte, informaron que altura de planta, período vegetativo y peso de 100 semillas tuvieron mayor influencia sobre el rendimiento.

Goksksoy et al. (2002) encontraron valores significativos de heredabilidad para diámetro de capítulo, peso de 1000 semillas y rendimiento de semilla por planta. Mishra et al. (2003), en un estudio sobre diversos caracteres, encontraron los mayores valores de heredabilidad para altura de planta, peso de 100 semillas y número de hojas por planta. Waseem et al. (2004), en un estudio sobre dos poblaciones, obtuvieron altos valores de heredabilidad para diámetro de capítulo, número de aquenios por capítulo, peso de aquenio y porcentaje de aceite, entre otros caracteres.

El objetivo del trabajo fue analizar variabilidad y evaluar la heredabilidad de cuatro caracteres de interés agronómico en familias S_1 elegidas al azar de dos poblaciones de libre polinización de la EEA Pergamino.

MATERIALES Y MÉTODOS

En la campaña 2005/06, se autofecundaron 100 plantas en el compuesto P3 (COMANGIR) de origen local con genes de girasol silvestre, tardío y un ciclo promedio a floración de 71 días. Un número similar fue autofecundado en el compuesto P6 de origen americano, precoz y de alto aceite de 65 días de ciclo a floración; ambos de la EEA Pergamino INTA.

Durante la campaña 2006/07, se sembraron las progenies S_1 en la EEA Pergamino en un diseño de bloques al azar con tres repeticiones. La parcela experimental fue de un surco de 6 metros de largo por 0.7 metros entre hileras. Se midieron cuatro caracteres: diámetro de capítulo (cm), peso de cien aquenios (g), número de aquenios por capítulo y contenido de aceite (%).

Se utilizó el paquete estadístico del S.A.S para el cálculo de los parámetros estadísticos: cuadrado medio entre familias S_1, cuadrado medio error, promedio, rango y coeficiente de variación.

Se estimó la variancia genética y ambiental, y se calculó la heredabilidad.
Las fórmulas empleadas fueron las siguientes:

Variancia genética = (Cuadrado medio familia – Cuadrado medio error) / repetición

Variancia fenotípica = Cuadrado medio familias S_1 / repetición

Variancia ambiental = Variancia fenotípica – Variancia genética

Heredabilidad (sentido amplio) = Variancia genética / Variancia fenotípica

RESULTADOS

En la Tabla 1 se presentan los parámetros estadísticos de los cuatro caracteres medidos en las compuesto P3 y P6. Los cuadrados medios entre familias S_1, utilizados en la estimación de las variancias genéticas, fueron estadísticamente significativos al 1% de probabilidad, indicando el grado de diferencia entre plantas derivadas de distintos individuos. El compuesto P3 tuvo en promedio mayor diámetro de capitulo, número y peso de aquenios, mientras que el P6 tuvo mayor porcentaje de aceite.

Tabla 1. Cuadrados medios entre familias, error, medias, rango y coeficiente de variación en familias S_1 de las poblaciones P3 y P6

	P3				P6			
	Diámetro de capitulo (cm)	Número de Aquenios	Peso 100 Aquenios (g)	Contenido de Aceite (%)	Diámetro de capitulo (cm)	Número de Aquenios	Peso 100 Aquenios (g)	Contenido de Aceite (%)
CM familia	14.082**	105461**	1.7552**	58.169**	12.023**	64156 **	1.810**	54.142 **
CM Error	4.752	45482	0.457	18.433	3.540	23867	0.594	16.00
Media	17.2	569	4.4	36.0	16.3	428	3.9	43.0
Rango	10-28	121-1680	1.8-7.2	14.4-48.7	7.5-25.0	82-1000	1.8-7.0	23.1-56.9
C.V. (%)	6.5	45.6	22.1	16.0	15.8	46.6	26.3	12.9

**Probabilidad al 1%

Los cuadrados medios del error fueron de similar magnitud para los caracteres evaluados, siendo el de número de aquenios extremadamente grande, reflejándose también en el coeficiente de variación.

En la Tabla 2, se presentan los parámetros genéticos estimados a partir del análisis estadístico. La variancia ambiental tuvo la mayor incidencia en número de aquenios por capítulo tanto en el compuesto P3 como en el P6 y representó el 43.7% y 37.2%, respectivamente en relación a la variancia total o fenotípica. Por otra parte, la menor incidencia se obtuvo en peso de 100 aquenios en el compuesto P3 y en diámetro de capítulo y porcentaje de aceite en P6.

Tabla 2. Estimación de la variancia genética y ambiental y calculo de la heredabilidad en familias S_1 de las poblaciones P3 y P6

	P3			P6		
	Variancia Genética	Variancia Ambiental	Heredabilidad	Variancia Genética	Variancia Ambiental	Heredabilidad
Diámetro de capitulo (cm)	3.110	1.584 (33.8)[1]	0.66	2.828	1.180 (29.4)	0.71
Número de Aquenios	19993.013	15160.58 (43.1)	0.57	13429.590	7955.803 (37.2)	0.63
Peso 100 Aquenios (g)	0.433	0.152 (26.0)	0.74	0.406	0.198 (32.8)	0.67
Contenido de Aceite (%)	13.245	6.144 (31.7)	0.68	12.630	5.417 (30.0)	0.70

[1]Los valores entre paréntesis corresponden en porcentaje a la relación entre la variancia ambiental y la variancia fenotípica

La proporción de la varianza fenotípica debido a las diferencias genéticas, denominada heredabilidad en sentido amplio, fue de similar magnitud en los dos compuestos para todas las variables; mayor en el compuesto P6 para diámetro de capítulo y contenido porcentual de aceite. Estos valores coincidieron con los de Waseem et al. (2003) para diámetro de capítulo, peso de aquenios y contenido porcentual de aceite y con los informados por Goksksoy et al. (2002) para diámetro de capítulo y peso de aquenio.

En líneas generales puede concluirse que en las poblaciones estudiadas existe variabilidad para ser explorada con altas probabilidades de lograr progresos por la aplicación de métodos de selección

AGRADECIMIENTOS

Los autores agradecen a Catalina Améndola de la Sección Estadística de la EEA Pergamino INTA, por su valiosa colaboración en la realización de los análisis estadísticos de este trabajo

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General combining ability analysis in sunflower maintainer lines using line x tester crosses

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ABSTRACT

A line×tester analysis including 15 B-lines and two testers of sunflower was conducted to estimate the general combining abilities of B-lines to be converted into CMS lines and, ultimately used in a hybrid production program. Significant differences in mean values for all of the studied traits (length of growing period, plant height, seed yield, thousand kernel weight and oil content) were noticed between genotypes. High heterosis expression for all traits was observed due to a highly significant contrast variance of parents versus crosses. The main role in the inheritance of most of the studied traits was played by an additive component of genetic variance, which was documented by significant differences between lines and between testers means. Among the lines, the best general combiner was the line B-F80-407, which expressed a significant positive effect of GCA on almost all the traits studied. Moreover, there were other lines with good general combining abilities with respect to individual traits. It could be concluded that the choice of B-F80-407 as a B-line to be used for the production of a CMS sister line would be promising for a successive hybrid production system.

Key words: B-line – combining ability – line×tester – sunflower

INTRODUCTION

Combining ability of parental lines is one of the basic parameters which determine the heterosis of hybrid progenies in genetically controlled traits. Sprague and Tatum (1942) introduced general and specific combining abilities, the former determined by additive gene effects, and the latter by dominance and epistatic effects. Other authors (Griffing, 1956; Falconer, 1967) had the same point of view. Since the combining abilities can be tested only in crosses, various methods are used, one of which is the line×tester analysis first developed by Kempthorne (1957).

Choosing suitable lines for breeding as a parental component of a hybrid variety is of great importance. It is clear that the use of good general combiner B-lines (maintainers) for backcross transferring of CMS trait will improve the performance of the resulting future hybrids.

The objective of this investigation was to assess the general combining abilities for length of growing period, plant height, seed yield, thousand kernel weight and oil content in sunflower inbred B-lines to choose them for CMS sister-line production through backcross breeding and subsequent F_1 hybrid production.

MATERIALS AND METHODS

Fifteen inbred sunflower B-lines (S_8) developed at the Seed and Plant Improvement Institute (SPII) of Iran in Karaj were crossed with two testers in 2004. Two CMS lines with good combining abilities were used as testers. Lines, testers and their F_1 hybrids were planted at the experimental farm of SPII in a randomized complete block design with two replications and examined during 2005 crop season. Each plot consisted of 4 rows with 22 plants per row. As the testers and resulting F_1 hybrids were sterile, alternating rows of an open-pollinated variety were used to provide a sufficient pollen load for seed setting. Distance between the rows was 60 cm and between plants 25 cm.

Forty plants in each plot were chosen for observations on length of growing period (days), plant height (cm), seed yield (t/ha), thousand kernel weight (TKW) (g), oil content (%), and oil yield (t/ha). The combining ability analyses were done according to the procedures described by Singh and Chaudhary (1976).

RESULTS AND DISCUSSION

Analysis of variance revealed significant differences between genotypes for all the characters at 1% probability level. Parents mean squares also differed significantly for growing period, plant height, head diameter, TKW and oil percent indicating a great deal of diversity between them. High heterosis expression for all traits was observed due to highly significant variance components of parents versus crosses. The variance due to a general combining ability of the males (lines) was significant for growing period, plant height and TKW, while that of the females (testers) was significant for all characters under study except seed yield. The line×tester component of variance for all traits was not significant except in the case of plant height (Table 1). This indicates that there is a predominant role of the additive rather than non-additive (epistasis and dominance) component for all characters under study.

Table 1. Analysis of variance for general and specific combining abilities of growing period, plant height, seed yield, TKW, oil percent and oil yield in a line×tester crossing scheme.

Source of variation		df	Mean Squares				
			Growing period	Plant height	Seed yield	TKW	Oil percent
Replication		1	80.929 **	75.781 ^{ns}	0.058 ^{ns}	182.564 *	1.89 ^{ns}
Genotypes		46	40.3259 **	1225.813 **	1.612 **	178.977 **	48.997 **
Parents		17	44.7706 **	604.248 **	0.523 ^{ns}	114.854 **	27.552 *
Parents vs. Crosses		1	700.55 **	40070.98 **	51.19 **	3163.63 **	1381.22 **
Crosses	Line	14	16.004 *	278.98 **	0.579 ^{ns}	82.055 *	9.11 ^{ns}
	Tester	1	30.33 *	423.79 **	0.289 ^{ns}	1336.704 **	98.74 **
	Line×Tester	14	9.926 ^{ns}	122.48 *	0.406 ^{ns}	45.094 ^{ns}	12.713 ^{ns}
Error		46	6.497	51.897	0.318	38.333	13.28

ns, *, **: non-significant, significant at 5% and 1% probability levels, respectively

The predominant role of the additive component in the study of inheritance of some traits in sunflower has been described by some other researchers for plant height (Rao and Singh, 1977), 1000 seed weight (Putt, 1965; Kovacik et al., 1972), oil content in seed (Putt, 1965; Fick, 1975; Sindagi et al., 1979), kernel content in seed (Marinković, 1985) and seed yield (Putt, 1965). However, other studies (Putt, 1965; Velkov, 1980; Kovacik et al., 1972; Rao and Singh, 1977; Sindagi et al., 1980) pointed out that the predominant role in inheritance of some of the studied traits is played by non-additive component.

Considering the growing period, the lines B-F80-407 and B-F80-411/1 had significant positive effects and the line B-F80-421/2 showed significant negative effects of general combining ability (GCA). Other lines had no significant positive or negative effects on general combining ability (Table 2).

With respect to plant height, the lines B-F80-438/1, B-F80-421/1, B-F80-407 and B-F80-421/2 had significant positive effects and the lines B-F80-409/2, B-F80-409/1 and B-F80-418/2 demonstrated significant negative general combining ability (GCA) effects (Table 2).

For seed yield, none of the lines had significant positive or negative effects of general combining ability (GCA), but the greatest positive value of GCA effects was attributable to the line B-F80-407 (Table 2).

Line B-F80-407 had significant positive effects of GCA on the TKW, while B-F80-428/2 and B-F80-408 had significant negative GCA effects on it. For oil percent, none of the lines had significant positive or negative effects of general combining ability (GCA), but the positive value of GCA effects was accounted by B-F80-438/2, B-F80-409/2, B-F80-407 and B-F80-428/2. On the contrary, the lines B-F80-418/1, B-F80-421/1, B-F80-408 and B-F80-421/2 showed negative but non-significant GCA effects (Table 2).

Table 2. General combining abilities of B-lines according to growing period, plant height, seed yield, TKW and oil percent

Lines	GCA				
	Growing period	Plant height	Seed yield	TKW	Oil percent
B-F80-407	4.262**	8.689*	0.560	10.861**	1.271
B-F80-408	0.399	-6.064	-0.575	-6.664*	-1.880
B-F80-409/1	0.782	-12.456**	0.227	-2.564	0.276
B-F80-409/2	-1.606	-13.039**	-0.565	-1.802	1.528
B-F80-410	0.267	-7.674	0.352	2.598	-0.597
B-F80-411/1	3.454*	4.371	0.144	3.761	0.901
B-F80-411/2	-0.051	0.224	-0.599	-1.114	-0.439
B-F80-418/1	0.797	0.244	-0.200	1.861	-2.632
B-F80-418/2	-1.566	-8.259*	0.113	2.798	0.088
B-F80-421/1	0.722	9.139*	0.133	-0.952	-1.962
B-F80-421/2	-3.416*	8.306*	-0.200	0.511	-0.994
B-F80-428/1	0.449	-0.531	0.456	1.623	-0.039
B-F80-428/2	-1.121	-1.689	-0.294	-8.239*	0.993
B-F80-438/1	-1.876	14.914**	0.269	0.436	0.308
B-F80-438/2	-1.498	3.824	0.178	-3.114	3.181
SE	**1.274**	**3.602**	**0.282**	**3.096**	**1.822**

Strictly speaking, the best general combiner on the whole was the line B-F80-407 which expressed a significant positive effect of GCA on growing period, plant height and TKW, and non-significant positive GCA effects on seed yield and oil percent (Table 2). It would be useful to include this line in backcrossing programs for CMS A-line production to be used in the development of highly productive hybrid cultivars.

There were different contribution of lines, testers and their interaction in expression of the studied traits (Table 3). Contribution of lines in the expression of growing period, plant height and seed yield was the greatest. More pronounced was the contribution of lines in expression of plant height (64.6%). Contribution of testers in expression of TKW was the greatest, while it had the minimum contribution in the expression of growing period, plant height and seed yield and moderate contribution with respect to oil content. Interaction between lines and testers expressed high contribution in oil content and moderate contribution in other traits.

Table 3. Component's contribution (%) to the total variance of growing period, plant height, seed yield, TKW and oil percent

Components	Traits				
	Growing period	Plant height	Seed yield	TKW	Oil percent
Lines	56.960	64.619	57.583	36.858	31.552
Testers	7.711	7.012	2.049	42.887	24.423
Line×Tester	35.329	28.369	40.368	20.255	44.025

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Selection of sunflower genotypes for Central Brazil

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ABSTRACT

Despite the large availability of suitable areas for sunflower cropping in Central Brazil, few adapted cultivars are available in the market. The objective of this work was to select sunflower genotypes adapted to this important production region. Experiment data from 2006 and 2007 were obtained by the National Sunflower Trials, coordinated by Embrapa Soja. The evaluated traits were seed and oil yields. Commercial hybrids M 734 (Dow AgroSciences) and Agrobel 960 (La Tijereta) were used as controls for hybrid comparison. The open pollinated variety Embrapa 122 (Embrapa) was used as control for variety comparison. Two criteria were used for selection of genotypes: 1) the general mean obtained from different environments; 2) partitioning of general mean in favorable and unfavorable environments (IDMG). The method of the IDMG showed similar results to general mean analyses. The hybrids EXP 1447 and EXP 1446 and the varieties BRSGira 02 e BRSGira 01 had general indication for oil yield, i.e. they showed superior performance in both favorable and unfavorable environments. These varieties had also general indication for grain yield.

Key words: genotype x environment – *Helianthus annuus* – sunflower breeding.

INTRODUCTION

There is an increasing utilization of sunflower in Brazil, due to its use as raw material for ensilage, oil production and to its potential as a new source of energy from biological fuel production. Therefore, the growing area and grain production increased to 94 and 82%, respectively, between 2002/2003 and 2006/2007 crop seasons (Embrapa Soja, 2007b). Most of the 99,000 ha cultivated in 2006/2007 were sown in Central Brazil, following the major summer growing period, mainly in the states of Mato Grosso (22%), Goiás (18%), São Paulo (10%) and Mato Grosso do Sul (10%) (Embrapa Soja, 2007b).

In some Brazilian States, a common agricultural practice is summer double cropping, meaning that the main crop is sown from October to early November, allowing its harvesting by February. Then, a second crop is sown in February/March, taking advantage of the adequate temperature and rainfall conditions. Sunflower is one of the crops suitable for being the second summer crop.

The expansion of the sunflower crop as the second summer crop in Brazil depends on a constant evaluation of new genotypes obtained by selection of superior materials able to express high yield and acceptable quality in the different regions. Thus, the genetic progress of sunflower in Brazil plays an important role to make the necessary economic returns compared to other summer crops more feasible. Despite the large availability of suitable areas for sunflower cropping in Central Brazil, few adapted cultivars are available in the market.

The evaluation and selection of hybrids and varieties of sunflower from several companies are being carried out through the National Sunflower Trials, coordinated by Embrapa Soja and supported by the contribution of public and private institutions. The objective of this work was to select sunflower genotypes evaluated in the Trial Network carried out in 2006 and 2007 in Central Brazil.

MATERIALS AND METHODS

Data from the National Sunflower Trials, coordinated by Embrapa Soja, were used for this work. Trials were installed in 2006 and 2007 in several locations of the states of Goiás, Mato Grosso, Mato Grosso do Sul, Minas Gerais, Rondônia, São Paulo and Distrito Federal (Table 1).

The genotypes were sown in February/March, in a randomized block design with four replicates. Each plot consisted of four rows 6.0 m long, spaced from 0.7 to 0.9 m. Only the two central rows were used for data collection. Plants located up to 0.5 m apart from the extremity of each central row were also

discarded, resulting in a useful area from 7 to 9 m^2 per plot, depending on the space adopted. All the recommended cultural practices were observed to allow an optimum plant development.

The evaluated genotypes were simple and triple hybrids and open pollinated varieties developed by the companies ACA, CATI, Embrapa Soja, Helianthus do Brasil, La Tijereta and SPS. Commercial hybrids M 734 (Dow AgroSciences) and Agrobel 960 (La Tijereta) were used as control for hybrid comparison. The open pollinated variety Embrapa 122 (Embrapa) was used as control for variety comparison. The evaluated traits were grain and oil yields (kg ha^{-1}). Genotype evaluation was carried out during two years in the Final Trials of the First Year of Evaluation (FTF) and in Final Trials of the Second Year of Evaluation (FTS).

Table 1. Altitude and geographical coordinates of the Brazilian Sunflower Trial locations in 2006 and 2007.

State	City	Institution	Latitude	Longitude	Altitude (m)
SP	Piracicaba	ESALQ	22°41'S	47°38'W	546
MT	Campo Novo dos Parecis	Farm	13°40'31S	57°53'31"W	572
	Campos de Júlio	AGROPLANT	13°56'1"	59°07'6"	668
	Campo Verde	UFMT	15°32'48"	55°10'08"	736
MS	Dourados	Embrapa Agropecuária Oeste	21°74'S	54°62'W	293
MG	Patos de Minas	EPAMIG	18°34'44"	46°31'04"	815
DF	Planaltina	Embrapa Cerrados	15°27'10"S	47°36'51"W	1060
RO	Vilhena	Embrapa Rondônia	12°47'12"S	60°03'39"W	600
GO	Rio Verde	FESURV	17°47'0S	50º57'2"	737

The analysis of variance was performed on grain and oil yields for each environment (location and year). As the locations of the trials included in the FTF were not exactly the same ones as those chosen for the FTS, a joint analysis of environment for each group of genotypes was carried out. For this, a test to verify the homogeneity of residual variances was applied. Trials with coefficients of variation higher than 20% (Pimentel Gomes, 1985) were not included in the joint analysis of variance.

Two criteria were used for selection of genotypes: 1) the general mean obtained from different environments; 2) partitioning of general mean in favorable and unfavorable environments. As favorable environments were selected those with a superior general mean and aunfavorable environments those with inferior general mean (Verma et al., 1978).

In the analysis of the general mean, Scott-Knott test at 5% of probability was performed to verify significance of differences between genotypes, as well as the comparison of means between each evaluated genotype and the controls.

The favorable and unfavorable environment means of each genotype were compared to the control mean in each environment, according to the IDMG method (Indication Method – Partitioning of General Mean) (Porto et al., 2007). When the mean of a certain genotype was higher than the control mean in favorable but not in unfavorable environments, this genotype was regarded as being a suitable one for favorable environments, and vice versa. On the other hand, if a certain genotype was superior in both environments, it received a general indication.

The statistical analyses were performed with the Genes software package (Cruz, 2001).

RESULTS AND DISCUSSION

The interaction genotypes x environments was significant in the joint analysis of variance, indicating a different performance of genotypes over the evaluated environments, and pointing out the importance of studies of the yield components in specific environments (Table 2). The experimental accuracy was satisfactory according to classification of Pimentel Gomes (1985), since the coefficients of variation (CV) were lower than 20% for yield components. General means for grain yield over the year were remarkably superior to the approximately 1,360 kg ha^{-1}, observed in Brazilian commercial sunflower crops (Embrapa Soja, 2007b).

In spite of the acceptable values of C.V., significant differences between genotypes, at 5% of probability level, were detected by Scott-Knott test only when a large difference between their means was observed for both evaluated traits (Table 3), as reported by Embrapa Soja (2004, 2006, 2007a). Therefore, selection of sunflower genotypes was made based on the difference between their performance and the mean of controls, so that selected materials were those with means higher than that of controls. None of the hybrids showed means greater than the control (M 734 and AGROBEL 960) for grain yield. For this

trait, the mean for open pollinated variety BRSGira 02 was greater than Embrapa 122 (control). The genotypes that presented a general mean higher than the controls for oil yield were the hybrids EXP 1447 and EXP 1446 and the varieties BRSGira 01 e BRSGira 02. In this analysis, none of the open pollinated varieties was greater than any hybrid. Nevertheless, the use of open pollinated varieties may be meaningful to the farmholders, due to low seed price and less environmental risk (water deficit), once sunflower crop is sown in February/March, at the end of the rainy season.

Table 2. Joint analyses of variance for grain and oil yields (kg ha^{-1}) of sunflower genotypes evaluated in the National Sunflower Trials, coordinated by Embrapa, in 2006 and 2007.

Source of variation	Df	MS (Grain yield)	MS (Oil yield)
Block/ environment	54	185,735.156	44,818.04
Genotype (G)	15	3,700,641.79**	742,973.81**
Environment (E)	17	22,279,347.24**	4,309,501.67**
GxE	255	355,287.11**	74,515.86**
Residue	810	80,284.86	16,737.52
Mean		2,021	885
C.V.(%)		14.01	14.61

** Significant at 1% of probability for F test.

Table 3. Partition of means of sunflower genotypes evaluated in favorable and unfavorable environments for grain and oil yields (kg ha^{-1}), from experiments carried out in 2006 and 2007.

	Grain Yield (Kg ha^{-1})				Oil Yield (Kg ha^{-1})		
Genotype	Mean	FM[5]	UM[5]	Genotype	Mean	FM[5]	UM[5]
M 734 (H)[1,2]	2,328 a[4]	2,950 a	1,830 a	Exp 1447 (H)[1]	1,059 a[4]	1,304 a	864 a
Agrobel 960 (H)[2]	2,289 a	2,830 a	1,856 a	Agrobel 960 (H)[2]	1,043 a	1,315 a	825 a
BRSG 10 (H)	2,243 a	2,774 a	1,818 a	Exp 1446 (H)	1,039 a	1,309 a	823 a
Exp 1447 (H)	2,200 a	2,752 a	1,759 a	M 734 (H)[2]	946 a	1,160 a	775 a
ACA 886 (H)	2,196 a	2,709 a	1,786 a	BRSGira 09 (H)	903 b	1,123 b	726 a
Exp 1446 (H)	2,183 a	2,725 a	1,750 a	BRSGira 11 (H)	902 b	1,166 a	691 b
SPS 4561 (H)	2,069 a	2,626 a	1,623 a	ACA 861 (H)	887 b	1,062 b	747 a
BRSG 09 (H)	2,054 a	2,497 b	1,700 a	BRSGira 10 (H)	884 b	1,105 b	707 b
BRSG 11 (H)	2,033 a	2,560 a	1,612 a	ACA 886 (H)	872 b	1,048 b	732 a
ACA 861 (H)	2,011 a	2,463 b	1,650 a	SPS 4561 (H)	863 b	1,069 b	699 b
HELIO 256 (H)	1,976 a	2,372 b	1,658 a	HELIO 256 (H)	854 b	1,046 b	701 b
BRSG 08 (H)	1,929 a	2,442 b	1,518 b	BRSGira 08 (H)	846 b	1,047 b	685 b
BRSGira 02 (V)	1,881 a	2,308 b	1,540 b	BRSGira 02 (V)	830 b	1,050 b	655 b
Embrapa 122 (V)[3]	1,735 a	2,214 b	1,351 b	BRSGira 01 (V)	788 b	1,002 b	617 b
BRSGira 01 (V)	1,671 a	2,079 b	1,345 b	Embrapa 122 (V)[3]	744 b	959 b	571 b
BRSGira 03 (V)	1,543 a	1,905 b	1,253 b	BRSGira 03 (V)	695 b	873 b	552 b
GM[6]	2,021	2,513	1,628	GM[6]	885	1,102	711
HCM[6]	2,308	2,890	1,843	HCM[6]	994	1,238	800
VCM[6]	1,735	2,214	1,351	VCM[6]	744	959	571

[1]H: hybrid and V: open pollinated variety.
[2]Control for hybrid comparison.
[3]Control for variety comparison.
[4]Means followed by the same letter did not differ significantly at the Scott-Knott test ($P \leq 0.05$).
[5]FM = mean in favorable environments; UM = mean in unfavorable environments.
[6]GM = general mean; HCM = hybrid control mean and VCM = variety control mean.

For selection of sunflower genotypes, the general means of grain and oil content from different environments are commonly used (Embrapa Soja, 2004, 2005 and 2006), although the specific adaptation of the favorable and unfavorable environments should be taken into account. In this study, the method of the IDMG showed results similar to general mean analyses. The hybrids EXP 1447 and EXP 1446 and the varieties BRSGira 02 e BRSGira 01 gave a general indication for oil yield. These varieties also gave a general indication for grain yield.

ACKNOWLEDGEMENTS

We acknowledge the researchers and institutions that carried out the experiments and made available the experimental data necessary for this work.

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Selection of sunflower genotypes for sowing dates in August/September in Southern region of Brazil

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ABSTRACT

The choice of adapted cultivars to different environments is meaningful to sunflower crop. The objective of this study was to select sunflower genotypes for sowing dates in August/September in Southern region of Brazil. Experiment data from 2005/2006 to 2006/2007 were obtained by the National Sunflower Trials, coordinated by Embrapa Soja. The evaluated traits were seed and oil yields (kg ha^{-1}). The criterion used for selection of genotypes was the general mean obtained from different environments. For grain yield, the open pollinated varieties Catissol (1,775 kg ha^{-1}), BRSGira 02 (1,687 kg ha^{-1}) and BRSGira 01 (1,531 kg ha^{-1}) surpassed the open pollinated varieties used as control (1,510 kg ha^{-1}). The general mean for grain yield of the hybrids Helio 253 (2,498 kg ha^{-1}) and Helio 360 (2,427 kg ha^{-1}) surpassed the hybrids used as control (2,368 kg ha^{-1}). For oil yield, the hybrids Exp 1441 (1,136 kg ha^{-1}), Helio 360 (1,129 kg ha^{-1}) and Helio 253 (1,127 kg ha^{-1}) surpassed the controls.

Key words: *Helianthus annuus* – interaction genotype x environment – release of genotypes – sunflower breeding.

INTRODUCTION

The interest of farmers from the Southern region of Brazil in sowing sunflower (*Helianthus annuus* L.) in August/September is increasing for many reasons. Sunflower plays an important role as a new option for crop rotation or succession. It also contributes with raw material to the production of biological fuel and also for high quality oils for human consumption. In the states of Rio Grande do Sul and Paraná, for example, between 2001/02 and 2004/05, the sowed area increased around 200% and 1000%, respectively (Embrapa Soja, 2005).

For being a relatively new culture in the country, it is very important to obtain information on the available genotypes to recommend the most suitable ones for specific producing regions. These conditions are necessary to increase the possibility of a successful sunflower production to obtain a more competitive economic response.

Since 1989, the Embrapa Soybean has been coordinating a network of evaluation and selection of hybrids and varieties developed by diverse companies through a program called National Sunflower Trials. This network counts with the contribution of several public and private institutions. The objective of this work was to select sunflower genotypes evaluated in the Trial Network carried out between 2005/06 and 2006/07 during the August/September sowing season.

MATERIALS AND METHODS

Experiment data were obtained from the National Sunflower Trials between 2005/2006 and 2006/2007, in several locations of the states of Rio Grande do Sul (RS) and Paraná (PR).

Sowing was done in August/September according to a randomized block design with four replications. Each plot consisted of four lines 6.0 m long, spaced from 0.7 m to 0.9 m. At harvest, two border lines and 0.5 m from each extremity were discarded, resulting in 7.0 m^2 to 9.0 m^2 per plot, depending on the spacing adopted. Soil fertilization, weed control and other agronomic management practices were provided in order to allow good plant development.

The evaluated genotypes were hybrids (simple and triple) and open-pollinated varieties (populations) developed by the companies Advanta, Dow AgroSciences, Embrapa Soja, La Tijereta, and Helianthus do Brasil. Commercial hybrids M 734 (Dow AgroSciences) and Agrobel 960 (La Tijereta) were used as controls. The evaluated traits were grain and oil yields (kg ha^{-1}). The groups of genotypes were evaluated

in the network during two years in Final Trials of the First Year of Evaluation (FTF) and in Final Trials of the Second Year of Evaluation (FTS). Thirteen genotypes were evaluated. In the FTF, the evaluated locations and the respective institutions responsible were Campo Mourão (Cooperativa Mista Agropecuária do Brasil) and Ijuí (Cooperativa Regional Tritícola Serrana Ltda). In the FTS, the locations and institutions were Santa Rosa (Cooperativa Mista São Luiz Ltda-Coopermil), Passo Fundo (Universidade de Passo Fundo), Encruzilhada do Sul (Fundação Estadual de Pesquisa Agropecuária-Fepagro).

The analysis of variance was performed on grain and oil yields for each environment (location and year). As the locations of the trials included in the FTF were not exactly the same ones as those chosen for the FTS, a joint analysis of environment was carried out for each group of genotypes. For this reason, a test to verify the homogeneity of residual variances was applied. In this test, variances were considered as homogeneous when the ratio between the larger and the smaller residual mean square was smaller than seven (Pimentel Gomes, 1985). Moreover, trials with coefficients of variation higher than 20% (Pimentel Gomes, 1985; Carvalho et al., 2003) and experiments with major problems (bird attacks, drought and serious incidence of plant diseases, such as Alternaria) were not included in the joint analysis of variance.

Two criteria were used for genotype selection: 1) the general mean obtained from different environments; 2) partitioning of general mean in favorable and unfavorable environments. A favorable environment was considered to be that with superior general means, and an unfavorable one those with inferior general means (Verma et al., 1978).

In the analysis of the general mean, Duncan test at 5% of probability was performed to verify significance of differences between genotypes, as well as the comparison of means among each evaluated genotype and the controls. The calculations were done by the computational program Genes (Cruz, 2001).

RESULTS AND DISCUSSION

Significant effect was observed for the genotype x environment interactions (Table 1). This demonstrates that performance of genotypes depends on the different environments where they are cultivated, so that an analysis in specific environments is required, as reported by Allard and Bradshaw (1964). The presence of GxE interaction in sunflower yield tests has also been reported by Embrapa Soja (1996, 1997, 1998, 1999, 2000), Lúquez et al. (2002) and De la Vega and Chapman (2006). The coefficients of variation (CV) of the analysis were between 14.58% and 16.54% for grain yield, and between 15.47% and 17.02% for oil yield. According to the criteria suggested by Pimentel-Gomes (1985) and Carvalho et al. (2003), those experiment precision levels were satisfactory. In most of the assessed years, the average yield of the trials was higher than the yields of commercial field crops, which were approximately 1,500 kg ha^{-1} (Conab, 2005).

Table 1. Joint analyses of variance for grain and oil yields (kg ha^{-1}) of sunflower genotypes evaluated in the National Sunflower Trials, coordinated by Embrapa, in the period from 2005/2006 to 2006/2007

Year[1]	Yield (kg.ha^{-1})					
	Grains			Oil		
	MSGE[2]	CV[3]	Mean[4]	MSGE[2]	CV[3]	Mean[4]
2005/2006	281,663,48**	14,58	2,328,58	90,933,65**	15,47	1,040,99
2006/2007	262,336,33**	16,54	2,003,16	60,302,97**	17,02	912,16

** Significant at 1% of probability by the F test.
[1]Evaluations made in 2005/2006 (sowing date on August/September) include the experimental data obtained in the Final Trials of First Year of Evaluation and Final Trials of Second Year of Evaluation 2006/2007.
[2]MSGE: Mean square for the interaction genotypes x environments.
[3]CV: Coefficient of variation (%).
[4]General mean, in kg ha^{-1}

Despite the acceptable values of CV, Scott-Knott test at 5% of probability only detected significant differences between genotypes when the distance between such means was very large (Table 2). In most cases, genotypes did not differ from each other for the two evaluated traits, as reported by Embrapa Soja (1996, 1997, 1998, 1999, 2000). Due to the absence of differences for this test, genotypes had been indicated through the comparison of the performance of each one of them in relation to the mean of the experimental controls. Therefore, the material whose general mean was higher than the controls was recommended. The discrimination of genotypes through this criterion is more rigorous than the one based on the Duncan's test, resulting in a smaller group of selected genotypes. This criterion is used by the

Brazilian Ministry of Agriculture, Cattle and Supplies for the new soybean, wheat and beans cultivar registration (Ministério da Agricultura, 2006), but it is important to emphasize that no criterion has been established yet for sunflower.

Table 2. Means of sunflower genotypes evaluated in the National Sunflower Trials, coordinated by Embrapa, 2005/2006 and 2006/2007, for grain and oil yields (kg ha^{-1}).

Grain yield (kg ha^{-1})						
Genotype	Mean	Ijuí[1]	Campo Mourão[1]	Encruz. do Sul[2]	Santa Rosa[2]	Passo Fundo[2]
M 734 (H)[3,4]	2,553 a[6]	2,390 (1)	2,220 (4)	2,360 (4)	2,975 (1)	2,821 (3)
HELIO 253 (H)	2,498 a	1,651 (7)	2,093 (6)	2,865 (1)	2,847 (2)	3,034 (1)
HELIO 360 (H)	2,427 a	1,768 (6)	2,456 (1)	2,692 (2)	2,562 (6)	2,655 (5)
EXP 1441 (H)	2,349 a	2,126 (2)	2,232 (3)	2,197 (6)	2,642 (5)	2,547 (6)
BRSGira 06 (H)	2,323 a	1,833 (5)	2,243 (2)	1,971 (7)	2,775 (3)	2,793 (4)
Agrobel 960 (H)[4]	2,183 a	1,548 (9)	2,127 (5)	2,202 (5)	2,644 (4)	2,393 (7)
HELIO 362 (H)	2,183 a	1,440 (11)	1,923 (7)	2,363 (3)	2,292 (7)	2,896 (2)
BRSGira 05 (H)	1,918 b	1,949 (3)	1,819 (10)	1,845 (9)	1,903 (10)	2,074 (13)
BRSGira 07 (H)	1,886 b	1,877 (4)	1,653 (12)	1,927 (8)	1,819 (12)	2,154 (11)
Catissol (V)	1,775 b	1,590 (8)	1,822 (9)	1,533 (12)	1,959 (8)	1,970 (15)
BRSGira 04 (H)	1,747 b	1,493 (10)	1,917 (8)	1,668 (11)	1,534 (14)	2,122 (12)
BRSGira 02 (V)	1,687 b	957 (14)	1,750 (11)	1,777 (10)	1,657 (13)	2,294 (8)
BRSGira 01 (V)	1,531 b	998 (13)	1,075 (15)	1,516 (13)	1,905 (9)	2,159 (10)
Embrapa 122 (V)[5]	1,510 b	1,351 (12)	1,416 (13)	1,263 (15)	1,240 (15)	2,280 (9)
BRSGira 03 (V)	1,473 b	927 (15)	1,088 (14)	1,428 (14)	1,855 (11)	2,066 (14)
GM[7/]	2,003,16	665	887	950	961	1,095
CHM[7/]	2,368,4	-	-	-	-	-
CVM[7/]	1,510,4	-	-	-	-	-
Oil yield (kg.ha^{-1})						
Genotype	Mean	Ijuí[1]	Campo Mourão[1]	Encruz, do Sul[2]	Santa Rosa[2]	Passo Fundo[2]
EXP 1441 (H)[3]	1,136 a[6]	942 (1)	1,140 (2)	1,123 (3)	1,259 (2)	1,218 (4)
HELIO 360 (H)	1,129 a	751 (5)	1,176 (1)	1,354 (1)	1,162 (6)	1,202 (5)
HELIO 253 (H)	1,127 a	684 (7)	1,003 (5)	1,353 (2)	1,284 (1)	1,312 (1)
Agrobel 960 (H)[4]	1,035 a	683 (8)	1,061 (3)	1,094 (5)	1,204 (4)	1,131 (6)
BRSGira 06 (H)	1,027 a	712 (6)	1,059 (4)	910 (9)	1,193 (5)	1,259 (3)
M 734 (H)[4/]	1,019 a	915 (2)	928 (7)	964 (6)	1,208 (3)	1,080 (8)
HELIO 362 (H)	977 a	601 (11)	887 (9)	1,096 (4)	996 (7)	1,306 (2)
BRSGira 05 (H)	893 b	821 (3)	895 (8)	951 (7)	844 (9)	956 (12)
BRSGira 04 (H)	864 b	664 (9)	986 (6)	880 (10)	718 (13)	1,071 (9)
BRSGira 07 (H)	855 b	752 (4)	827 (11)	931 (8)	818 (12)	948 (13)
Catissol (V)	780 b	662 (10)	829 (10)	694 (13)	821 (11)	895 (15)
BRSGira 01 (V)	771 b	485 (13)	558 (14)	811 (12)	891 (8)	1,107 (7)
BRSGira 02 (V)	751 b	386 (14)	827 (12)	854 (11)	690 (14)	1,001 (11)
BRSGira 03 (V)	665 b	381 (15)	506 (15)	670 (14)	838 (10)	930 (14)
Embrapa 122 (V)[5/]	646 b	540 (12)	629 (13)	563 (15)	495 (15)	1,003 (10)
GM[7/]	912	665	887	950	961	1,095
CHM[7/]	1,027	-	-	-	-	-
CVM[7/]	646	-	-	-	-	-

[1]Final Trials of the First Year of Evaluation 2005/2006.
[2]Final Trials of the Second Year of Evaluation 2006/2007
[3] H: hybrid, V: open pollinated variety.
[4]Test genotype to compare hybrids.
[5]Test genotype to compare open pollinated variety.
[6]Means followed by the same letter did not differ significantly by the Scott-Knott test (P≤0.05).
[7]MG = General mean; CHM= Control hybrids mean; CVM = Control open pollinated varieties mean.

The hybrids selected for having presented superior general means in relation to the mean of controls for grain yield were HELIO 253 and HELIO 360 (Table 2). The hybrids EXP 1441, HELIO 253 and HELIO 360 showed the best performances for oil yield. Thus, only the hybrids HELIO 253 and HELIO 360 presented good results for both evaluated traits. The open pollinated varieties selected for having presented superior general means in relation to the mean of controls for grain yield were Catissol, BRSGira 02 and BRSGira 01 (Table 2). All the open pollinated varieties presented better performances for oil yield than the controls. When a genotype is superior for only one of these characteristics, the choice of cultivars to be sown by farmers should be based on the commercial policy practised by sunflower industry. Currently, genotypes with oil contents above 40% receive a bonus at the moment of purchasing. Therefore, the higher the bonus, the greater the preference for genotypes with outstanding oil content instead of giving priority to grain yield.

ACKNOWLEDGEMENTS

The authors wish to thank the research scientists and institutions that evaluated the trials with sunflower genotypes and provided the experiment data used in this study.

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Identification of a new CMS cytoplasm and localization of its fertility restoration gene in sunflower

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ABSTRACT

Cytoplasm male sterility (CMS) and its fertility restoration (Rf) genes are critical tools for hybrid seed production. To broaden the genetic diversity of sunflower hybrid breeding materials, a new CMS, designated as CMS GIG2, was identified from an interspecific cross between *Helianthus giganteus* accession 1934 and *H. annuus* cv. HA 89. We also identified the fertility restoration gene for CMS GIG2 from an amphiploid involving *H. maximiliani* accession 1631. The CMS GIG2 and its fertility restoration gene were introduced into HA 89 background through recurrent backcross and single plant selection techniques. Genetic analysis revealed that the CMS GIG2-fertility restoration system is controlled by a completely dominant gene, designated as Rf_4, in a HA 89 background. The gene Rf_4 was mapped onto linkage group 3 and tightly links with the markers ORS1114 and ORS13, based on 576-plant F_2 and F_3 populations. The CMS GIG2-Rf_4 system tagged by molecular markers provides an alternative genetic source for hybrid sunflower breeding.

Key words: cytoplasm male sterility – interspecific hybridization – male fertility restoration – sunflower.

INTRODUCTION

Cytoplasm male sterility (CMS) has been reported for over 150 plant species (Kaul, 1988) and used to produce commercial hybrid seed for several crops. Since the first sunflower CMS was reported in 1969, 72 CMS sources have been identified in the *Helianthus* genus (Serieys, 2005). So far, the CMS PET1 developed by Leclercq (1969) from *H. petiolars* Nutt. is the only one that has been extensively used in commercial hybrid sunflower production. Genetic analyses indicated that almost all CMS restoration lines currently used in sunflower breeding carry the same restorer gene Rf_1 (Serieys, 1996, 2005). In order to prevent cytoplasmic uniformity and reduce genetic vulnerability, alternative CMS and restorer sources are needed for sunflower breeding programs.

Sunflower male fertility restoration is generally controlled by a single dominant gene (e.g., Rf_1), or two complementary dominant genes (e.g., Rf_1 and Rf_2) plus modifiers (Serieys, 1996, 2005). Kinman (1970) discovered the male fertility restorer gene Rf_1 in the line T66006-2-1-B. Since then, many restoration lines, such as all the USDA-ARS RHA lines, RHA 271, 272, 273, 274, 275, 276, 278, 279 and 296 carry the Rf_1 gene derived from T66006-2-1-B (Korell et al., 1992; Serieys, 2005; Jan et al., 2002). An allelic test between T66006-2-1-B and MZ01398, an obsolete local cultivar, led to the discovery of a second major dominant gene Rf_2 from MZ01398 (Vrânceanu and Stoenescu, 1971, 1978). Recently, a fertility restoration gene Rf_3, which is different from both Rf_1 and Rf_2, was assigned to the confection restorer RHA 280 (Jan and Vick, 2007).

The current research was devoted to identification of a new CMS and fertility restoration genes, and also localization of the fertility restoration genes on the sunflower genetic map.

MATERIALS AND METHODS

Plant materials

The wild sunflower *H. giganteus* accession 1934 (Ames 1934, PI 503250), *H. maximiliani* accession 1631 (PI 468750), cultivated *H. annuus* cv. HA 89, and HA 89 nuclear male sterility mutant NMS HA 89 were used as parents for this study. An initial F_2 population of 113 individuals was generated from a single F_1 plant from the cross *H. giganteus*/7*HA 89/3/*H. giganteus*/ 6* HA 89// *H. maximiliani* 1631 (amphiploid) and this population was used to develop the genetic model for the new CMS and fertility restoration system. Some of F_2 heterozygous plants were selected to produce F_3 lines to confirm the genetic model and to map the Rf gene. Plants were grown in the field or greenhouse conditions in Fargo, USA.

Phenotype identification
Male fertility or sterility for individual plants was identified visually examining anther morphology and evaluating pollen stainability (Alexander, 1969). Plants with normal, dehiscent anthers and abundant pollen were scored as male-fertile, otherwise as male-sterile. To evaluate stainability, about 500 pollen grains were counted under a microscope to estimate the percentage of fertile pollen grains.

Marker genotyping
Genomic DNA was extracted from fresh young leaves of individual F_2 and F_3 plants according to the protocol described by Zhang et al. (2006). The bulked segregant analysis strategy (Michelmore et al., 1991) was used to screen for markers polymorphic for the segregation populations. Equal amounts of DNA from 10 male-sterile and 10 male-fertile F_2 plants were bulked to form the sterile and fertile pools, respectively. In total, 200 simple sequence repeat (SSR) markers relatively evenly distributed among the 17 linkage groups (Tang et al., 2002; Yu et al., 2002, 2003) were selected to screen for polymorphism between male-fertile and male-sterile pools. The polymorphic markers were used to genotype F_2 and F_2-derived F_3 populations. PCR amplification was performed according to Tang et al. (2002). PCR products were displayed on 6.5% polyacrylamide denaturing gel at 60 W for 2.0 h (0.5× TBE) and then scanned with a Typhoon 9410 variable mode imager (Molecular Dynamics Inc., CA, USA) after staining with GelRed nucleic acid gel stain (Biotium, Inc., CA, USA).

RESULTS

Isolation of a male sterility cytoplasm
The wild sunflower *H. giganteus* acc.1934 was crossed with HA 89 and the resulting F_1 seedlings were recovered through an embryo rescue technique. All F_1 plants had 2n=34 chromosomes and were treated with colchicine to induce double haploid progenies (2n = 68). One of the F_1 plants was male-sterile and the remaining were male-fertile, based on morphology of the anthers and pollen stainability. The male-sterile F_1 plant was backcrossed with HA 89 repeatedly and the chromosome numbers of the hybrids were reduced with each successive backcross. After five cycles of backcrossing, plants with 2n=34 were selected from the BC_5F_1 generation. All plants from the BC_1 to BC_5 generations were male-sterile, indicating that the CMS trait from *H. giganteus* acc. 1934 was introduced into the HA 89 background.

BC_5F_1 plants were pollinated by a set of 19 cultivars, i.e., Armavir, HA 89, HA 290, P21, HA 821, Hopi Dye, Issanka, Luch, RCMG1, RCMG2, RCMG3, RHA 266, RHA 274, RHA 280, RHA 294, RHA 801, Seneca, Smena, and VNIIMK. This standard set of restoration lines was used to test the fertility restoration pattern for the new CMS from *H. giganteus* because of their diverse genetic background. Hybrids from all these crosses were male-sterile, suggesting that the tester lines had no restorer genes for the new CMS from *H. giganteus* acc. 1934. Thus, the new CMS is different from all previously reported CMS types, including CMS GIG1 which can be restored by RHA 280 and RHA 801 (Serieys, 1996). Therefore, we concluded that the *H. giganteus* acc. 1934-derived CMS was a new type. According to the FAO codification, the new CMS source was designated as CMS GIG2.

Identification of the CMS GIG2 fertility restoration gene
To search for the fertility-restoration gene for CMS GIG2, we crossed the BC_5F_1 plants with seven interspecific amphiploids: NMS HA 89 × *H. maximiliani* 1631, *H. cusickii* × P21, *H. atrorubens* × HA 89, *H. mollis* × P21, *H. grosseserratus* × P21, *H. pumilus* × P21, and *H. angustifolius* × P21. Four amphiploids involving wild species *H. maximiliani* 1631, *H. atrorubens*, *H. grosseserratus*, and *H. angustifolius* restored fertility of CMS GIG2. It was noticed that a single male-fertile plant with 2n=34 was obtained after crossing a BC_5F_1 plant with the amphiploid NMS HA 89 × *H. maximiliani* 1631, while all other progenies of the same cross were also male-fertile, but had 2n=51 chromosomes. This particular male-fertile plant was used to pollinate a male-sterile BC_6F_1 plant, and the resulting F_1 plant was used to develop an F_2 mapping population. The standard set of 19 cultivated lines used above, including RHA 266 and RHA 274 carrying the Rf_1 gene, HA 89 and HA 821 carrying the Rf_2 gene, and RHA 280 carrying the Rf_3 gene (Jan and Vick, 2007), were not able to restore CMS GIG2, indicating that this fertility restoration gene derived from amphiploid *H. maximiliani* 1631 is different from Rf_1, Rf_2, and Rf_3.

Inheritance of the CMS GIG2 fertility restoration system
The population of 113 F_2 plants was classified into fertile and sterile groups (Fig. 1) and the segregation ratio fit a monogenic ratio of 3:1 (Table 1). The segregation pattern was confirmed by the F_3 population

(Fig. 1 and Table 1). The data from the two generations demonstrated that the CMS GIG2 fertility restoration system is controlled by one locus. This new fertility restoration locus was designed as *Rf*$_4$.

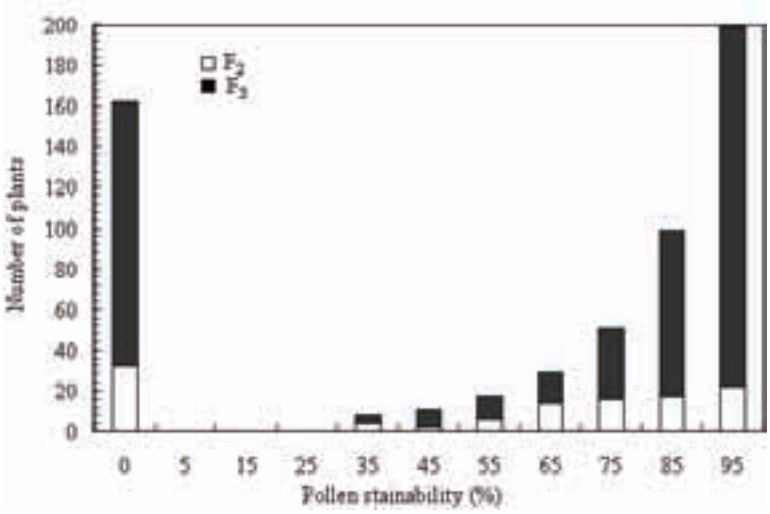

Fig. 1. Distribution of pollen stainability in the F_2 and F_2 heterozygote-derived F_3 populations.

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Table 1. Fitness test for segregation ratios of pollen fertility and its associated marker OSR1114 or ORS13 in the initial F_2 and F_2 heterozygote-derived F_3 populations

Population	Trait or marker	Number of plants[1]			Expected ratio	χ^2 - value	Probability
		Fertile		Sterile			
		Rf$_4$*Rf*$_4$	*Rf*$_4$*rf*$_4$	*rf*$_4$*rf*$_4$			
F_2	Pollen fertility	81		32	3:1	0.6	0.42
	ORS1114/ORS13	32	49	32	1:2:1	2.0	0.37
F_3	Pollen fertility	333		130	3:1	2.3	0.13
	ORS1114/ORS13	106	226	131	1:2:1	2.9	0.23

[1]Individual plants were visually and microscopically examined for pollen fertility/sterility based on the morphology of the anthers and the pollen stainability at flowering time. The *Rf*$_4$/*rf*$_4$ locus is the proposed fertility restoration gene based on the expected segregation pattern and this locus co-segregated with the marker ORS1114 or ORS13 in the F_2 population.

Mapping of the Rf$_4$ *locus*

Of 200 simple sequence repeat (SSR) markers selected from the linkage maps (Tang et al., 2002; Yu et al., 2002, 2003), seven (ORS13, ORS294, ORS349, ORS502, ORS822-3, ORS1114, ORS1146) were polymorphic between the fertile and sterile pools, respectively, and were used to genotype the whole F_2 population. The genotyping data showed that only ORS13 and ORS1114 (Fig. 2) on linkage group 3 were associated with pollen fertility and co-segregate with the *Rf*$_4$ locus. There were no recombinants between the markers ORS13 and ORS1114, and both fit a monogenic segregation ratio of 1:2:1 in the F_2 population (Table 1). The marker-trait association demonstrated that *Rf*$_4$ is a completely dominant gene, because there was no difference in pollen stainability between the *Rf*$_4$*rf*$_4$ and *Rf*$_4$*Rf*$_4$ genotypes (Fig. 1).

Fig. 2. Co-segregation of the marker ORS1114 with pollen fertility and the fertility restoration locus (*Rf*$_4$/*rf*$_4$) in the F_2 population. The letters MW indicate molecular weight (base pair or bp) ladder, and FP (fertile pool) and SP (sterile pool) indicate DNA samples bulked from 10 fertile and 10 sterile F_2 plants, respectively.

Of the 463 F_3 plants genotyped with ORS13 and ORS1114, no recombinants were detected between the two markers, while four recombinants were identified between the marker and Rf_4 loci. Combining the two populations of 576 individuals, it is estimated that the genetic distance between Rf_4 and ORS13 or ORS1114 loci is 0.69 cM.

DISCUSSION

The fertility restorer gene Rf_1 of CMS PET1 is universally used in hybrid seed production and this gene was assigned to linkage group 13 of the public SSR genetic map (Gedil et al., 2001; Yu et al., 2003; Horn et al., 2003; Kusterer et al., 2005), or linkage group 2 of the RFLP genetic map (Jan et al., 1998). We identified CMS GIG2 from wild species *H. giganteus* and incorporated the CMS into cultivated sunflower. It represents a new source of male-sterile cytoplasm different from CMS PET1. We also identified the fertility restoration gene Rf_4 for CMS GIG2 from *H. maximiliani* 1631. The Rf_4 gene is completely dominant and tightly links to the markers ORS13 and ORS1114 on linkage group 3 of the public SSR genetic map. ORS13 and ORS1114 were reported to be 11 cM apart (Tang et al., 2003), but co-segregated in our F_2 and F_3 populations of 576 plants. The possible reason for the difference could be the multi-allelic differentiation at one of the marker loci.

The estimated genetic distance between Rf_4 and the cosegregated markers is less than 1 cM. Therefore, the markers ORS13 and ORS1114 can be used to track the linked Rf_4 gene in sunflower hybrid breeding programs. Further fine mapping covering the Rf_4 gene region and screening of the BAC/BIBAC libraries will allow us to clone the Rf_4 gene in the future (Feng et al., 2006).

ACKNOWLEDGEMENTS

We thank Lisa Brown for assistance in greenhouse and field experiments, and Jinguo Hu, Bing Yue, Chenggen Chu, Caifeng Li, and Ana Capatana for technical assistance and valuable discussion. We also thank Gerald J. Seiler and Brady A. Vick for critical review of the manuscript. This work was supported by funding from the USDA-ARS National Sclerotinia Initiative grant awarded to CCJ.

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Germoplasma mejorado de girasol de la EEA Pergamino

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RESUMEN

Se evaluaron 85 líneas estabilizadas con valor agregado en algún carácter o con un grado de mejora en caracteres de interés para los fitomejoradores. Las mismas fueron producto del programa de mejoramiento de la E.E.A. Pergamino INTA, obtenidas por endocria, cruzamiento y retrocruzas. Las plantas se seleccionaron por características agronómicas (altura, ciclo, vuelco, etc.), contenido aceite y calidad de aceite, y reacción a enfermedades comunes o difundidas en el cultivo de girasol en Argentina. El objetivo fue evaluar las características del material obtenido como resultado del programa y proyectar la re-orientación del mismo y la introducción de germoplasma. Se obtuvieron 26% de líneas de alto aceite, 21% de alto contenido de ácido oleico; 32% de las líneas fueron de buen comportamiento frente a *Verticillium*, 24% de buen comportamiento a Albugo. Se destacaron 2 líneas por su buen comportamiento a *Verticillium* y a Sclerotinia, 2 líneas de buen comportamiento frente a la raza 5 (770) de mildiu recientemente difundida en área de siembra de Argentina y 2 líneas con resistencia a imidazolinonas.

Palabras clave: calidad – enfermedades – germoplasma - girasol – mejoramiento.

ABSTRACT

Eighty five lines stabilized with value-added in some characters or with some degree of improvement in traits that are interesting for breeders were evaluated. These lines were part of a breeding program carried out at the E.E.A. Pergamino INTA. They were obtained by selfing, crosses and backcrosses. The plants were selected for agronomics traits (plant height, cycle, etc.), oil content and oil quality, and reaction to diseases that are either common or spread in sunflower culture in Argentina. The objective was to evaluate the traits of lines through the sunflower program, and plan new objectives and germplasm introduction. The lines obtained were 26% with high oil, 21% with high oleic acid content; 32 % with Verticillum resistance, and 24 % with good performance to Albugo. In addition, another four lines were obtained: two of them resistant to Verticillium and Sclerotinia, two resistant to Downy mildew race 5 (770) lately spread in the sowing area (Argentina) and another two with resistance to imidazolinones.

Key words: breeding – diseases – germplasm - quality – sunflower.

INTRODUCCIÓN

La introducción de germoplasma provee variabilidad, condición indispensable para el desarrollo de un programa de mejoramiento genético, dando una oportunidad para la selección de genotipos útiles para el desarrollo de cultivares.

El mejoramiento de girasol en la EEA Pergamino comenzó en el año 1938 (Bertero de Romano y Vázquez, 2003) con el desarrollo de variedades de polinización abierta, a partir de poblaciones introducidas por los inmigrantes, adaptadas a las condiciones locales y las nuevas introducciones de Europa Central. En la década del 70 con la aparición de la andro-esterilidad citoplasmática, se orientó a la obtención de líneas endocriadas para el desarrollo de híbridos (González y Mancuso, 2004). Se amplió la base genética combinando la rusticidad de las poblaciones nativas con la introducción de germoplasma que aportó fundamentalmente contenido de aceite y precocidad.

Se estudiaron hasta nueve caracteres mejorados en ochenta y cinco líneas estabilizadas obtenidos a partir de diferentes fuentes y diferente metodología de selección del material en un período de 8 años.

El objetivo fue evaluar las características del material obtenido como resultado del programa mejoramiento de girasol de la EEA Pergamino, y proyectar la re-orientación del mismo y la introducción de germoplasma.

MATERIALES Y MÉTODOS

Se evaluaron 85 líneas estabilizadas con valor agregado en algún carácter. Las mismas fueron producto del programa de mejoramiento de la E.E.A. Pergamino INTA, obtenidas por endocria, cruzamiento y retrocruzas.

Las plantas se seleccionaron por características agronómicas (altura, ciclo, vuelco, etc.), contenido y calidad de aceite, y reacción a enfermedades.

Se establecieron 3 grupos de líneas:

1. Obtenidas por autofecundación de poblaciones
2. Obtenidas por cruzamiento de líneas endocriadas
3. Obtenidas por endocría y selección a partir de un cultivar

En todas las líneas se evaluó contenido de aceite y ácido oleico, reacción a Verticillium y Albugo. En las derivadas del grupo 1 y 2 se evaluó reacción a Sclerotinia y precocidad; en las derivadas de poblaciones también reacción al mildiu de girasol producido por producida por *Plasmopara halstedii* (Farl.)Berl. & de Toni y a imidazolidonas además de rendimiento. En las del grupo 3 se evaluó también reacción al mildiu.

El contenido de aceite fue medido por resonancia magnética nuclear (NMR), considerándose de alto contenido cuando el porcentaje era superior a 50%. El contenido de oleico fue medido por cromatografía gaseosa, considerando de alto contenido cuando el porcentaje era superior a 80%

El comportamiento a *Verticillium dahliae* se evaluó por el método de inoculación artificial en plántula en invernáculo (Bugbee y Presley, 1967) de alta correlación con la incidencia a campo.

La reacción a Albugo o Roya blanca fue evaluada por apreciación visual de las pústulas a campo.

La reacción a Sclerotinia se evaluó por apreciación visual del la podredumbre del capitulo al estado de madurez fisiológica (R8) en infectación natural y por inoculación artificial de ascosporas.

La reacción al mildiu de girasol, raza 5 (770) difundida en área de siembra (Ivancovich et al., 2001), fue evaluada por el método de infección de radícula.

La evaluación de la resistencia a imidazolinonas se efectuó tratando a los materiales con una dosis de 2X de Imazamox + surfactante no iónico para facilitar la penetración del herbicida (Miller and Al-Khatib, 2002). El tratamiento se realizó al estado de 4 a 6 hojas, efectuándose la evaluación entre 7 y 10 días después de la aplicación, siguiendo la metodología de Basf.

Se evaluó el rendimiento individual en grano de las plantas “per se”, considerándose como de alto rendimiento aquellas que superaron al promedio en un 20%.

Se consideró precoz cuando el ciclo de emergencia a floración fue hasta 4 días más tardío que la línea americana HA89.

Los materiales seleccionados para cada objetivo se condujeron en sucesivos ciclos de recombinación y selección por características agronómicas (altura, ciclo, vuelco, etc.), sanitarios y calidad industrial. En una etapa posterior fueron evaluados y re-seleccionados para otros caracteres.

Para efectuar las cruzas entre líneas mantenedoras se empleó el método de castración química con ácido giberélico (Miller and Fick, 1978).

RESULTADOS Y DISCUSIÓN

En el Fig. 1, se presenta el porcentaje de líneas obtenidas para cada objetivo de selección en el programa de mejoramiento de INTA- EEA Pergamino.

Del total de 85 líneas, se obtuvieron 26% de líneas de alto contenido en aceite. Cuando se las evaluó para los otros caracteres, 36 % son de buen comportamiento a *Verticillium* y un 19% de alto contenido de oleico. Se destacaron 2 líneas: una con buen comportamiento a *Verticillium* y precoz, y otra de buen comportamiento a *Verticillium* y Albugo.

Se lograron 18 líneas (21%) de alto contenido de ácido oleico; de las cuales, el 17 % tuvo buen comportamiento a Scelerotinia y otro 17%, resistente a mildiu.

Un 32 % de las líneas fueron de buen comportamiento frente a *Verticillium*. De 27 líneas logradas, el 30% tuvo alto contenido de aceite, el 37% buen comportamiento a Albugo. Dos de estas líneas se destacaron por su buen comportamiento a Sclerotinia.

Se obtuvieron 8 líneas (9 %) de buen comportamiento frente a la raza 5 (770) de mildiu recientemente difundida en área de siembra de Argentina, destacándose 2 con resistencia a imidazolinonas.

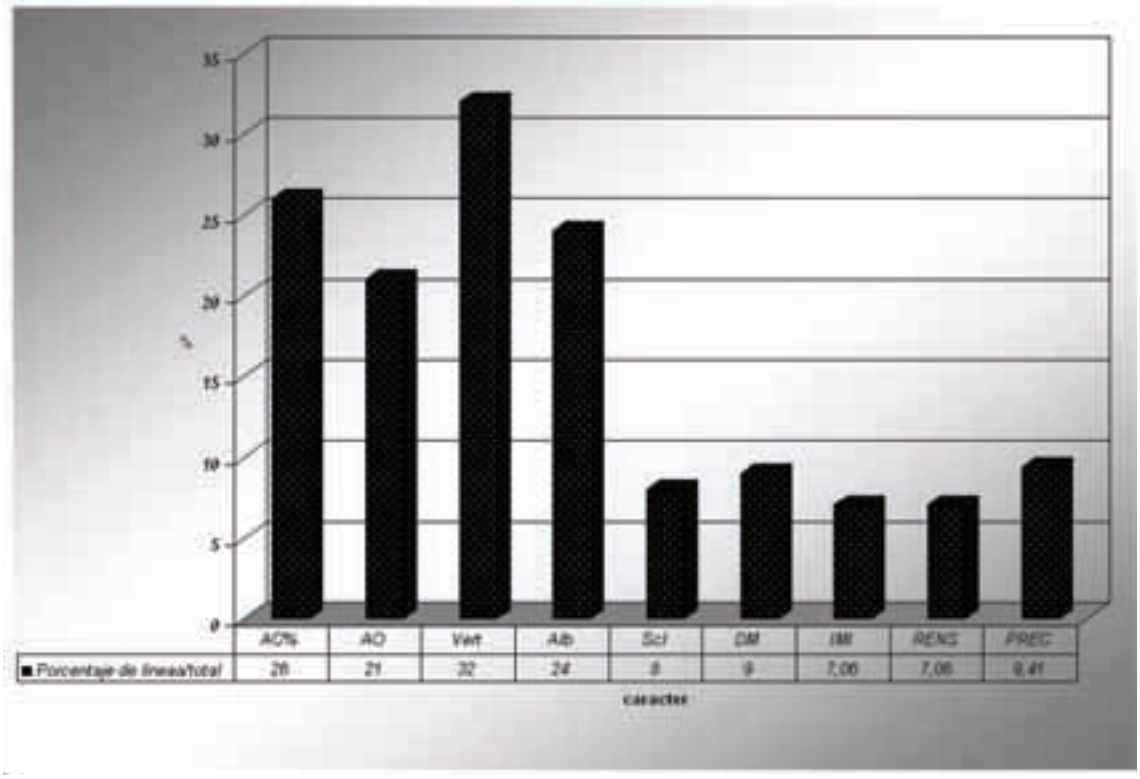

Fig. 1. Proporción de líneas obtenidas para cada carácter principal de selección. AC%: alto contenido porcentual de aceite, AO: alto contenido de ácido oleico, Vert: buen comportamiento frente a Verticillium, Alb.: buen comportamiento frente a albugo, Scl: buen comportamiento frente a Sclerotinia de capítulo, DM: buen comportamiento frente a mildiu, IMI: resistencia a imidazolinonas, Rens: alto rendimiento de semilla, Prec: ciclo corto a floración.

Un 24% de líneas fue de buen comportamiento frente a Albugo. Dentro del grupo hubo un 45 % de buen comportamiento frente a Verticillium, 15% de alto contenido en ácido oleico y 11% de buen comportamiento frente a Sclerotinia y mildiu. El 8 % de las líneas tuvo buen comportamiento frente a Sclerotinia de capítulo. Dentro de este grupo el 43 % tuvo alto contenido de ácido oleico; el 29 % buen comportamiento frente a Albugo y el 14 % buen comportamiento frente a Verticillium.

El 7 % de las líneas, 6 en total, presentó resistencia a imidazolinonas, 2 fueron resistentes a mildiu y de buen comportamiento frente a Albugo.

Es de destacar que todas las líneas tienen un rendimiento aceptable de producción de semilla “per se”, pero el 7 % se destacó por su alto rendimiento.

De las 8 líneas seleccionadas por precocidad, ciclo a floración, 3 fueron de buen comportamiento a Verticillium y una de alto contenido de aceite

En la Tabla 1 se muestran las líneas obtenidas por endocría de poblaciones que permitieron obtener resultados en el mejoramiento de todos los caracteres.

Las líneas derivadas del compuesto P1C2 se caracterizaron por alto rendimiento de semilla, resistencia a mildiu y buen comportamiento frente a Albugo. Las líneas derivadas del compuesto P2 se caracterizan por su precocidad (ciclo a floración).

De VNNIMK1646 se obtuvo resistencia a Verticillum.

La población ND 01 se caracterizó por derivar líneas de alto contenido de oleico y alto contenido de aceite.

A partir de la población local PGRK se obtuvieron líneas de alto rendimiento de semilla.

El Compuesto P4 aportó líneas de buen comportamiento sanitario, especialmente con relación a Verticillium y Albugo así como alto contenido de aceite.

El compuesto restaurador PRII permitió obtener líneas restauradoras de alto contenido de aceite y de buen comportamiento frente a Verticillium.

La población que dio origen a las líneas con resistencia a imidazolinonas fue obtenidas por cruzamiento y retrocruza de *Helianthus annuus* silvestre por líneas de North Dakota.

En la Tabla 2 se muestran las líneas derivadas de endocría de cruzamientos de líneas estabilizadas (BxB).

Tabla 2. ***Líneas obtenidas a partir de cruzamiento de líneas estabilizadas***

	AC.%	AO	Vert	Alb	Scl	Precoz	Total
DxT, HA 300; V112; E..	1					1	2
AXB, DXT, HA 300.	1		1			1	3
HA 343 x Hib F1 nº2		10		3	1		14
AxB /BXC			3	3			6
MP 557/N.Bellocq/HA 89			1			1	2
KLM 280/HA 300			1			1	2
RK 416/HA 89			1	1	1		3
DxT/HA 89.				1	2		3
MP 83/2/HA 89				1	1		2
Total	2	10	7	9	5	4	37

(1) Referencias: AC% alto contenido porcentual de aceite; AO: alto contenido de ácido oleico; Vert: buen comportamiento frente a Verticillium; Alb.: buen comportamiento frente a albugo; Scl.: buen comportamiento frente a Sclerotinia de capítulo; Precoz: ciclo corto a floración

Se destaca el cruzamiento AXB / BXC, del cual se derivaron el mayor número de líneas con resistencia a Verticillium y buen comportamiento frente a Albugo. HA 343x hib F1nº2 permitió lograr líneas de alto contenido de ácido oleico, buen comportamiento frente a Sclerotinia y a Albugo. Las cruzas derivadas de DXT permitieron mejorar la precocidad y el contenido de aceite.

Los resultados obtenidos a partir de la endocría y selección de fuentes de origen diverso, muestran al comportamiento frente a Verticillium y al alto contenido de aceite como los caracteres en los que se lograron líneas de las fuentes más diversas (Tabla 3).

Tabla 3. ***Líneas obtenidas a partir de endocría y retrocruzas de diferentes fuentes***

	AC.%	AO	Vert	DM	Alb	Total
M731	3		2			5
ACA 860	2		4		1	7
AGR 379	1					1
G 105	1		2		2	5
SB	2					2
9021		1	1			2
9014		1	1		1	3
2341				5		5
Total	9	2	10	5	4	30

(1) Referencias: AC%: alto contenido porcentual de aceite; AO: alto contenido de ácido oleico; Vert: buen comportamiento frente a Verticillium; Alb.: buen comportamiento frente a albugo; DM: buen comportamiento frente a Downy mildew;

En la Fig. 2 se presenta el total de líneas obtenido en el período de tiempo considerado

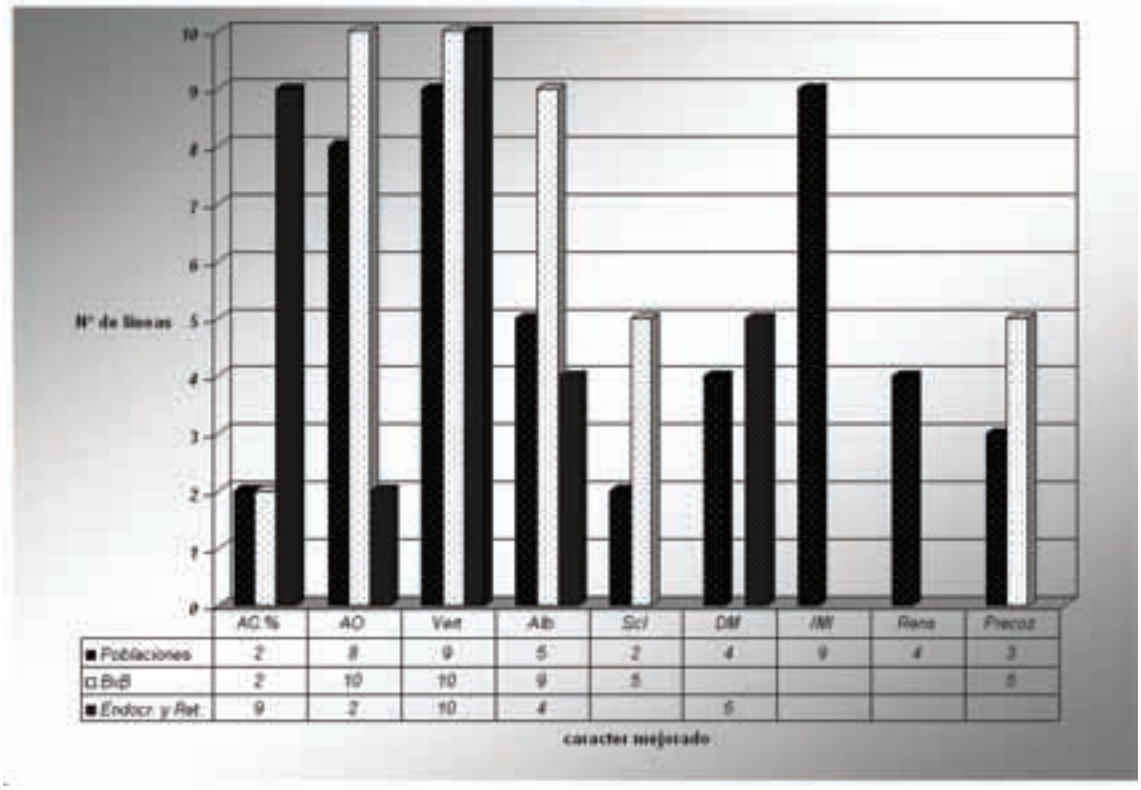

Fig. 2. Resultados de los tres grupos. AC%: alto contenido porcentual de aceite; AO: alto contenido de ácido oleico; Vert: buen comportamiento frente a Verticillium; Alb.: buen comportamiento frente a albugo; Scl.: buen comportamiento frente a Sclerotinia de capítulo; DM: buen comportamiento frente a mildiu ; IMI: resistencia a imidazolinonas; Rens: alto rendimiento de semilla; Precoz: ciclo corto a floración.

El mayor número de líneas se obtuvo a partir de poblaciones y de cruzas de líneas estabilizadas, algunas de las cuales tienen en su pedigrí líneas derivadas de estas poblaciones.

La introducción de fuentes de Alto Oleico y resistencia a imidazolinonas desde North Dakota permitieron el desarrollo de numerosas líneas con estos caracteres en distintos fondos genéticos.

El alto número de líneas con resistencia a Verticillium, se explica por el hecho que esta enfermedad endémica acompaño la expansión del cultivo en la región girasolera argentina. La resistencia a Sclerotinia y mildiu provino de fuentes de diferente origen.

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Heterosis for yield and oil content of sunflower lines developed from bi-parental populations

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ABSTRACT

Improvement of grain yield and oil content remains the major objective of many sunflower breeding programmes worldwide. Different sets of S_3 CMS lines developed from bi-parental populations were evaluated in hybrid combination at two sites to estimate heterosis for oil content and grain yield. Results showed that standard heterosis of testcross hybrids of S_3 CMS lines ranged from -14.4 to 16.0% and -5.9 to 11.4% for yield and oil content, respectively, indicating their potential for obtaining specific combinations of superior hybrids between the S_3 CMS lines and the testers in comparison to the current checks. High panmictic and positive heteroses were obtained, 49.8 to 114.9% for yield and 6.1 to 28.4% for oil content suggesting that if open-pollinated varieties (OPVs) were to be developed from the bi-parental populations, their performance would be inferior to their corresponding hybrids for the traits evaluated.

Keywords: Bi-parental - cytoplasmic male sterility (CMS) - heterosis - panmictic.

INTRODUCTION

Sunflower production in South Africa is done under rainfed conditions and is mainly concentrated in the Northwest and Free State provinces, which are characterised by low rainfall, shallow and/or sandy soils. The major breeding objectives of the public sector sunflower programme in South Africa have remained unchanged, namely, continued improvement for yield, yield stability and oil content (Chigeza, 2007). Quantitative evaluations of traits such as yield require vigorous testing in two or more environments with a view to either managing or exploiting genotype-environment interaction (Eisemann et al., 1990). In early stages of selection, testing in two or more environments is constrained by the limited amount of seed and large numbers of entries involved. Thus, multi-environment trials are normally carried out with advanced generation materials.

In sunflower, the cytoplasmic male sterility (CMS) system, which involves the use of CMS line (A), maintainer line (B) and fertility restorer line *(Rf)*, has allowed breeders to exploit heterosis through the development of three-way and single-cross hybrids (Miller et al., 1980). Heterosis, which is defined as some measure of F_1 performance relative to its parental performance (Shull, 1952), has extensively been exploited in cross-pollinated crops although its genetic basis is still not well understood (Lamkey and Edwards, 1998). Several types of heterosis have been proposed. In studies involving cross-pollinated crops, such as maize and sunflower, estimation of heterosis for yield and other agronomic traits is either based on mid-parent (MP) or better parent (BP) heterosis and is often expressed as a percentage (Duvick, 1999). The major drawback of these approaches is that they lack relevance in applied plant-breeding approaches since inbreds are normally not the final product grown by the farmers. Nonetheless, they can be useful in determining the price ratio of seed and grain in seed production. Estimation of heterosis based on MP or BP requires that both parental lines are also included in the trials or planted adjacent to the testcrosses, which may not be practically possible when large number of testcross hybrids are involved. Another measure of heterosis is the relative standard heterosis, defined as the superiority of experimental F_1 hybrids as compared to the performance of the checks (Patnaik et al., 1990). The use of standard heterosis is based on the argument that in developing new hybrids, the aim is to surpass the performance of existing commercial hybrids in the trait of interest. While the argument is practically valid, the check may not be in any way related to the experimental hybrids being developed, making an interpretation of heterosis based on the genetic background of the parental populations difficult (Lamkey and Edwards, 1998). Panmictic-midparent heterosis, defined as the difference between the mean of the F_1 hybrid and the mean of the two random-mating parental populations (Lamkey and Edwards, 1999), can be used complementary to standard heterosis if the genetic background of the parental population is to be interpreted. In sunflower, instead of using the mean performance of both populations, mid-parent

heterosis, it is more appropriate to use the female parent population because female parents are single-headed, a trait required in sunflower production. On the other hand, the male parents are multi-headed so as to ensure a long period of pollen availability to the female during seed production. Hence, direct improvement for yield for the male parent is not practiced, so that to interpret heterosis using the male line or parental population will not be relevant.

Thus the objectives of this study were to quantify standard and panimictic heterosis of testcrosses formed from lines derived from different bi-parental populations of sunflower.

MATERIALS AND METHODS

A total of 240 genotypes divided into seven unequal sets based on source of the seed or genetic relationships were used for study as indicated in Table 1. The populations (Pop1, Pop 2, Pop3 and Pop4), from which the S_3 CMS lines were derived, were formed by crossing two B lines with different genetic backgrounds. The male testers T1 and T2 were randomly selected from the improved male lines in the ARC sunflower breeding programme.

Table 1. Genetic material used for the study.

Set	Number of genotypes	Description of the material
1	88	Testcross hybrids formed by crossing two male testers T1 and T2 to 44 S_3 CMS lines developed from Pop 1, S_3 CMS inbreds coded Pop1-1 CMS, …Pop1-44 CMS.
2	24	Testcross hybrids formed by crossing two male testers T1 and T2 to 12 S_3 CMS lines developed from Pop 2, S_3 CMS inbreds coded Pop2-1 CMS, …Pop2-12 CMS.
3	52	Testcross hybrids formed by crossing two male testers T1 and T2 to 26 S_3 CMS lines developed from Pop 3, S_3 CMS inbreds coded Pop3-1 CMS, …Pop3-26 CMS.
4	54	Testcross hybrids formed by crossing two male testers T1 and T2 to 27 S_3 CMS lines developed from Pop 4, S_3 CMS inbreds coded Pop4-1 CMS, …Pop4-27 CMS.
5	12	Testcross hybrids formed by crossing two male testers T1 and T2 to 6 parental inbreds, H55, H52, HA89, KB61, KB16 and KB189 mated in pairs to produce the bi-parental populations Pop1, Pop2, Pop3 and Pop4.
6	4	Bi-parental populations Pop1, Pop2, Pop3 and Pop4
7	6	Six commercial checks AGSUN8251, AGSUN5551, PAN7033, PAN7355, Mydelo and DKF 68-22
Total	240	

The 240 entries were then planted in an alpha (0,1) design with two replications at two locations, Potchefstroom, 26.745°S, 27.083°E situated in the Northwest Province and Bothaville, 27.235°S, 26.67°E located in the Free State Province, South Africa. Both trials were machine planted in January 2007 and then thinned at three weeks after emergence. The plant population at Potchefstroom was 36,000 plants/hectare, while that of Bothaville was 28,000 plants/hectare. Recommended agronomic practices were followed at both sites, include basal application of 150 kg/ha fertilizer (3N:2P:1K) incorporated into the seedbed before planting. A further 28 kg/ha N was applied at four weeks after emergence. Grain oil concentration was determined on 12-g, air-dried achenes samples by nuclear magnetic resonance with a Newport Analyzer (Newport-Oxford Instruments Ltd, New-port Pagnell, Buckinghamshire, England).

Data were analysed using GENSTAT version 9, adopting the restriction maximum likehood (REML) methodology (Paterson and Thompson, 1971). The analysis was done using the mixed model procedure based on the reasoning given by Piepho and Möhring (2006), where genotypes within sets were regarded as random. Using the notation of de la Vega and Chapman (2006) the phenotypic observation y_{ijkmp} is the performance of genotype *i* nested within set *j*, in incomplete block *n*, of replicate *m* of environment *k*., was given by the following mixed model:

$$y_{ijkmn} = \mu + e_k + (r/e)_{km} + (b/r/e)_{kmn} + s_j + (es)_{jk} + (g/s)_{ij} + (eg)_{ik}\,(s_j) + \varepsilon_{ijkmn}$$

where μ is the grand mean; e_k the fixed effect of the environment *k;* $(r/e)_{km}$ the random effect of the replicate *m* nested within the environment *k*; $(b/r/e)_{kmn}$ the random effect of the incomplete block *n* nested within the replicate *m* of the environment *k*; s_j the fixed effect of the set *j*; $(es)_{jk}$ is the fixed effect of the interaction of environment *k* and set *j*; $(g/s)_{ij}$ the random effect of genotype *i* nested within set *j;* $(eg)_{ik}\,(s_j)$ the random effect of the interaction of the environment *k* with genotype *i* nested within set *j* and ε_{ijkmn} is the random error term.

Relative standard heterosis was estimated as the percentage increase or decrease in the performance of genotypes in comparisons to the mean of checks while panmictic heterosis was estimated as the

percentage increase or decrease in the performance of the testcross hybrids compared to the corresponding bi-parental population mean.

RESULTS AND DISCUSSION

Variance components

The combined analysis across the two environments showed significant variation among the genotypes nested within the sets for oil content and yield. The environment main effect was significant for oil content (P<0.01) but not significant for yield (data not shown). The genotypes nested within the sets and their interaction with the environment were the largest source of variation for both percent oil content and yield as indicated by the relative magnitude of the variance components, Table 2.

Table 2. Estimated variance components (±SE) estimates for yield and percent oil content of the sunflower genotypes across the two environments**.**

Parameter	Variance components estimates	
	Yield	Oil Content
$\sigma^2_{r/e}$	1712 ± 2883*	1.082 ± 1.086**
$\sigma^2_{b/r/e}$	2849 ± 4104ns	0.006 ± 0.020ns
$\sigma^2_{g/s}$	29624 ± 12459**	0.352 ± 0.207**
$\sigma^2_{(eg)s}$	39769 ± 16505**	2.367 ± 0.26**
σ^2_{ε}	235132 ± 15533	0.846 ± 0.058

* P<0.05; **P<0.01; for corresponding mean square; ns-not significant.

Genotype-environment interaction is normally caused by the magnitude of the variance of genotype performance across environments and changes in genotypic ranks between environments (Allard and Bradshaw, 1964). In this study genotype-environment interaction was a result of the magnitude of variance for yield but for oil content, the genotype-environment interaction was a result of both magnitude and change in rank order of the genotypes in the different two environments as the environment main effect was also significant (data not shown). The ratio of $\sigma^2_{(eg)s}$ to $\sigma^2_{g/s}$ was 1.34 for yield and 6.7 for oil content indicating also that genotype-environment interaction is more pronounced for percent oil content than yield.

Mean grain yield and heterosis

The mean grain yield ranged from 1040 kg/ha (Pop1, Set 6) to 2357 kg/ha (Pop1-1 CMS x T1, Set 1), Table 3. The commercial checks had a mean yield range of 1565 to 2124 kg/ha.

Table 3. Mean performances, range for yield, relative standard heterosis and panmictic heterosis of the genotypes nested within sets across the two locations

	Sets						
	1	2	3	4	5	6	7
Yield performance (kg/ha)							
Mean	1932	1947	1908	1811	1252	1058	1864
Range	1609–2357	1635–2235	1626–2223	1596–2164	1076–1556	1038–1076	1524–2160
SE[1]	168.6	171.4	169.3	169.3	175.1	189.2	183.2
Relative standard heterosis (%)							
Mean	3.6	4.5	2.4	-2.8	-32.8	-43.2	
Range	-13.7–26.4	-12.3–12.8	-12.8–19.3	-14.4–16.0	-42.3– -16.5	-42.4– -44.2	
Panmictic heterosis (%)							
Mean	79.9	87.2	78.2	69.7			
Range	49.8–119.5	57.2–114.9	51.8–107.6	82.5–109.5			

[1]SE based on the means of genotypes within sets

The relative standard heterosis ranged from -44.2% to 26.4%. The mean standard heterosis was negative for sets 4, 5 and 6 indicating better performance of commercial checks in comparisons to genotypes in the stated sets. The panmictic heterosis for yield ranged from 49.8% to 119.5% indicating low yields of the bi-parental populations compared to the testcross hybrids.

Mean percent oil content and heterosis

The mean percent oil content ranged from 33.1% (Pop3, Set 6) to 43.3% (testcross hybrid, Pop3-8 CMS x T1, Set 3), Table 4. Within the sets the range for oil content was small indicating some level of similarity of the genotypes and the past efforts on selection for high oil content.

Table 4. Mean performances, range for oil content, relative standard heterosis and panmictic heterosis of the genotypes nested within sets across the two locations

	Sets						
	1	2	3	4	5	6	7
Oil content performance (%)							
Mean	39.8	39.7	40.2	39.7	39.2	34.0	38.9
Range	36.6–41.7	37.6–41.0	37.2 43.3	37.3–41.4	36.5–40.3	33.1–34.5	38.0–40.0
SE[1]	0.43	0.43	0.43	0.43	0.43	0.43	0.43
Relative standard heterosis							
Mean	2.3	2.0	3.3	2.0	0.8	-12.5	-
Range	-5.9–7.4	-3.3–5.5	-4.4–11.4	-4.1–6.5	-6.1–3.5	-11.3– -13.2	-
Panmictic heterosis							
Mean	15.4	17.4	19.1	16.4			
Range	6.1–21.1	11.2–21.3	10.1–28.4	9.3–21.4			

[1]SE based on the means of genotypes within sets.

The commercial checks had a mean range of 38.0 to 40.0% with a set mean of 38.9%. The relative standard heterosis ranged from –13.2 to 11.4. The mean standard heterosis was negative for set 6 but positive for other sets including the set with inbreds that were used for developing the bi-parental populations. The panmictic heterosis for percent oil ranged from 6.1 to 28.4% indicating that testcross hybrids had a significant advantage over their populations for yield.

In conclusion, the study revealed a high and moderate significant variance for environment-genotype nested within sets interaction for percent oil content and yield, respectively. The relative standard heterosis estimates showed that 3 out of 4 sets from which the S_3 CMS lines were derived had positive mean heterosis indicating the potential of developing new hybrids from specific line x tester combination that would do better for yield and percent oil content as compared to the current commercial hybrids. The panmictic heterosis was highly positive for all the four S_3 CMS line sets when crossed to the two testers. In cross-pollinated crops the panmictic heterosis is useful in whether to develop hybrids or open-pollinated varieties (OPVs). A low and negative panmictic heterosis for yield will favour development of OPVs, while it will be logical to develop hybrids if the panmictic heterosis is high and positive.

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A modifying gene affecting gamma-tocopherol content in sunflower

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ABSTRACT

High levels of gamma-tocopherol confer greater oxidative stability to sunflower oil. Several lines with high gamma-tocopherol content have been developed and in all of them the trait has been found to be controlled by recessive alleles at the *Tph2* locus, underlying a gamma-tocopherol methyltransferase. Genetic studies involving crosses between standard lines and lines with high gamma-tocopherol content reported bimodal segregation patterns with clear-cut classes including low gamma-tocopherol (<10%) and high gamma-tocopherol (>85%) levels, respectively but not intermediate levels. Intermediate gamma-tocopherol content ranging from 10 to 85% has only been reported in the isolation process of the line IAST-1, derived from a mutagenic treatment. The objective of this research was to investigate the occurrence of intermediate gamma-tocopherol content in crosses involving the high gamma-tocopherol line IAST-1. Plants of the high gamma-tocopherol lines T2100 and IAST-1, and the standard line HA89 were crossed and the F_2 seed generation was analysed for seed tocopherol profile. F_2 seeds from all the $F_{1:2}$ families from the crosses between HA89 and T2100 followed bimodal distributions with clear-cut classes fitting a 3:1 (<5%:>90%) ratio, corresponding to the expected segregation of the recessive alleles *tph2*. In addition to the 3:1 ratio, a 13:3 (<80%:>90%) ratio was identified in $F_{1:2}$ families from the crosses between HA89 and IAST-1, which included F_2 seeds with intermediate levels of gamma-tocopherol (5 to 80%). Intermediate levels of gamma-tocopherol were also observed in some $F_{1:2}$ families derived from the crosses between T2100 and IAST-1. The results suggested the presence of a modifying gene that produced intermediate gamma-tocopherol levels in combination with the *tph2* alleles.

Key words: gamma-tocopherol – modifying gene – oil quality – tocopherols

INTRODUCTION

Conventional sunflower seeds mainly contain alpha-tocopherol, which accounts for more than 90% of the total tocopherols. Several lines with modified tocopherol profiles have been developed. Demurin (1993) reported the lines LG-15 and LG-17, with increased levels of beta-tocopherol (50%) and gamma-tocopherol (95%), respectively. Both lines were developed from segregating accessions identified in a germplasm collection. Also in the course of germplasm evaluation, Velasco et al. (2004a) identified variations for beta- and gamma-tocopherol content, which allowed the development of the lines T589 and T2100, with increased levels of beta-tocopherol (>30%) and gamma-tocopherol (>85%), respectively. Additional variation for gamma-tocopherol content was created in sunflower by using chemical mutagenesis (Velasco et al., 2004b). The authors identified two M_2 seeds, derived from different M_1 plants, with increased gamma-tocopherol contents of 19.2% and 96.7%, respectively. M_3 progenies from the M_2 seed with 96.7% bred true for high gamma-tocopherol content, containing more than 90% gamma-tocopherol, which led to the development of the line IAST-540. M_3 progenies from the M_2 seed with 19.2% gamma-tocopherol segregated from zero to 84.6% gamma-tocopherol. Selection for high gamma-tocopherol content produced the line IAST-1, with stable high gamma-tocopherol content.

Genetic studies conducted by Demurin et al. (1996) concluded that the increased levels of beta-tocopherol were produced by recessive alleles at the *Tph1* locus, whereas the increased levels of gamma-tocopherol were the result of recessive alleles at the *Tph2* locus (Demurin et al., 1996). Similarly, Velasco and Fernández-Martínez (2003) reported the presence of recessive alleles at a single locus underlying the increased beta-tocopherol content in T589 and the high gamma-tocopherol content in T2100 seeds. Comparative genetic studies concluded that *tph1* alleles were present in both LG-15 and T589 lines (Demurin et al., 2004; Vera-Ruiz et al., 2005), and *tph2* alleles were present in the high gamma-tocopherol lines LG-17, T2100, IAST-540, and IAST-1 (Demurin et al., 2004; García-Moreno et al., 2006). The *Tph2* gene underlies a gamma-tocopherol methyltransferase (Hass et al., 2006). Genetic studies involving crosses between lines with high gamma-tocopherol content and lines with wild-type high alpha-tocopherol content have reported bimodal segregation patterns with clear-cut classes including low gamma-tocopherol (<10%) and high gamma-tocopherol (>85%) levels, respectively (Demurin et al., 1996; Velasco and Fernández-Martínez, 2003). Intermediate gamma-tocopherol content ranging from 10

to 85% in germplasm segregating for *tph2* alleles has only been reported so far in the isolation process of IAST-1. The objective of this research was to investigate the occurrence of intermediate gamma-tocopherol content in crosses involving the high gamma-tocopherol line IAST-1.

MATERIALS AND METHODS

The study included the sunflower lines T2100 and IAST-1, with high gamma-tocopherol content (>85%), and the standard line HA89, with high alpha-tocopherol content (>95%). T2100 was developed from an accession of the open pollinated cultivar 'Peredovik' (Velasco et al., 2004a). IAST-1 was isolated after chemical mutagenesis on seeds of several 'Peredovik' accessions (Velasco et al., 2004b). HA89 is an oilseed maintainer line released by the Texas Agricultural Experiment Station and the USDA-ARS in 1971.

Twenty-four half seeds of HA89, T2100, and IAST-1 were nondestructively analyzed for tocopherol profile, germinated and planted in pots under open air conditions in spring 2005. Plants of the three lines were crossed following an incomplete diallel design. Half seeds of the parents as well as F_1 half seeds were analysed for tocopherol profile. F_1 and parent half seeds were sown in March 2006 and the corresponding plants were grown in pots under open air conditions. F_1 plants were self-pollinated to obtain the F_2 generation.

Twenty-four to 96 F_2 half seeds from 12 to 24 F_1 plants from each cross were analysed for tocopherol profile following the procedure reported by Velasco et al. (2004b).

RESULTS AND DISCUSSION

Seeds of the high gamma-tocopherol lines T2100 and IAST-1 showed uniformly high gamma-tocopherol content (>95% of the total tocopherols). Seeds of the standard line HA89 showed uniformly high alpha-tocopherol content (>95%). F_2 seeds from all the $F_{1:2}$ families from the crosses between HA89 and T2100 followed bimodal distributions with clear-cut classes characterized by low (<5%) and high (>90%) gamma-tocopherol content that fitted a 3:1 (low:high) segregation ratio (Fig. 1), corresponding to the expected segregation of the recessive alleles *tph2* (Demurin et al., 1996; Velasco and Fernández-Martínez, 2003).

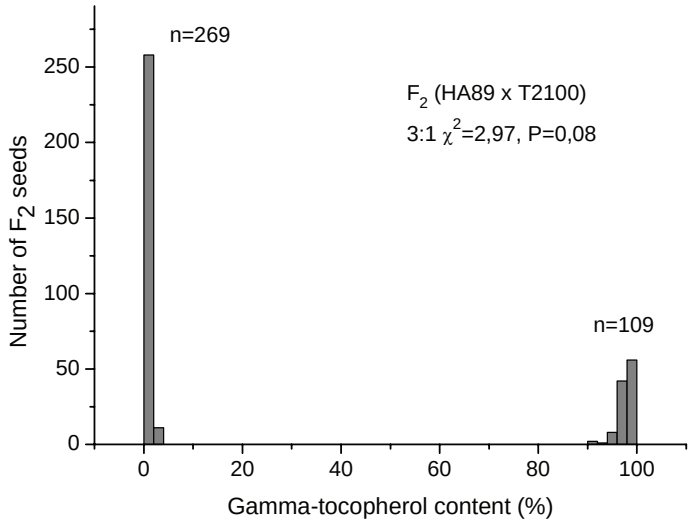

Fig. 1. Gamma-tocopherol content (% of the total tocopherols) in F_2 seeds from the cross between the high gamma-tocopherol line T2100 and the standard line HA89.

Two different segregation patterns were identified in $F_{1:2}$ families from the crosses between the standard line HA89 and the high gamma-tocopherol line IAST-1. The first segregation pattern was similar to that observed for the cross between HA89 and T2100, with F_2 seeds distributed into low and high gamma-tocopherol classes that fitted a 3:1 segregation ratio (Fig. 2A). The second segregation pattern showed the particularity of the presence of F_2 seeds with intermediate levels of gamma-tocopherol content (5 to 80%). The high gamma-tocopherol (>90%) class included 3 out of every 16 F_2 seeds (Fig.

2B), suggesting the presence of a second recessive gene that produced intermediate gamma-tocopherol levels in combination with the *tph2* alleles in a homozygous condition.

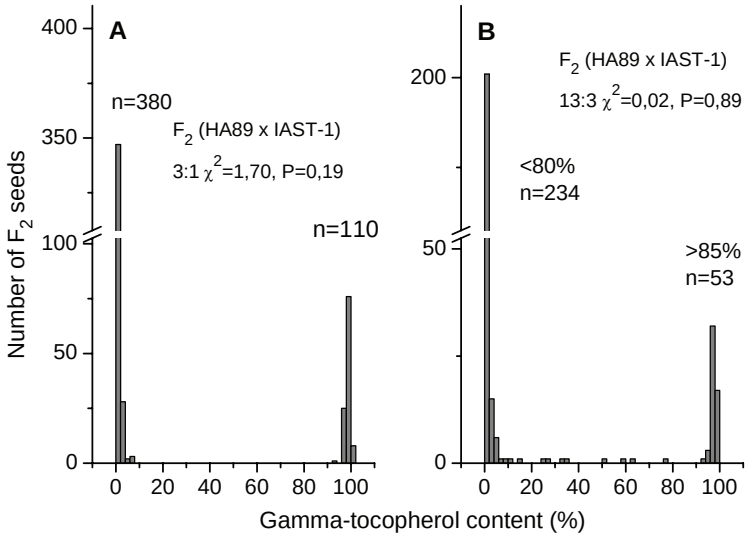

Fig. 2. Gamma-tocopherol content (% of the total tocopherols) in F_2 seeds from the cross between the high gamma-tocopherol line IAST-1 and the standard line HA89.

Two different patterns of gamma-tocopherol distribution were also identified in F_2 seeds from $F_{1:2}$ families derived from the crosses between the high gamma-tocopherol lines T2100 and IAST-1. F_2 seeds had uniformly high gamma-tocopherol content in some $F_{1:2}$ families (Fig. 3A), whereas other families showed segregation for a wide range of intermediate gamma-tocopherol levels (Fig. 3B).

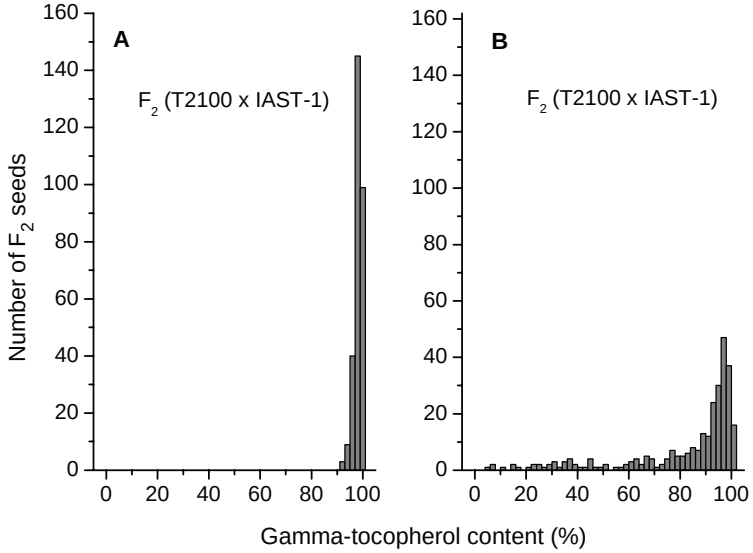

Fig. 3. Gamma-tocopherol content (% of the total tocopherols) in F_2 seeds from the cross between the high gamma-tocopherol lines T2100 and IAST-1.

A previous genetic study concluded that the high gamma-tocopherol lines T2100 and IAST-1 shared the alleles *tph2*, as both the F_1 and F_2 seed generations from crosses between them showed uniformly a high gamma-tocopherol content (García-Moreno et al., 2006). The present research work suggested the presence of a modifying gene affecting gamma-tocopherol content in IAST-1. The modifying gene produced a reduction in gamma-tocopherol content from high (>90%) to intermediate (5 to 80%) levels in

seeds with expected allelic configuration *tph2tph2*. This effect was observed in some F_1 plants from the crosses of IAST-1 with HA89 and T2100, but not in others. Additionally, the genetic effect of the modifying gene was not expressed in seeds of the IAST-1 parent grown in the same environment. Modifying genes affecting high oleic acid content have been reported in sunflower, leading to supression of the trait (Lacombe et al., 2001) or a strong distortion of segregation patterns (Fernández-Martínez et al., 1989). Further characterization of the modifying gene affecting high gamma-tocopherol content in sunflower is currently under way.

ACKNOWLEDGEMENTS

The research was supported by Ministerio of Educación y Ciencia, research project AGL2007-62834.

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QTL for capitulum resistance to *Sclerotinia sclerotiorum* in sunflower

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ABSTRACT

Quantitative trait loci (QTL) for sunflower capitulum resistance to head rot (*Sclerotinia sclerotiorum*) were studied on a population of recombinant inbred lines (RIL), by infection at flowering with suspensions of ascospores. In addition, hybrids made between the RIL and 2 tester lines were observed under natural *Sclerotinia* attack in yield trials and under re-enforced *Sclerotinia* attack in trials made specially to observe disease reaction**.** The inbred lines were genotyped with RFLP and SSR markers which were mapped to give correspondence between different widely published maps and so permit comparison of QTL results in different sunflower populations. Two or three QTL were shown for each character, generally each representing only 8-15% of phenotypic variance. Mostly the same QTL were found for percent infection and latency index as in results for F_3 families of the same population or other populations of F_3 progenies or RIL. They were also observed in the hybrids, but the latter also showed significant QTL on other linkage groups.

Key words: ascospores – branching – hybrids – natural attack – recombinant inbred lines – tester.

INTRODUCTION

Improved knowledge of the inheritance of quantitative characters is most often obtained by studies of QTL, number, proportion of phenotypic variance explained and whether they are the same or different in different genotypes and for characters observed in different environments.

From 1995 to 2000, a recombinant inbred line (RIL) population was developed from 2 inbred sunflower lines, XRQ and PSC8, which not only showed considerable polymorphism for resistance to diseases including *Sclerotinia sclerotiorum*, Phomopsis (*Diaporthe helianthi)* and downy mildew (*Plasmopara halstedii*) and to drought but also gave some of the best yields of varieties registered in France in that period. An AFLP map, anchored to the Cartisol map (Gentzbittel et al., 1995) with RFLPs was developed for the F_2 generation and permitted mapping of the downy mildew resistance gene *Pl5* (Bert et al., 2001) and of QTL for a wide range of agronomic and disease resistance characters of the F_3 families (Bert et al., 2002a, 2002b). To enable more detailed studies, in particular of yield characteristics of hybrids, RIL were obtained, and to develop a map of this cross comparable with other sunflower genetic maps, these lines were genotyped with SSR, but also with RFLP to enable comparison with, and continued use, of QTL and genes mapped previously. This paper reports QTL for resistance to *Sclerotinia* head rot, not only concerning reactions of inbred lines to a resistance test but also for hybrids under natural attack, for which little data has so far been published.

MATERIALS AND METHODS

Sunflower recombinant inbred lines. 279 RIL were obtained by single seed descent from a cross of INRA lines XRQ (bred from a cross of USDA line HA89 and the Russian open pollinated variety Progress) and PSC8 (bred from a population under recurrent selection for *Sclerotinia* resistance). These RIL were studied *per se* and were crossed with 2 tester lines representing maintainer (F) and restorer (R) sunflower populations, according to their restorer (*Rf1*) and recessive branching (*b1*) gene status, in order to obtain male fertile unbranched hybrids. Tester F- CMS line PGF650 (Soltis) was crossed with branched and unbranched RIL carrying *Rf1* and 181 hybrids were obtained. Tester R- Branched genic male sterile line 83HR4gms (INRA) was crossed with unbranched RIL and 130 hybrids were obtained.

Measurements of Sclerotinia resistance. The RIL were subjected to *Sclerotinia* ascospore infections at flowering (2 replications of 25 plants) following the method described by Tourvieille and Vear (1984).

Observations of first symptoms were made twice a week to obtain the mean percentage attack for each line and a latency index, which is the mean delay from infection to symptom appearance compared with check lines infected at the same time. QTL were calculated for the 2 characters. The hybrids were observed in 2001 and 2002 in trials (2 replications of 50 plants) in a field devoted to *Sclerotinia* observations, with natural attack re-enforced by provision of sclerotia in the soil and irrigation at flowering to maintain liquid water on the florets for at least 48h for all plants, whatever their flowering date (Vear and Tourvieille, 1982). Observations were made at maturity of percentage attack, compared with check hybrids (trials 01ROPGF, 01RO83HR4, 02ROPGF and 02RO83HR4). In addition, in 2001, observations were made of a significant natural attack on yield trials (2 replications of 100 plants) in Eure et Loir department, near Paris (trials 01RNPGF and 01RN83HR4). The 3 series of data were analysed for QTL, but the mean was studied only for hybrids with tester 83HR4 since not all the hybrids with PGF650 were present in the re-enforced *Sclerotinia* field.

Marker analysis. DNA extraction was performed from young leaves of greenhouse-grown plants using a CTAB method (Rogers and Bendich, 1985). Digestion by restriction enzyme (*Eco*R1 and *Hin*d3) and Southern hybridization were carried out as described by Gentzbittel et al. (1999). RFLP probes and candidates genes (anther specific gene SF3, heat shock protein HSP70, Ubiquitin, Calmodulin, protein-kinase-like PK, NBS-LRR type Resistance Gene analogues L3) were chosen from the F2 XRQ × PSC8 linkage map (Bert et al., 2002) for their location in order to assign chromosomes according to the Cartisol consensus map (Gentzbittel et al., 1999). Therefore, only 48 RILs were genotyped with these markers. Morphological markers *Pl2* and *Pl5* loci (downy mildew resistance) *Rf1* (male fertility restoration) and *b1* (apical branching) were also added to marker data. A set of 212 SSR markers named 'ORS', 'SSL' and 'SSU', provided by GIE Cartisol (public and available upon request) was genotyped on the RIL population. Microsatellite amplifications and detections were obtained either by standard PCR method or by an M13 tailing scheme. In the first procedure, amplification reactions were performed using a Perkin Elmer 9600 or Biorad thermocycler, in a final volume of 20µl containing 50ng of template DNA, 1x PCR reaction buffer, 1.5mM MgCl2, 0.2mM dNTPs mix, 250nM of each primer, and 0.5 units of *Taq*-DNA polymerase (Qiagen). Amplification conditions consisted of 95°C for 2min, 37 cycles of denaturing at 94°C for 30s, annealing at 54°C for 30s, and elongation 25s at 72°C. Microsatellite pattern were then visualised on polyacrylamide gel by silver-nitrate staining method (Tixier et al., 1997). In the second procedure, M13 tailing required adding the M13 forward consensus sequence to the 5' end of each forward primer (Boutin-Ganache et al., 2001). Then, the M13-forward primers were used in combination with a 10-fold excess of a fluorescently labelled M13 forward primers. The PCR conditions were in 13µl mixes containing 50ng of genomic DNA, 1x PCR reaction buffer, 1.5mM MgCl2, 0.2mM dNTPs mix, 500nM of fluorescently labelled M13 forward primer, 50nM of M13-tailed forward primer and 500nM of reverse primer and 0.2 units of *Taq*-DNA polymerase (Qiagen). Thermal conditions included 5 min denaturation at 95°C, 30 cycles of 30s at 95°C, 30s at Tm°C and 30s at 72°C, followed by 8 cycles 30s at 95°C, 30s at Tm-4°C and 30s at 72°C, and a final extension of 5min at 72°C. Amplified fragment sizes polymorphisms were detected using fluorescent capillary electrophoresis on an ABI PRISM 3100 Genetic Analyzer. The use of three different dyes (Pham, Hex and Ned) allowed for the pool-plexing of samples during separation and allele sizing.

QTL analysis. The map built for QTL detection included 39 RFLP, 162 SSR, and 4 Mendelian traits (*Pl2, Pl5, Rf1, b1*). It was developed with the CARTHAGENE software (de Givry et al., 2005) with the commands [group 0.4 4], then [buildfw 3 3 {} 0] to build a framework for each of the groups identified, and finally [build] to add the remnant markers. It spans over 1666cM, with an average of 12.2 markers per linkage group. QTL detection was performed with the software MCQTL ® (Jourjon et al., 2005) under the "forward" algorithm and with the "iQTL" option (Charcosset et al., 2001). The level of significance was determined through 3000 permutations for each trait. As several traits related with resistance to *S. sclerotiorum* were recorded, we used the software BIOMERCATOR ® (Arcade et al., 2004) to map the different QTL and to check the hypothesis of a unique QTL associated with different related traits, recorded either on RIL's ("per se" value) or on testcrosses.

RESULTS

Fig. 1 presents distributions for the *Sclerotinia* resistance observations. For the RIL, the mean percentage of plants showing *Sclerotinia* symptoms after ascospore infections was 70.8% and the latency index, calculated thus on a mean of 18 plants per plot, was 1.09. Distribution of RIL percent attack was not normal, but since no transformation rendered the data normal, QTL analyses were carried out on the raw data. Latency index data were distributed normally, and significantly negatively correlated with percent attack (r= -0.674**), so that both characters were considered as representing resistance to *Sclerotinia* head rot. In 2001, mean attacks in the re-enforced *Sclerotinia* trials were greater for hybrids with 83HR4gms (41%) than for those with PGF650 (25%) whereas in 2002 levels were very similar (83HR4: 52%, PGF650: 55%). In contrast, under 2001 natural attack, 83HR4 hybrids were less attacked (15%) than those made with PGF650 (37%). The data were normal for hybrids with the tester line PGF650, and close to normal for 83HR4gms. For each series of hybrids, the 3 observations were highly significantly correlated (PGF650: r= 0.375, 0.377, 0.378; 83HR4gms: r= 0.372, 0.397, 0.516). In contrast, the results for the 80 RIL crossed with the two tester lines in the yield trial with natural attack were not correlated, and in the re-enforced *Sclerotinia* trials, there were only 11 pairs of hybrids with the same RIL. In 2001, their reactions were not correlated, but were significantly in 2002 (r= 0.807**).

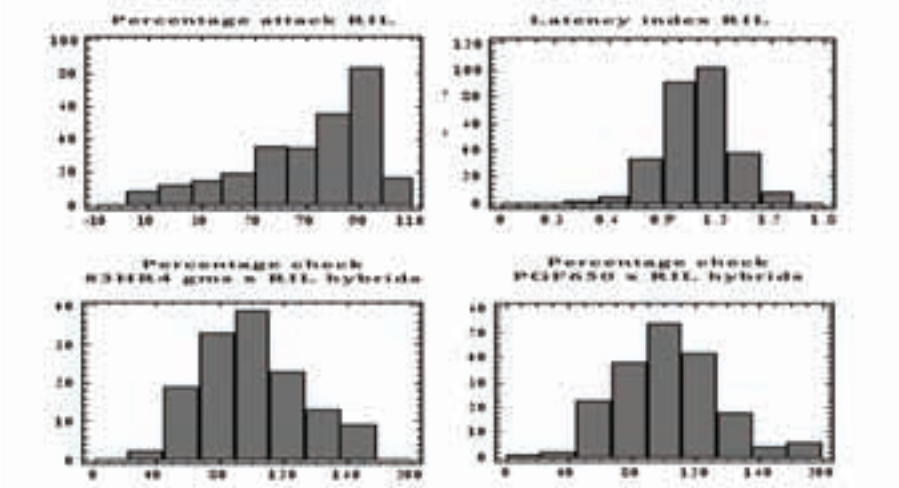

Fig. 1. Distributions of reactions of RIL in *Sclerotinia* ascospore tests and their hybrids under natural capitulum infection.

Table 1 presents correlations between mean data for the hybrids and either all the RIL or only the unbranched lines, for the hybrids made with the tester PGF650 and only for unbranched lines for the 83HR4gms hybrids, since only these were made. Coefficients were weak and similar for hybrids compared with unbranched RIL, but highly significant when both types of RIL are included. For the 3 locations taken separately, for the PGF650 hybrids, the reduced correlation for unbranched RIL was true for both locations in 2001 but not apparent for 2002.

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Table 1. Correlations coefficients between results of *Sclerotinia* ascospore tests on RIL and means of re-enforced (2001, 2002) and natural (2001) *Sclerotinia* capitulum attack on hybrids between the RIL and with tester lines 83HR4 and PGF650

Ascospore test	Percentage attack	Latency index
	Unbranched plants	unbranched plants
Mean percent check of 83HR4 hybrids	0.187*	- 0.177*
Mean percent check of PGF650 hybrids	*0.145ns*	- 0.172*
	all plants	all plants
Mean percent check of PGF650 hybrids	**0.251****	**- 0.309****

Table 2 presents the equivalent of Cartisol (1995 and 1998) and Tang et al. (2002) linkage groups. Significant QTL for RIL and hybrid data are presented in Fig. 2, and their details in Table 3. For each character, one to 3 QTL were identified, explaining from 9 to 29% of phenotypic variation. The parental line PSC8 provided generally the allele with better resistance (lower percent attack, longer latency period). For percentage attack and latency index, the same 2 QTL were shown, on LG 1 and 10 (Tang et

al. 2002), but they explained only up to 30% of phenotypic variance. Checking with BIOMERCATOR a unique MetaQTL position for the QTL involved on LG10 for Latency Index and for Percent attack on RIL, and for some of the susceptibility indices observed on hybrids, showed a lightly strongest likelihood for one QTL, with an Akaike criterion (AIC value) of 38.53, when compared with two different positions (AIC=38.68). For LG1 and LG2, the same approach produced rather two different positions for RIL and hybrids, but the AIC value bkaike criterion (AIC value) is not very different between the hypothesis "One QTL" versus "Two QTL". QTL on LG16, 5 and LG 11 appear for both two sets of test crosses although with LOD of 2.7 to 3.3.

Table 2. Correspondence between Linkage Groups from Cartisol (Gentzbittel et al., 1995 and Mestries et al., 1998) and those of Tang et al., 2002)

	LG								
Gentzbittel et al. (1995)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Tang et al. (2002)	8	14	11	17	6	13	10	9	16
Mestries et al. *(1998)*	*G*	*F*	*E*	*D*	*C*	*B*	*A*	*H*	*I*
Gentzbittel et al. (1995)	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	
Tang et al. (2002)	2	5	7	1	4	12	3	15	
Mestries et al. *(1998)*	*J*	*K*	*L*	*M*	*N*	*O*	*Q*	*P*	

Table 3. Significant QTL for capitulum resistance to *Sclerotinia* in XRQ x PSC8 RIL and hybrids.

	TRAIT	Linkage Group	LOD	R^2	Local max. position	INF position	SUP position	PSC8 "allele" value
RIL	Latency Index	10	13.90	21%	88	84	92	8.3
		1	6.10	10%	15	3	22	5.5
	Percent Attack	2	5.40	9%	54	23	54	-9.2
		10	7.20	12%	88	82	96	-8.2
		1	4.60	8%	15	0	66	-6.6
Hybrids: % attack compared with checks								
Trials	01RNPGF650	16	3.20	8%	92	78	105	-5.0
		10	2.90	8%	90	75	153	-4.7
	01ROPGF650	10	2.67	12%	71	5	88	-9.0
	02ROPGF650	5	2.70	12%	12	2	38	-5.3
		11	2.74	12%	69	10	86	-5.7
	01RN83HR4	16	3.60	13%	80	48	105	-8.7
		3	2.93	11%	35	9	83	-7.2
		1	2.96	11%	49	23	67	-7.2
	01RO83HR4	5	3.89	14%	19	11	31	-8.1
	02RO83HR4	2	3.04	11%	29	0	54	-5.2
		10	2.76	10%	50	19	86	6.5
	Mean83HR4	5	3.15	12%	19	4	32	-4.7
		2	5.06	29%	29	26	54	-5.7
		16	3.32	13%	69	47	104	-4.9
		11	2.83	11%	67	20	98	-4.5

DISCUSSION

With a mean of 70% attack, and only 2 replications available, the percentage attack from the ascospore tests was not very precise, whereas the latency index, calculated from a mean of 2 replications of 18 diseased plants gave a good idea of the resistance levels of the RIL. For the 2 series of hybrids, the 3 locations of trials were all correlated highly significantly, so it could be expected that they also gave a satisfactory representation of the *Sclerotinia* reaction.

Bert et al. (2002a), on F_3 of the same population demonstrated for ascospore tests the same QTL on LG 10 and 1 for percent attack, but not that on LG 2 or for latency index on LG1. They reported significant QTL on LG 6, 9 and 13. The LG most frequently shown in this and other populations to carry

significant QTL for capitulum resistance is that carrying the branching gene *b1* (Mestries et al., 1998; Bert et al., 2003; Ronicke et al., 2005) and it appeared possible that the morphology of branched inbred lines compared with unbranched lines directly influenced results of the resistance tests. However, the hybrids made between the tester line PGF650 and both branched and unbranched RIL showed closer correlations with results of the ascospore test than the hybrids made only with unbranched RIL and either of the tester lines. The QTL on LG10 carrying the recessive branching gene thus appears more likely to be a genetic linkage than a pleiotropic effect. Jouan et al. (2000) suggested the same conclusion from tests of *Sclerotinia* mycelium extension on capitula of F_3 families of the same population, since in this case PSC8 provided a susceptible allele linked to *b1*.

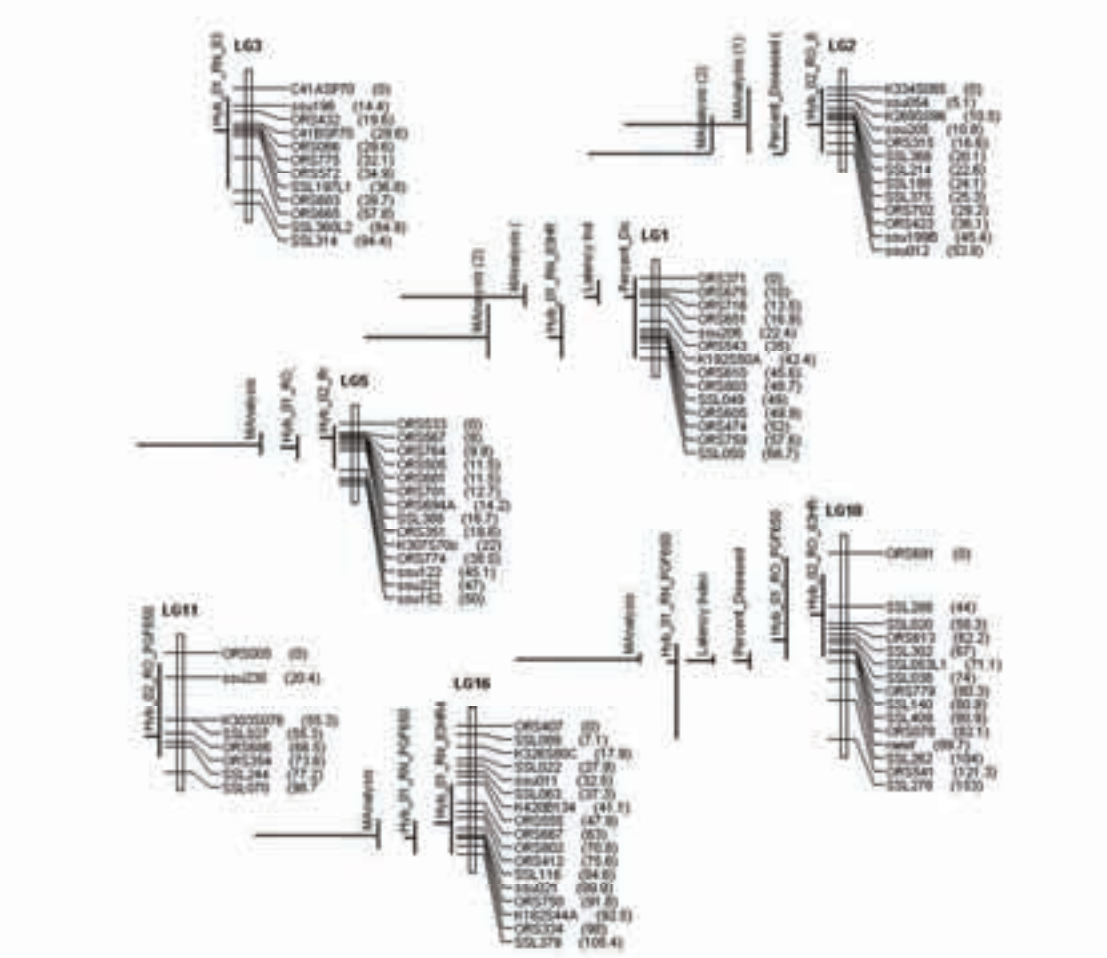

Fig. 2. Linkage groups with QTL and MetaQTL analysis

Many of the QTL were only significant at $p<0.05$ or $p<0.1$ and only a relatively small part of phenotypic variance was explained, as has generally been the case in the past (Bert et al., 2002a; Ronicke et al., 2005), except for the strong QTL shown by Gentzbittel et al. (1998), but which appears to be specific to the line PAC1. The XRQ x PSC8 RIL segregated for the PK locus linked with this strong QTL, but showed no linkage with *Sclerotinia* reaction. Overall, the large number of LG which have been shown to carry QTL for *Sclerotinia* resistance are evidence that capitulum resistance to *Sclerotinia* is truly "polygenic", and it may be that, with a large number of small QTL, it is difficult to show significant linkages with genetic markers.

Since the ascospore test represents only part of "natural attack", it could be expected that there would be more QTL for hybrids, although with smaller effects since the tester lines provide half of the genotype. In the present study, QTL on LG 5, 11 and 16 were observed on both series of hybrids but not on the RIL and it will be interesting to make further studies on them, first to check whether these effects are linked to characters such as height, maturity date or capitulum size or whether they concern some part of host-parasite relations which are not measured by artificial ascospore infections on inbred lines.

ACKNOWLEDGEMENTS

This study was supported by a French "Agence Nationale pour la Recherche" programme "Génoplante 2001-2004". We would like to thank the personnel of the INRA farm at Clermont-Ferrand for help in trial organisation and collaboration with R2N for observations of natural *Sclerotinia* attack in yield trials.

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HeliaGene, a bioinformatics portal for *Helianthus* sp. genomics

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ABSTRACT

A bioinformatics portal, called HeliaGene (http://www.heliagene.org) has been developed for in-depth analyses of *Helianthus sp.* EST data. This portal uses the same approach as that already developed for *Medicago truncatula* (MENS database, http://medicago.toulouse.inra.fr/MENS), and provides a variety of pre-computed analyses and tools for EST clusters and for exploring gene function and protein families in a user-friendly fashion. The prediction of EST cluster-encoded peptides is supported by FrameD, a program originally developed for prokaryotic gene prediction. Analyses at the protein level, such as signature and domain searches, can be helpful to make predictions about gene function and to annotate EST clusters. The HeliaGene portal provides interactive access to the annotation of tens of thousands of clusters and their corresponding peptides. Generic workflows for similarities searches versus plant databases or protein family phylogenies are provided as well as specific workflows, like the detection of potential SNPs, based on the between and within *Helianthus* species sequence polymorphisms. In the future, HeliaGene will integrate more tools and results, including genetic maps, characterization of genetic resources and core collections, and integration of sequence-based expression data with transcriptomics experiment results.

Key words: annotation – bioinformatics.

INTRODUCTION

Due to its global adaptation to a wide range of southern European water-scarce environments as well as to the introduction by breeding of a quality trait now being required for biofuel production ("high oleic" type), the sunflower crop *Helianthus annuus* is able to occupy an increasing place among the environmentally safe crops devoted to the production of raw material for the "first generation" biofuels. *Helianthus annuus* is not a model plant, and less genomic resources than for other agronomic crops like corn have been developed. But particularly thanks to an important effort by U.S. laboratories (Compositae Genome Project, http://compgenomics.ucdavis.edu/) but also by French teams (Genoplante program, https://gpi.versailles.inra.fr/), a relatively large number of *Helianthus* sp. ESTs are available in the public databases (284,251 at NCBI by January 18th, 2008). Besides *Helianthus annuus*, six other *Helianthus* species have been used to produce these ESTs, which are derived from a variety of cDNA libraries, providing information on gene transcription in a number of developmental and physiological contexts: various organs at different developmental stages (buds, roots, stems, leaves, seeds…), responses to abiotic stresses, and interaction with various pathogens, etc.

Using the EST-clusters consensus dataset generated by Mike Barker (http://msbarker.com/data.htm), which is the current reference set of sequences on which is based the design of the first generation of sunflower chips, we have developed a user-friendly portal, "HeliaGene", which provides a variety of pre-existing or specifically developed tools and pre-computed searches to conduct in-depth analyses at different levels. The navigation system provided makes it possible (i) to rapidly visualize EST cluster characteristics, (ii) to explore gene function, (iii) to analyse gene and protein families, (iv) to detect potential SNPs, based on the between and within *Helianthus* species sequence polymorphisms.

Whilst graphical representations are provided for immediate access to analysis summaries, raw results, as well as a number of links, are also provided to conduct in-depth searches whenever necessary. This important feature enables the HeliaGene user to examine the validity of annotations that have been automatically entered for thousands of EST clusters, and to propose a different annotation wherever judged appropriate.

The scope of this paper is to present the HeliaGene navigation system.

MATERIALS AND METHODS

Implementation

The web server has been developed with PERL/CGI and is run on a linux cluster. Sequence data and corresponding annotation sheets are indexed using a lucene-based search engine to allow complex queries.

Similarity searches and automatic annotation

Sequence comparisons against the protein databases UniProt (Apweiler et al., 2004), ProDom (Bru et al., 2005) and HuSep2007 were performed using NCBI-BLASTX and NCBI-BLASTP release 2.2.13 (Altschul et al., 1997) with default parameters, except for the penalty values to create a gap (-G) (set to 9 instead of 11) or to extend a gap (-E) (set to 2 instead of 1), and the threshold for the expected value (-e) (set to 0.1 instead of 10). InterproScan (Quevillon et al., 2005) software has been executed with default parameters on the peptide database in order to identify InterPro (Mulder et al., 2007) domains and families. Then, raw results have been analysed to generate, whenever possible, a synthetic description of the peptide function based on InterPro domain content.

RESULTS

General organization of EST data mining system

The system is organized around two databases, corresponding to EST cluster DNA sequences and predicted protein sequences, respectively. The set of 87,237 EST-clusters was primarily generated by Mike Barker at http://msbarker.com/data.htm from a total set of 284,251 ESTs available on GenBank (9 Sep 2007), most of them having been produced in the frame of the Compositae Genome Project (http://compgenomics.ucdavis.edu/) and by Genoplante (https://gpi.versailles.inra.fr/), and additional sequences being provided by Steve Knapp lab. The results of various analyses, conducted both at the DNA and protein sequence levels, are provided and can be used to annotate EST clusters and corresponding gene products (see below).

The system can be entered in a variety of ways: via queries based upon annotations, keywords (using a lucene-based retrieval system) as well as similarity searches. Results are presented with links allowing for an easy navigation through different sources of information.

EST cluster analysis at the DNA level

An overview of the various types of information provided for EST cluster analysis is shown in Fig. 1. A general control panel gives access to a synthetic summary of similarity results, to the predicted peptide annotation sheet and to several workflows enabling the execution of more complex pipelines on the current sequence.

The cluster annotation is found below the control panel. Summaries of WU-blastn searches (Gish, W. (1996-2002) http://blast.wustl.edu) using HuSep2007 consensus sequences against other HuSep2007 clusters and of NCBI-blastx searches against the UniProt protein database are then shown, with links to complete raw results and database entries.

Prediction of EST coding regions

The starting point for the prediction of coding regions is the FrameD program originally designed for prokaryotic sequences (Schiex et al., 2003). Prediction of coding regions from eukaryotic transcripts is somewhat similar to prokaryotic gene prediction, but additional difficulties arise from the fact that (i) EST clusters are of different sizes, with depth from 1 to almost 100 for a given nucleotide, and, consequently, of a variable robustness in the consensus cDNA sequence prediction. To manage this heterogeneity in consensus quality, FrameD was repeatedly applied to each cluster using similarity information and several combinations of parameters aiming at handling different frameshift sensitivities. By this means it was possible to predict a protein sequence for 83% of the assembled clusters, which corresponds to 72,372 peptides from 87,237 EST-clusters (Table 2). Prediction failures were mainly due either to a too short coding fragment (threshold 29 aa) or to the absence of a parameter set fitting the sequence.

Table 1. Summary of the peptide predictions

Total	72,372
predicted full-length CDS	24,799
N-term fragment (translation start only)	14,504
C-term fragment (translation stop only)	23,145
Fragment (start and stop are missing)	9,924
Number of frameshifts detected/corrected	24,053
Min-Max peptide length	29 aa-1,466 aa
Mean/Median peptide length	181 aa-155 aa

Protein sequence analyses

Protein prediction allows searches of structural or functional domains and motifs to be conducted (Fig. 1), which can be particularly informative when trying to decipher gene function. Queries of InterPro (Integrated Resource of Protein Domains and Functional Sites) were carried out to look for protein domains and amino acid signatures. Information about possible subcellular location and overall protein structure are provided with results from SignalP (Bendtsen et al., 2004) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM/).

Fig. 1. Typical annotation sheet providing a synthetic view of the functional annotation, and a summary of the similarities with access both to raw results (database icon) and to database entries (hypertext link).

Remora Workflows

The user interface provides access to several analysis pipelines based on Remora, which is a workflow manager (Carrere and Gouzy, 2006) able to create and run workflows based on BioMoby web-services. From the protein annotation sheet, the system provides the user with three Remora workflows:

- for searching SNPs "CandidatesToSNPs": as ESTs have been produced and made available on seven different species (*H. annuus, H. petiolaris H. argophyllus, H. paradoxus, H. exilis, H. tuberosus, H. ciliaris*), which is quite unusual in public databases, HeliaGene proposes a workflow starting from an amino-acid sequence, for example of candidate genes with a proven function in a model crop like *Arabidopsis*, to exploit the between and within species sequence polymorphism of ESTs to try to detect potential positions of SNPs.
- for identifying similar EST on other plants "Plant ESTs tblastn"
- and for aligning the current protein with other members of the same family "Protein Family".

From the Cluster annotation sheet an additional workflow is provided permitting the identification and the alignment of plant proteins belonging to the same family ("Family analysis").

Functional annotation overview

Table 2 lists the 25 top domains according to their representation in the predicted *Helianthus* protein sequences. When compared to the representation of the same domains in *Arabidopsis thaliana* (Uniprot database) some discrepancies appear: IPR009072 (Histone-fold, dominant role in regulating transcription), IPR000425 (involved in plant tonoplast intrinsic proteins), IPR001344 (involved in light-harvesting complex which delivers excitation energy to photosystems I and II) are over-represented. On the contrary, IPR001611 (Leucine-rich repeat:signal transduction, cell adhesion, DNA repair, recombination, transcription, RNA processing, disease resistance, and apoptosis) is clearly under-represented. These discrepancies are probably due to the differences in complexity between *Helianthus* and *Arabidopsis* genomes and/or to the specificities of cDNA libraries. Concerning the lack of representation of the LRR associated ESTs, it has to be mentioned that besides these ESTs, there is about 820 NBS-LLR resistance like fragments in the CoreNucleotide section of the NCBI database.

Table 2. InterPro top 25 entries. In the last column, the ratio between the number of predicted peptides in *Helianthus* having each domain and its occurrence in *Arabidopsis thaliana* proteins is calculated.

InterPro Accession	Number of occurrence	InterPro description	% of Arabidopsis in UniProt
IPR011009	1783	Protein kinase-like	97%
IPR001128	570	Cytochrome P450	130%
IPR001810	553	Cyclin-like F-box	74%
IPR013083	524	Zinc finger, RING/FYVE/PHD-type	62%
IPR000719	497	Protein kinase, core	27%
IPR012336	452	Thioredoxin-like fold	114%
IPR009057	425	Homeodomain-like	52%
IPR012677	339	Nucleotide-binding, alpha-beta plait	64%
IPR011046	334	WD40 repeat-like	72%
IPR009072	327	Histone-fold	234%
IPR002885	275	Pentatricopeptide repeat	42%
IPR001611	266	Leucine-rich repeat	20%
IPR000425	257	Major intrinsic protein	367%
IPR001344	246	Chlorophyll A-B binding protein	390%
IPR002213	244	UDP-glucuronosyl/UDP-glucosyltransferase	118%
IPR015609	243	Molecular chaperone, heat shock protein, Hsp40	116%
IPR002198	236	Short-chain dehydrogenase/reductase SDR	120%
IPR011992	235	EF-Hand type	96%
IPR000608	224	Ubiquitin-conjugating enzyme, E2	202%
IPR001087	223	Lipolytic enzyme, G-D-S-L	114%
IPR001806	221	Ras GTPase	140%
IPR001471	214	Pathogenesis-related transcriptional factor	140%
IPR008972	210	Cupredoxin	154%
IPR011050	207	Pectin lyase fold/virulence factor	77%
IPR003593	192	AAA+ ATPase, core	39%

DISCUSSION

The HeliaGene navigation system should prove useful for carrying out high throughput characterization of a large number of cDNA clones, and for collecting information complementary to expression profiling data. Indeed a major challenge in the coming years will be to determine the function of a vast number of genes by integrating as many sources of information as possible. As a first step towards this objective, we have developed a navigation system which links information derived from raw data, sequence analysis and database searches. Sequence annotation is facilitated by rapid access to various sources of documentation, and thus does not merely rely upon sequence homology detection and automated transfer of pre-existing annotation.

In future, we plan to integrate both expression profiling data from microarray experiments and genetic maps. Finally, tools for comparative genomics will be improved, especially to be able to transfer more information from other well studied model plants (*Arabidopsis thaliana*, *Medicago truncutula*, etc.).

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Mapping a novel fertility restoration gene in sunflower

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ABSTRACT

A novel fertility restoration factor for the PET1 cytoplasm, coming from the public line RHA340, was evaluated together with resistance genes to downy mildew (*Plasmopara halstedii*) *Pl8* and black rust (*Puccinia helianthi*) in a mapping population segregating for the three characters. Linkage between the resistance genes and the fertility restoration was not detected, showing that the restoration factor present in the line RHA340 is different from *Rf1*. Bulk segregant analysis with microsatellite markers allowed to detect markers corresponding to linkage group 7, and this location was confirmed in the mapping population. A map was built showing the order and the distance of these loci.

Key words: bulk segregant – CMS – fertility restoration – mapping – microsatellites – restoration *Rf1*

INTRODUCTION

Hybrid breeding in sunflower is based on a single source of cytoplasmic male sterility (CMS), the so-called PET1 or French cytoplasm, which originates from an interspecific cross between *Helianthus petiolaris* and *Helianthus annuus* (Leclercq, 1969). This cytoplasm is present in all the female, sterile plants. To produce the hybrid seed, the CMS plants are crossed with restorer plants that have the *Rf1* gene, permitting the obtention of fertile plants.

The inbred line RHA340 is a public line that carries the *Pl8* gene (Miller and Gulya, 1991), which was introgressed from *Helianthus argophyllus* using the line HA89 as recurrent parent (Miller and Gulya, 1988). This line is also resistant to black rust and it also has a fertility restoration gene probably introduced during the backcrossing process.

Our objective is to investigate the linkage between the fertility restoration factor and the disease resistance loci; and to establish a linkage map of the region spanning the fertility restoration locus using SSR and Indel markers.

MATERIALS AND METHODS

Plant material

A segregating population was built by crossing the line RHA340 with the line ZENB8, manually emasculated. The latter is a proprietary maintainer line susceptible to downy mildew and black rust. The F_1 plants were sown in a greenhouse, and bagged in order to self-pollinate and obtain F_2 seeds. The F_2 population was sown in the field in Orán, Salta (Argentina). Leaf tissue was collected and reserved for DNA extraction. The F_2 plants were self-pollinated and F_3 seeds were harvested. At the flowering time, pollen was collected and used to pollinate plants of the line ZENA8, the CMS version of the ZENB8 line, with the aim of generating a test cross population. One hundred and seventy individuals from F_2 population were used to produce F_3 families

Pathological tests

Genotyping for both fungal diseases was done by progeny test in groups of 20 seedlings per family. Families with low number of individuals in the evaluations were not considered for the analysis.

Downy mildew evaluations

The whole seed immersion technique described by Mouzeyar et al. (1993) was used for resistance tests using zoosporangia from race 730 (Tourvieille de Labrouhe et al., 2003) maintained in Advanta Semillas, Balcarce, Argentina.

Observations were made 10 days after infection, after the plants had been kept for 24 hs in a saturated atmosphere. Plants were considered susceptible when sporulation in the cotyledons was observed. Plants with low rate of sporulation were transplanted to pots to observe the progress of the disease. All observations were made without knowing the genotype in question.

Black rust evaluation
Two-week-old plants at two pairs of true leaves stage were infected by spraying with a suspension of uredospores (50000 u/ ml). Each plant received an application of approximately 1 ml of inoculum suspension. The uredospores used as inoculum were collected from leaves of plants naturally infected located in a field in Venado Tuerto, Santa Fe, Argentina. The evaluation of this inoculum with differential lines corresponding to the American set showed a pathogenic pattern similar to race 4.

After inoculation with the uredospores suspension, the plants were kept in growing chamber for 24 hs. at 18º C and 100 % humidity to allow the infection to proceed. After this period, the plants were transferred to a greenhouse with 25 º C temperature, and 13 h light photoperiod.

Two weeks after inoculation plants were evaluated for the presence of pustules (uredosores) on the leaves. Plants showing pustules were considered susceptible and plants without signs or symptoms were considered resistant. In all the families, the number of susceptible and resistant individuals was scored and used to classify the families. All observations were made without knowing the genotype in question.

Fertility restoration evaluation
In order to determine the genotype of the F_2 plants from the mapping population, a progeny test was done using the test cross families [(ZenB8/RHA340)F_2/ZenA8] in rows of 20 plants sown in the field in Orán, Salta, Argentina, in June 2003. The families were evaluated at flowering time by counting the number of sterile plants (no pollen) in each of them. The proportion of sterile plants in the F_3 families was used to infer the genetic constitution of the F_2 plant.

SSR genotyping
Leaf tissue was collected from F_2 plants before flowering and dehydrated for preservation until DNA extraction. DNA was extracted from dehydrated leaves using the procedure described by Haymes (1996).

Microsatellites were amplified by PCR using this DNA and primer pairs corresponding to previously mapped molecular markers (Yu et al., 2003; Tang et al., 2002). PCRs were performed as described by Tang et al. (2002).

Amplicons labelled with 6-FAM, HEX and NED were separately amplified, pooled and diluted 50-fold. Samples were prepared for analysis by combining 2 µl of diluted pool, 0.2 µl of Genescan 500 internal lane standard labelled with ROX, and 10 µl of formamide. Electrophoresis was performed in an ABI 3100-avant capillary sequencer with 36 cm capillars and POP4 matrix. Electrophoretograms were analyzed with the software Genemapper 3.0 to assign genotypes following vendor's instructions.

Bulk segregant analysis
To perform the bulk segregant analysis, DNA from plants corresponding to the non-segregant genotypes (100% plants fertile or 100% plants sterile) was quantified and combined in bulks of four plants. The bulk DNA and DNA from the parental lines were amplified with selected primer pairs and the products were analyzed as previously described.

Linkage analysis
Linkage analysis and maps for the linkage group 7 in the mapping population were constructed using Joinmap 3.0 software (Van Ooijen and Voorips, 2001).

RESULTS

Phenotype evaluations
In the entire evaluations susceptible and resistant parents were included as controls, and they always showed 100% and 0 % of diseased plants, respectively.

In all the experiments single dominant genes appear to be controlling the resistances and the fertility restoration as can be observed in Table 1

Table 1. Segregation ratios in the F_2 population regarding downy mildew and black rust resistance following the test of F_3 families.

Trait	Seg.	n (A) Zen B8	n (H)	n (B) RHA340	χ 2	Prob.	Total
Black rust	1:2:1	42	73	40	2.96	0.23	155
Downy mildew	1:2:1	49	74	31	4.4	0.10	154
Rf	1:2:1	42	91	29	4.56	0.10	162

As Table 2 and Table 3 show, black rust and downy mildew resistance are independent of fertility restoration. Independence tests showed no evidence of linkage (χ^2= 0.303, P = 0.999 for downy mildew-restoration and χ^2= 0.97, P = 0.998 for black rust-restoration).

Table 2. Cosegregation between fertility restoration and downy mildew resistance

	Fertility restoration			
Downy mildew resistance	A (sterile)	H (segregant)	B (fertile)	Total
A (susceptible)	11	26	10	47
H (segregant)	14	43	13	70
B (resistant)	13	15	5	33
Total	38	84	28	150

Table 3. Cosegregation between fertility restoration and black rust resistance

	Fertility restoration			
Black rust resistance	A (sterile)	H (segregant)	B (fertile)	Total
A (susceptible)	11	27	8	46
H (segregant)	15	35	13	63
B (resistant)	11	21	7	39
Total	37	83	28	148

This lack of correlation was unexpected because the *Rf1* gene is located in linkage group 13, a few centimorgans away from *Pl8*. (Yu et al., 2003). These results suggest that the restoration factor segregating on this population is not an allele of *Rf1*.

Molecular assays

The genotype of the restoration locus was converted to a codominant score and integrated with molecular data corresponding to 63 loci distributed in all the linkage groups of sunflower genome. The data were analyzed with Joinmap software to detect possible linkages but no markers were grouped with the restoration locus.

In this marker set were included 10 different markers located in linkage group 13. The lack of cosegregation among any of these loci and the fertility restoration shows that this locus is not an allele of *Rf1*, which was mapped in linkage group 13 (Yu et al., 2003) but a different one, located in a different part of the genome.

In order to detect regions of the genome not covered by the selected markers, bulk segregant analysis was conducted using 23 polymorphic markers not included in the original mapping population. Out of all these, only ORS-004, located on linkage group 7, showed linkage disequilibrium, with all the bulks of fertile plants having the allele corresponding to RHA340 and those of the sterile plants the allele of ZenB8. This marker together with IN0182, a proprietary INDEL marker also located in linkage group 7, were analyzed and scored for all the individuals of the population. The linkage analysis showed that the fertility restoration grouped with the two loci with LOD > 9. Table 4 shows the maximum linkage observed for these three loci. The corresponding linkage map was calculated and is shown in Fig. 1.

Table 4: Recombination frequency between *Rf* and selected markers.

Locus1	Locus2	Recombination frequency	LOD
ORS-004	Rf	0.1161	23.19
IN0182	Rf	0.2125	9.10

Fig. 1. Map of restoration factor and associated markers.

DISCUSSION

Up to now, two restoration factors have been described in sunflower; *Rf1*, located in linkage group 13 and *Rf2*, which is complementary to *Rf1* in certain genotypes, but is usually fixed in maintainer lines, so it usually does not segregate. Both genes seem to have been originated in *H petiolaris* (Miller and Fick, 1997). The restoration factor present in the line RHA340 is not any of these two, because it is located in linkage group 7 (difference with *Rf1*) and it restores the fertility in PET1 cytoplasm even in the absence of *Rf1* (difference to *Rf2*) being inherited as a simple gene. This novel gene is a fertility restoration that could be traced to the wild *H. argophyllus* used as source of downy mildew resistance during the creation of the line RHA340. We propose the name *Rf3* for this new locus.

Further experiments are being done to include more markers in the map and eventually saturate it. This may allow a cloning of the restoration factor and an explanation its mode of action. Another line of investigation will include the cross of RHA340 with other CMS systems to see if other male sterile cytoplasm can be restored.

This finding could alleviate the genetic bottleneck caused in sunflower due to the exclusive use of *Rf1* to restore PET1 CMS in the production of hybrid cultivars.

The availability of molecular markers linked to this locus facilitates the introgression of this gene in the different lines of breeding programs, to develop new restorer lines.

Summarizing, we studied the linkage of the fertility restoration locus present in the line RHA340 with the resistance genes to downy mildew *PL8* and black rust as well as with molecular markers. The lack of correlation with the resistance genes (linked in repulsion with *Rf1*) and the linkage with markers of linkage group 7 demonstrate that this is a novel restoration gene.

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Verification of positive BAC clones near the *Rf1* gene restoring pollen fertility in the presence of the PET1 cytoplasm in sunflower (*Helianthus annuus* L.) and direct isolation of BAC ends

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ABSTRACT

Cytoplasmic male sterility (CMS) plays an important role in the production of hybrid seeds. In sunflower, commercial hybrid breeding is based on a single CMS-inducing cytoplasm, PET1. In this particular male sterility, which was derived from an interspecific cross between *Helianthus petiolaris* Nutt and *Helianthus annuus* L., CMS is caused by the expression of an aberrant mitochondrial gene that prevents the development of viable pollen. The restoration of pollen fertility in the presence of the PET1-cytoplasm has been reported to be controlled by two dominant nuclear genes (*Rf1*and *Rf2*). In a previous work, a linkage map has been constructed around the *Rf1* gene that consisted of 35 AFLP (amplified fragment length polymorphism) markers, 7 RAPD (random amplified polymorphic DNA) markers, and 1 SSR (simple sequence repeat) marker. These markers represent a good opportunity and an excellent basis for a map-based cloning approach. Markers tightly linked to the restorer locus *Rf1* have been used as overgo probes to be hybridized against sunflower BAC libraries. Positive BAC clones were identified and a putative closed contig around *Rf1* was constructed by fingerprinting. Here we report on: (i) the verification of the identified BAC clones near the *Rf1* gene and (ii) the inability of the DNA fingerprinting methodology alone for identifying overlapping DNA fragments that can be assembled into contigs, showed by using different restriction enzymes, and (iii) the description of a fast and efficient method to clone BAC ends into a high copy number vector based on double antibiotics selection.

Key words: BAC end – CMS – contig – fertility restoration – *Helianthus annuus* L.

INTRODUCTION

Cytoplasmic male sterility (CMS) is often associated with mitochondrial DNA rearrangements, resulting in the expression of chimeric genes believed to interfere with normal pollen development (Horn, 2006). In sunflower, commercial hybrid breeding is based on a single source of cytoplasmic male sterility, PET1, obtained from an interspecific cross between *Helianthus petiolaris* Nutt and *Helianthus annuus* L. (Leclercq, 1969). Detectable alterations in the mitochondrial genome of CMS and fertile lines are limited to a 17-kb-region and consist of two mutations: a 12-kb-inversion and a 5-kb-insertion/deletion, which lead to an altered transcript pattern of the *atp*A gene (Siculella and Palmer, 1988). CMS is associated with the expression of a novel open reading frame, *orf*H522 (Köhler et al., 1991), which encodes a 16-kDa polypeptide (Horn et al., 1991; Laver et al., 1991). Male fertility can be restored by the introduction of nuclear *Rf* (restorer of fertility) genes that compensate for this deficiency (Schnable and Wise, 1998). The isolation of *Rf* genes in sunflower may help to clarify the mechanism behind the expression of the CMS-associated mitochondrial gene.

The *Rf1* gene, responsible for fertility restoration in the presence of the PET1-cytoplasm, was mapped based on AFLP, RADP and SSR markers (Kusterer et al., 2002; Horn et al., 2003). A linkage map surrounding the restorer gene *Rf1*, which consists of 43 markers (35 AFLPs, 7 RAPDs, and 1 SSR) and covering 250.3 cM, has been constructed (Kusterer et al., 2005). These markers were used in a map-based cloning strategy as probes against the sunflower BAC library RHA325 (Özdemir et al., 2002, 2004) to identify positive BAC-clones near the restorer gene *Rf1*. This allowed the development of a preliminary putative contig around the restorer gene by fingerprinting (Kusterer et al., 2004). This contig could have provided a starting point for cloning the *Rf1* gene restoring pollen fertility in the presence of the sunflower PET1 cytoplasm.

Our objectives were: (i) To establish the resources and protocols necessary to verify the previously identified putative closed contig around the restorer gene using different restriction enzymes. We show

here that DNA fingerprinting alone is not a sufficient criterion for identifying overlapping DNA fragments that can be assembled into a contig. (ii) To present a method for isolating BAC ends from positive BAC clones. The use of BAC-end sequences (sequences adjacent to the insert sites) has been proposed as a means for selecting minimally overlapping clones for sequencing large genomic regions (Venter et al., 1996). Our aim was to develop a fast and efficient tool to obtain cloned BAC ends by ligating restriction fragments of BAC clones digested with *BamH*I into a universal cloning vector, followed by double antibiotics selection.

MATERIALS AND METHODS

The first BAC library reported here has been constructed from the restorer line RHA325 (an American public restorer line carrying the PET1 cytoplasm) using *Hind*III fragments and the pBeloBAC11 vector. The library has a 1.9 fold genome coverage and an average insert size of 60 kb (Özdemir et al., 2004). The second BAC library, constructed from the maintainer line HA383, was developed for Steven Knapp and is distributed by Clemson University Genomic Institute (CUGI, http://www.genome.clemson.edu/capabilities/bacCenter.shtml).

For cloning BAC ends, we used a modified plasmid end rescue method (Kelley et al., 1999). Ten µl of DNA from the positive BAC clones were digested with the restriction enzyme *BamH*I for 3 hours at 37°C. Four µl of the digested DNA was ligated into pUC18 in 20 µl final solution of 10 x ligase buffers, sterile H_2O, and T4 DNA ligase for 16 hours at 4°C. Prior to ligation, the vector pUC18 had been digested with the restriction enzyme *BamH*I and dephosphorylated with calf intestinal alkaline phosphatase. In the mean time, *E. coli* (DH5α) bacteria were treated with calcium chloride to make them competent. For the transformation of *E. coli*, 4 µl ligation was added to 100 µl of competent *E. coli*, incubated on ice for 20 min, and heat shocked at 42°C for exactly 60 sec. The tubes were immediately returned to ice for a minimum of 5 minutes. Transformed cells were incubated in 250 µl SOC media for 1 hour at 37°C, plated on LB agar medium containing X-Gal, IPTG, and two antibiotics for selection: chloramphenicol (12 µg/ml) for the pBeloBAC11 vector and ampicillin (50 µg/ml) for the pUC18 vector. The cultures were incubated at 37°C overnight to allow the colonies to grow. Only colonies containing pBeloBAC11 and pUC18 could grow on both antibiotics. To estimate the insert size of the cloned BAC end, DNA from the obtained clone was isolated by the alkaline lysis method, resuspended in 100 µl H_2O (for sequencing purpose), completely digested with *BamH*I and separated on a 0.8% agarose gel. For cycle sequencing, we used the SP6 universal primer for the promoter that flanks one cloning site of the pBeloBAC11 vector.

RESULTS AND DISCUSSION

In previous studies, a linkage map for the *Rf1* gene was constructed using a segregating F_2 population of the cross between RHA325 (restorer line carrying the PET1 cytoplasm) and HA342 (maintainer line). Forty-three markers (7 RAPD-markers, 35 AFLP-markers and 1 SSR-marker) were identified that were localized on both sides of the restorer gene (Fig. 1). This corresponds to an average marker distance of 5.8 cM for this linkage group and a marker density of 1/0.56 cM in an area of 3.9 cM around the restorer gene *Rf*1. This area consists of two RAPD and five AFLP markers.

The closest markers from both sides of the restorer gene *Rf1*, OP-K13_454 and E33M61_136 were used as probes against the BAC library RHA325 to identify positive BAC clones (Kusterer et al., 2004). Five positive BAC clones were identified. BAC fingerprinting using *Hind*III as restriction enzyme was performed. The BAC clones 67I5 and 67N4 showed an identical banding pattern, which overlapped with the smaller BAC clone 59J13 from one side. From the other side, the BAC clones 22408 and 22407 also shared *Hind*III fragments. The banding pattern was confirmed by Southern hybridization using the *Hind*III digested BAC clone 67N4 as probe. A 3.7-kb-fragment shared between the clones 224O8, 67N4 and 67I5 was identified. This procedure allowed the development of a preliminary putative contig at the restorer gene *Rf*1. This could also be an indication that the contig around the restorer gene *Rf*1 might be closed (Fig. 2)

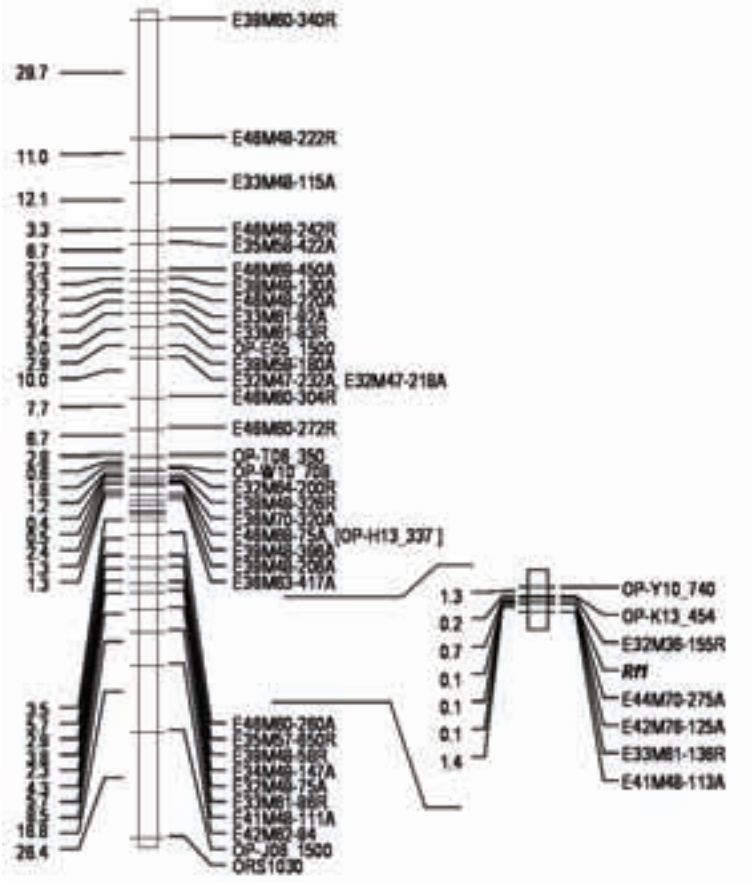

Fig. 1. Marker-saturated map of the linkage group surrounding the restorer gene *Rf1* of sunflower. A region of 3.9 cM around the *Rf1* gene is shown enlarged (Kusterer et al., 2005).

The putative closed contig around the *Rf1* gene of the PET1 cytoplasm needed to be verified. The 3.7 kb-fragments from the BAC clones 67N4 and 22408 were cloned. Four restriction enzymes *Hind*III, *Pst*I, *Eco*RI and *Kpn*I were used to verify the identity of the putative overlapping 3.7-kb-fragment (Fig. 3). Surprisingly, the restriction patterns obtained for these clones with *Pst*I and *Eco*RI were not identical. These digests proved that we had cloned two different fragments and have no closed contig around the *Rf1* gene, so far (Fig. 2).

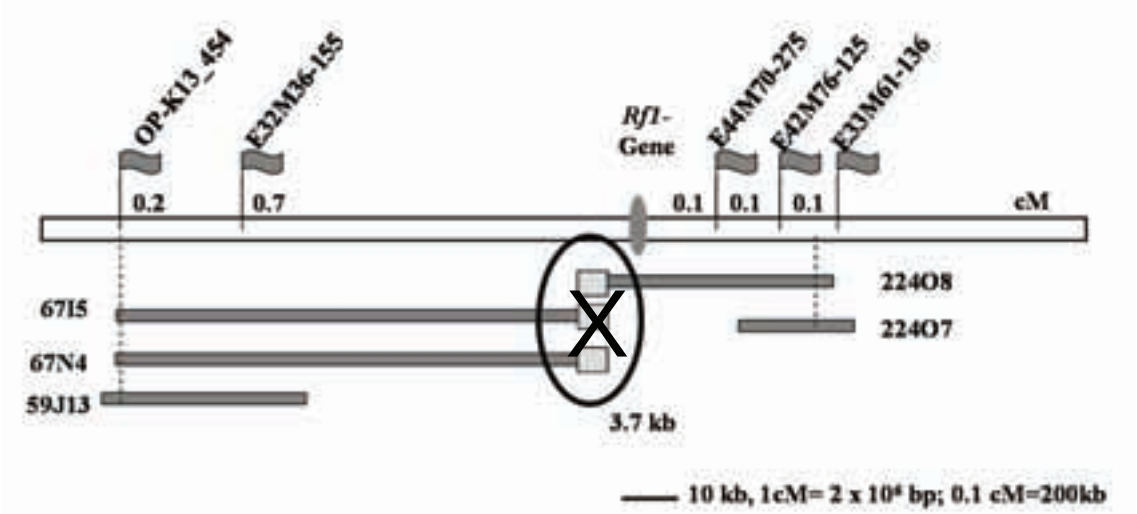

Fig. 2. Preliminary putative contig around the restorer gene *Rf1*.

In addition, we sequenced the 3.7-kb-fragment from the 67N4 BAC clone. The analysis showed homology to a retrotransposon, which might explain the observed cross hybridization between the 3.7-kb-fragments of the two BAC-clones 67N4 and 22408. It has been reported that overlaps can be detected simply by hybridization but it is not a satisfactory criterion because dispersed repeats can generate false-positive (Hong et al., 1997). We believe that to obtain reliable fingerprints, digestion with several restriction enzymes should be part of the whole process.

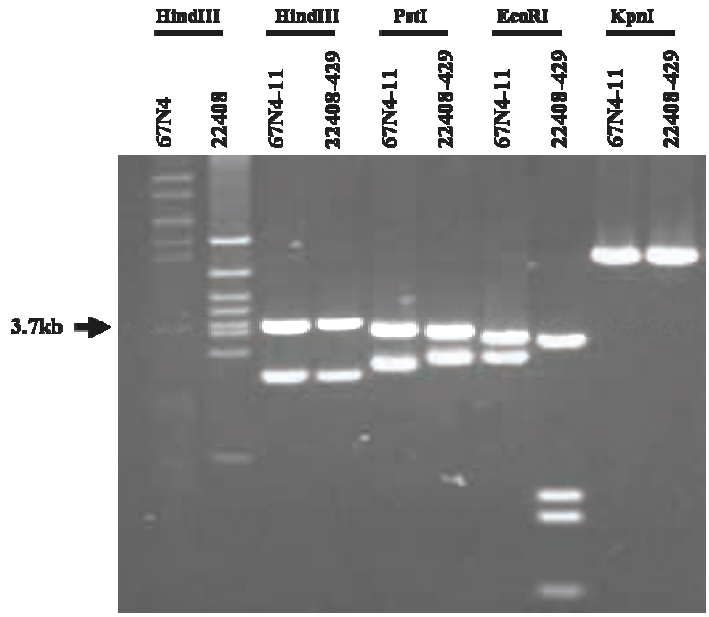

Fig. 3. Investigation of the putative closed contig. The cloned 3.7-kb-fragments from 67N4 and 22408 BAC clones were digested with four restriction enzymes. The intensity of the sub-cloned fragments is due to the high copy number of the vector pUC18.

The RAPD marker OP-K13_454 was also used for hybridization against the BAC library of the line HA383 (maintainer line), developed for Steven Knapp and distributed by Clemson University Genomic Institute (CUGI, http://www.genome.clemson.edu/capabilities/bacCenter.shtml). Four positive BAC-clones 216F17, 307N02, 225D09 and 401E15 were identified that, according to the obtained *Hind*III-BAC-fingerprinting data, belonged to one contig.

The first objective of this study was to identify positive BAC clones near the *Rf1* gene restoring male fertility in sunflower in order to conduct a map-based cloning strategy for the isolation of the gene. This involves screening of BAC libraries with cloned markers and the assembly of contigs. Since chromosome walking requires the isolation of the BAC ends to be used as probes for further steps, we developed a method for an efficient and reliable isolation of BAC ends.

Fig. 4 shows a scheme to isolate BAC ends by cloning restriction fragments of BAC clones. In our case, we used *BamH*I that cuts several times within the insert and once within the BAC vector, followed by ligation into the pUC18 vector, transformation of *E.coli*, and plating on double antibiotics selection media. When using two antibiotics: ampicillin resistance for the pUC18 vector and chloramphenicol resistance for the pBeloBAC11 vector, we could be sure that the resulting plasmid in pUC18 contained the BAC vector pBeloBAC11 and a *BamH*I-BAC-end as insert.

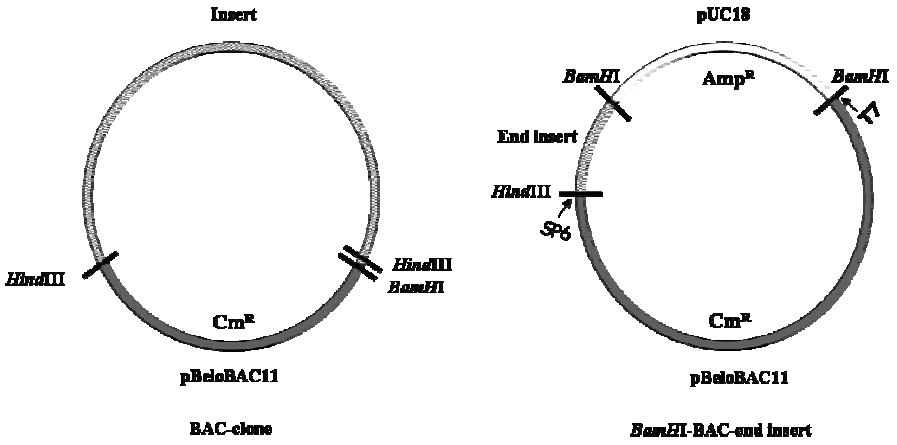

Fig. 4. Schematic diagram of the generation of end-specific probes from BAC clones. Digestion of the BAC clone with the restriction enzyme *BamH*I cuts the insert of the BAC clone into several fragments, one containing the *BamH*I-BAC-end together with the pBeloBAC11 vector as *BamH*I has only one restriction site within pBeloBAC11. Ligation of the *BamH*I-BAC-end into pUC18 combined with a double antibiotics selection results in clones with a high copy number vector construct inside.

During chromosome walking at the restorer locus, using this strategy for BAC-end cloning we were able to isolate 17 BAC-ends out of 21 positive BAC-clones, which had been identified by various overgo probes.

The cloned *BamH*I-BAC-ends were released from the pUC18 vector by complete digestion with *BamH*I (Fig. 5A). For the failure of cloning the four remaining BAC ends, possible explanations could be that the restriction fragments generated by *BamH*I digestion were either too large for an efficient ligation or too small to be detected. To check the reliability of our method, we performed additional hybridizations using pBeloBAC11 as probe against the cloned BAC ends. The results confirmed that all obtained sub-clones contained the pBeloBAC11 vector and a BAC end (Fig. 5B) as all obtained fragments gave a hybridization signal and had a size larger than the size of the pBeloBAC11 vector (7.5 kb). Knowing the sequence of the pBeloBAC11, the BAC-end sequence could be easily separated from the vector sequence.

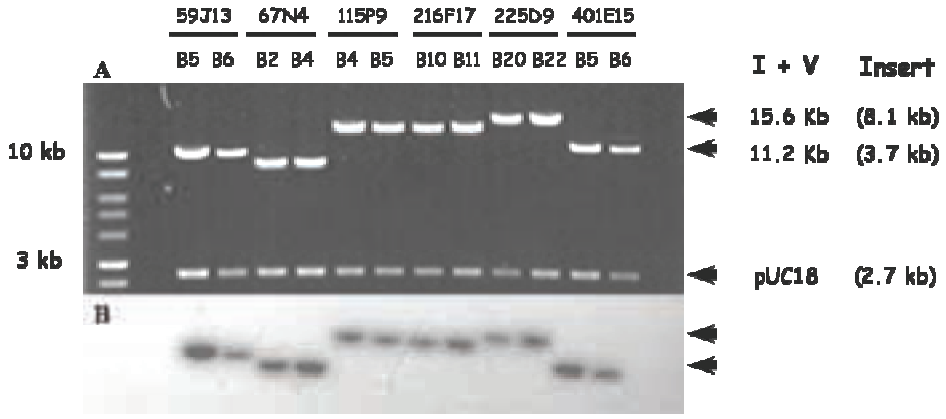

Fig. 5. *BamH*I digest of six cloned BAC ends. **(A)** For higher reliability, at least two sub-clones for each BAC end were analysed. Two fragments were obtained in each digest: one for the pUC18 vector (2.7 kb) and a second fragment with a size larger than the vector pBeloBAC11 (7.5 kb). **(B)** Hybridization of the BAC ends 59J13, 67N4, 115P9, 216F17, 225D9, and 401E15 digested with *BamH*I against pBeloBAC11 vector as probe. The larger *BamH*I-fragments harbouring the cloned BAC ends hybridized to the pBeloBAC11 vector probe, which means that the pBeloBAC11 vector is definitely part of these inserts. I= *BamH*I-BAC-end, V= pBeloBAC11

We believe that this method to clone BAC ends, despite having just one end, is simple, fast, very reliable, and not costly. First, there is no risk of cloning non-target fragments because of the double antibiotics selection. Second, due to cloning into pUC18, the cloned BAC end is present in a high copy vector, which makes minipreparations using the simple alkaline lysis method much more efficient.

The sequences of the BAC ends are now used to develop markers that allow the back mapping of these BAC clones and contigs to the restorer gene *Rf1*. Markers developed from the BAC end of 216F17 proved that the contig of the BAC clones 216F17, 307N02, 225D09 and 401E15 is not localized on the linkage group of the *Rf1* gene.

Our study proved that there are a lot of pitfalls in map-based cloning. However, more steps of chromosome walking will be performed by verification of the new obtained BAC clones. Furthermore, new AFLP markers will be identified and mapped to the restorer gene to saturate the region of the gene with more markers, especially with markers cosegregating with the *Rf1* gene.

ACKNOWLEDGEMENTS

This work is supported by the Deutsche Forschungsgemeinschaft (DFG) (HO 1593/5-1).

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The efficiency of different molecular indices in sunflower breeding

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ABSTRACT

The sunflower heterosis breeding is justified by the possibility of obtaining increased yield. In order to obtain a yield increase in first generation hybrids, it is necessary to determine the genetic diversity of selected parental lines. Genetic distances between sunflower genotypes employed in breeding processes could improve the inbred line selection efficiency for obtaining higher yielding hybrids.

Key words: genetic diversity – hybrid vigor– sunflower – UPGMA.

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the main oil crops in the Republic of Moldova, and it is planted every year on about 180-200 thousands ha. Varieties characterized by their high content in edible oil, elaborated by V.S. Pustovoit in 1976-1978, were gradually substituted by hybrids, due to their higher productivity and technological effectiveness.

The hybrid vigor phenomenon refers to the capacity of first generation hybrids to exceed by certain traits the best parents. Heterosis has been applied to obtain a high yield and represents one of the most important phenomena of plant morphogenesis. Hybrid vigor assures an optimal viability and increased yield of crops (Anashenko, 1974; Gundaev, 1971).

Many hypotheses have been postulated with the purpose of explaining the hybrid vigor process, but, up to today, its nature has not been elucidated. Usually, the determination of a heterosis effect is based on certain morpho-genetical indices such as morphological, functional and biochemical ones (Konarev, 1993). In addition, the quantitative value of heterosis can be established both by comparison of the average value of homozygous breeding genotypes (parents) and by comparison of first hybrid generation with homozygous breeding lines (Vrânceanu, 2000). The increasing practical implementation of heterosis requires the accomplishment of new fundamental studies at the level of molecular and supramolecular systems of the cell (Boppenmaier et. al., 1993; Konarev, 1993). Hence, it is necessary to study comparatively the breeding sunflower genepool in order to disclose different molecular indices related to a heterosis effect.

Our objective was to determine the efficiency of different molecular indices, such as total protein, hydro-soluble protein, salt-soluble protein, and RAPD amplicons in an estimation of their association with heterosis effect.

MATERIALS AND METHODS

Sunflower hybrids (Performer, Valentino, Xenia, Oxana) and their parental forms are annual, diploid sunflower genotypes (2n=34), used within a commercial scope. The male sterile genotypes analyzed (Performer, Valentino, Xenia, Oxana) possess cytoplasmic male sterility (CMS) PET1. The male fertile parental lines (Performer, Valentino, Xenia, Oxana) carry a fertility restorer gene (*Rf*).

Total soluble proteins from peeled seeds were isolated in buffer: 0.628 mM Tris-HCl, pH=8.0 (1g tissue: 5 ml), 0.03% ascorbic acid, 1 mM EDTA. Water-soluble proteins were extracted in: 0.01 % Trilon B, 0.5 % ascorbic acid, 1 % mercaptoethanol. Salt-soluble proteins were isolated in buffer: 50 mM Tris, pH 8.0, followed by 10% NaCl (1:15). Electrophoresis was carried out according to Laemmli, in 1mm gel of polyacrylamide (GPAA), in denaturant conditions (Laemmli, 1978; Duca et.al., 2001). Post-electrophoresis processing was carried out according to the standard method (Duca et.al., 2001). The relative molecular mass of the polypeptide fractions was established by stepladder - SDS Protein Standards for Capillary Electrophoresis (USA).

Genomic DNA was isolated from sunflower plants, at the stage of 2-leaves, in buffer: 133 mM Tris-HCl, pH 7.8; 6.7 mM Na_2EDTA; 0.95 M NaCl; 1.33% Na sarcosyl; 1.33% mercaptoethanol.

RAPD (Random Amplified Polymorphic DNA) analyses were performed with five arbitrary primers (P2, P6, P8, P37, P39). The PCR was carried out using the following profile: 95ºC for 5 min; 45 cycles: 95ºC for 1 min, 37-42ºC for 1-2 min, 72ºC for 2 min, and the final extension was at 72ºC for 7 min.

Gel electrophoresis was performed using 2% agarose gel. Determination of products of amplification molecular masses was done by Smart stepladder 200-3000 pb.

The starting point of the analysis was the presence of amplified fragments at a given level designated as "1", and its absence, "0". The amplified fragments were quantified from the printed images.

Based on scored data, the following indices (Nei et.al., 1983; Lynch, 1990; Sivolap et.al., 1998) were calculated:

* Similarity coefficient: $GS = \frac{2 \times N_{ij}}{N_i + N_j}$, where N_{ij} – the number of common bands for two samples, N_i and N_j - the number of bands in samples *I* and *J*.

* Genetic distance: $GD = -\ln(GS)$, where GS – similarity coefficient (Gentzbittel et.al., 1992).

Matrices of genetic distances were constructed. Furthermore, these matrices were subjected to cluster analysis (unweighted pairwise method with arithmetic mean - UPGMA) (Michener et.al., 1957).

RESULTS

The heterogeneity of the breeding material was demonstrated by cluster analysis (UPGMA), based on the values of the genetic distances and the genetic similarity calculated from protein and genomic DNA polymorphisms.

Total protein. Analysis of total sunflower proteins from seed revealed a high level of polymorphism (Duca et.al., 2005a). The analyzed genotypes were grouped in two clusters: homozygous (with similarity level of 84-88%) and heterozygous (83-96% of genetic similarity). Only the fertile line Valentino was characterized by an average similarity of 77% in comparison with hybrids and 73% in comparison with analyzed lines (Fig. 1A).

Water-soluble protein. The genetic distances (GD) of sunflower lines, based on albumin pattern analysis, showed values of between 0.05 and 0.12 (Duca et.al., 2005b). The greatest genetic distances were revealed in the homozygous genotypes Performer, while the smallest GD was typical for Valentino lines. Homozygous lines Xenia and Oxana were characterized by similar distances (GD=0.07).

A dendrogram of albumin was made based on the genetic distance analysis of homozygous and heterozygous sunflower genotypes. Three main clusters represented are A, B and C (Fig. 1B). The A cluster included five sunflower genotypes: male fertile line Performer, Performer F_1, Oxana F_1, Valentino Rf line and Valentino F_1, with genetic similarity of 0.91-1.

Male sterile lines Xenia, Valentino and Oxana, characterized by a 99% similarity, represented the B cluster. The CMS line Xenia was very similar to male sterile genotype Valentino (94%). Grouped in the last cluster were male sterile line Performer, Rf line Xenia and Xenia F_1, which were characterized by a genetic similarity of about 0.90.

Elaboration of an albumin dendrogram of sunflower homozygous and heterozygous genotypes based on genetic distances analysis revealed the separation of sterile lines from Rf genotypes.

Salt-soluble proteins. The study of the relations of salt-soluble proteins in sunflower homozygous and heterozygous genotypes (Duca et.al., 2005c) revealed the presence of three main clusters: A, B and C, which, in general, sum up the genotypes belonging to the breeding combinations analyzed - Performer, Valentino, Oxana, Xenia (Fig. 1C).

The cluster A was characterized by the presence of the following genotypes: male sterile line Performer, Rf line Performer, Performer F_1 and CMS-line Oxana, which shared values of genetic similarities between 0.91-1.00. The first generation hybrid Performer (100%) proved to have high similarities with its parental lines. Cluster B was represented by male sterile genotypes Oxana, Xenia and Xenia F_1, with similarities of 85%. In C cluster, the Valentino genotypes and the fertile line Xenia, GS=0.78-0.84 (Fig. 1C) were grouped.

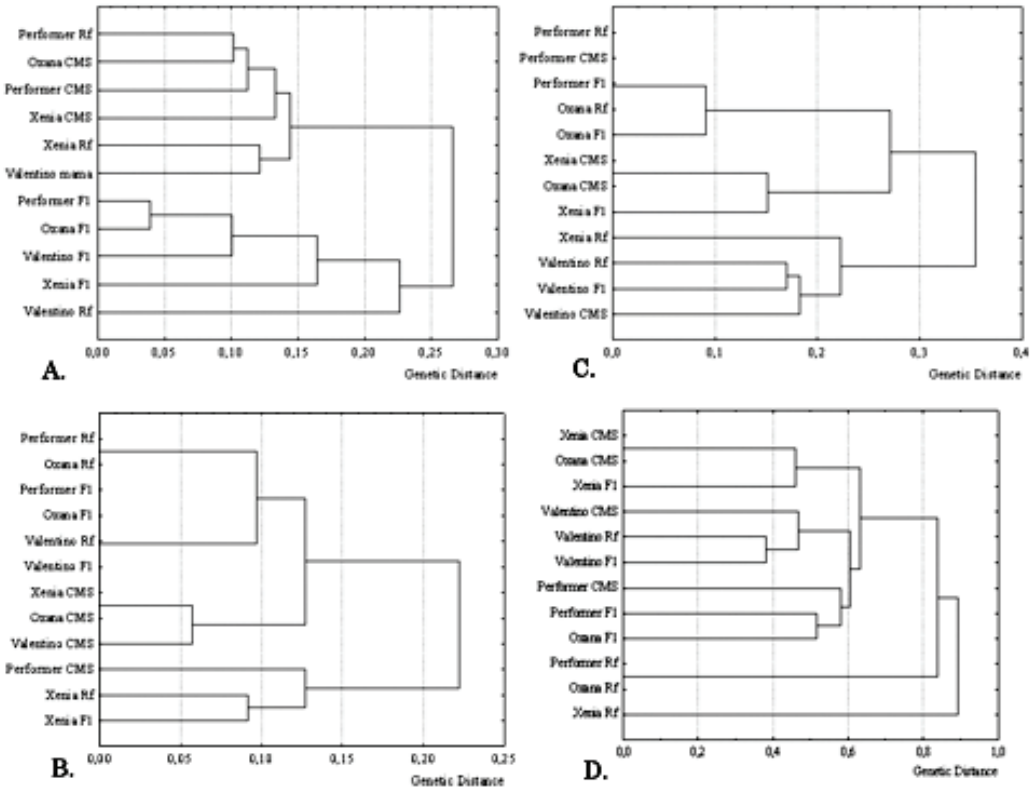

Fig. 1. Cluster analysis of sunflower genotypes based on:
A. total proteins, B. water-soluble proteins, C. salt-soluble proteins, D. RAPD sequences.

The study of genotypic relations based on electophoretic mobility of salt-soluble proteins revealed the presence of three main clusters, which, in general, sum up the genotype belonging to analyzed breeding groups - Performer, Valentino, Oxana, Xenia. Therefore, grouping of genotypes in this way was according to breeding groups.

RAPD analysis. Random amplified polymorphic DNA analysis on different sunflower inbred genotypes and on their hybrids (Duca et.al., 2005d), revealed the presence of 69 loci. PCR amplified sequences were represented by amplicons with lengths of 90-3000 bp. Number of amplified fragments differed depending on the primer used, and varied between 3 and 11 bands. The number of polymorphic loci varied within the experiments, by between 5 and 20 loci depending on the primer and the genotype (Table 1). Genotype polymorphism within analyzed families varied in the range of 5-46 %, while the average value of polymorphism was about 31%.

Table 1. Genetic polymorphism in the framework of different sunflower genotypes.

Primer	Nucleotide sequence	GC composition	Loci	Polymorphism, %
P2	gAc AgA cAg AcA	50	20	45.65
P6	gAg cAA gTT cAg ccT g	56	13	28.57
P8	cAg gAA AcA gcT gAc	53	9	37.5
P37	cTg Acc Agg Agc	67	12	5.26
P39	ccA ggT cgc c	80	15	39.47
Total			69	31.29

The dendrogram of RAPD loci on sunflower genotypes highlighted five main clusters (Duca et.al., 2008): A, B, C, D and E (Fig. 1D). Cluster A is composed of three sunflower genotypes: male sterile Xenia, Xenia F_1, and male sterile Oxana, which are characterized by genetic similarity values of 0.57-0.81, with an average of 0.69. B cluster was represented by the genotypes of Valentino breeding group, with a similarity of about 60%. The hybrid Valentino was similar to Rf line Valentino (62%) and to its sterile line (55%). The cluster C was represented by male sterile line Performer, Performer F_1 and Oxana

F_1, which were characterized by genetic similarity values of 0.41-0.44. The male fertile genotype Performer represented cluster D, which differed considerably from the other genotypes from clusters A, B and C. Cluster E was represented by the Rf line Xenia and it showed a resemblance of 12% with all the genotypes analyzed.

The analysis of RAPD amplicons (GD=0.41) disclosed that the genetic distance within homozygous lines (Rf and CMS) of hybrid Valentino was reduced, a fact that could contribute to explain its reduced productivity (2500 kg/ha) in comparison with the yield of the hybrid Oxana (2900 kg/ha). RAPD sequence pattern clusterization separated the heterozygous genotype Oxana from both parent genotypes with a great distance between them ($DG_{F1-♀}$=0.62 and $DG_{F1-♂}$=0.82).

DISCUSSION

Heterosis estimation. Increasing crop yields based on the heterosis phenomenon is a never-ending problem and permanently in force, and contributes to the realization of society's food program. One of the main problems in crop breeding consists of the evaluation and classification of the material implicated and in the selection of parental lines with the aim of obtaining high yield hybrids. The unravelling of the heterosis mechanisms and their estimation will open up new and important prospects in crop breeding.

Development of crop varieties and hybrids is based on traditional breeding methods (Siminel, 1998; Vitalis and Couvet, 2001) or on the use of genetic engineering (Tracy, 2003; Duca et.al., 2007). Both of these require the application of some efficient techniques that ensure the monitoring of breeding material used in breeding programs.

The specific biological peculiarities of plants can be determined by molecular markers such as DNA markers (Mohan et.al., 1997; Vrânceanu, 2000) and protein markers (Anisimova et.al., 2004; Durante et.al., 1989; Konarev, 1993). These can be applied for the evaluation of genetic diversity during the selection of quantitative and qualitative traits, for the choice of parental genotypes, for determination of hybridization, etc. Heterosis effect can be appreciated by the increase in the amount of DNA in somatic cells, DNA replication rate (Fedin, 1980) and cell division rate (Capatana, 2004).

Molecular markers, such as proteins, have contributed to the elucidation of the nature and origin of different crop genomes. This allowed the prognosis of certain cross breeding and the analysis of uniform populations with respect to their morphological traits. In addition, protein markers allowed the direct appreciation of hybridization level (Konarev, 1993).

In our experiments, the total sunflower seed proteins proved to have a high protein polymorphism (Duca et.al., 2005a; Capatana, 2006), the analyzed genotypes being grouped into two clusters: homozygous (with similarity level of 84-88%) and heterozygous (83-96% of genetic similarity). Therefore, from these results we can confirm that total proteins cannot be associated with crop productivity, and cannot be used in heterosis prognosis.

The total hydro-extractable proteins from seeds are represented by different protein fractions such as enzymes, enzyme inhibitors etc. (Anisimova, 1992; Norton, 1989). The seed albumins were used for electrophoretic patterns analysis (Duca et.al., 2005c; Capatana, 2006). The elaboration of an albumin dendrogram based on genetic distance analysis of sunflower genotypes revealed the separation of cytoplasmic male sterile from Rf genotypes. Thus, the absence of any association with the heterosis effect was verified.

In sunflower seeds, the main storage proteins are represented by globulins, especially by the major globulin fraction heliantinin (Anisimova et.al., 2004; Schwenke et.al., 1975; Vonder Haar et.al., 1988). Our results related to salt-soluble proteins (Duca et.al., 2005c) showed three main groups: A (Performer group of genotypes), B and C (Valentino group of genotypes), which in general sum up the genotypes representing the breeding groups studied (Fig. 1C). In this way, the association of less productive hybrid genotypes (Valentino and Performer) with small genetic distance between their parental lines and their location in the same cluster was shown, while hybrids that are more productive (Oxana, Xenia) were located apart from their parental lines.

The cluster analysis of RAPD products of amplification on sunflower genotypes revealed five main clusters (Capatana, 2006; Duca et.al., 2008): A, B, C, D and E (fig. 1D), with the separation being made on the basis of their genetic distance. Analyzing data of the breeding group Valentino by RAPD sequences (DG=0.41) it was disclosed that the genetic distance within homozygous lines (Rf and CMS) of hybrid Valentino was low, a fact that could contribute to explain the lower productivity (2500 kg/ha) in comparison with the yield of hybrid Oxana (2900 kg/ha). Clusterization of RAPD amplified sequences separated the heterozygous genotype Oxana from both parent genotypes, and these results were demonstrated by the large distance between them ($DG_{F1-♀}$=0.62 and $DG_{F1-♂}$=0.82).

Thus, UPGMA clusterization method of proteins and nucleic acids polymorphic spectra proves that salt-soluble proteins and RAPD markers could be used as a basis for the prognosis and selection of lines with different combining abilities. The methods of heterosis evaluation against genome peculiarities can serve as a basis for the elaboration of a heterosis prognosis method and the selection of breeding material.

ACKNOWLEDGEMENTS

The authors are grateful to Dr., Rotaru T. (SRC ``Magroselect``, Soroca, Republic of Moldova) for the provision of sunflower seeds and discussion during the investigation and to Dr. N. Barbacar for detailed discussions on RAPD analysis. The paper was realized with the support of National Grant Nr.32006C and MRDA/CRDF Travel Project MTFP-1017A.

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Differential gene expression in SuCMoV-tolerant and susceptible sunflower lines

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ABSTRACT

Sunflower chlorotic mottle virus (SuCMoV) is one of the most widely distributed viruses affecting sunflower (*Helianthus annuus* L.) in Argentina. Symptoms include yellow blotches, reduced and distorted leaves and plant stunting. SuCMoV-susceptible (20016) and tolerant (B-133) sunflower lines were mechanically inoculated with SuCMoV and total RNA was isolated from infected leaf tissue, amplified and hybridized to an *Arabidopsis* oligonucleotide-microarray containing 22,000 genes. The gene expression profile in inoculated plants from both lines was statistically different from non-inoculated leaf samples. Eighty-eight genes were differentially expressed in the tolerant sunflower line.

Key words: *Arabidopsis* – genomics – *Helianthus annuus* – microarrays – oligonucleotide – *Sunflower chlorotic mottle virus*

INTRODUCTION

Several viruses infect commercial sunflower hybrids, including *Cucumber mosaic virus*, *Tobacco streak virus*, *Potato virus Y* and *Tomato spotted wilt virus*, whereas *Tobacco ringspot virus* and *Sunflower mosaic virus* have been found in wild sunflowers (Gulya et al., 2002). In Argentina, *Sunflower chlorotic mottle virus* (SuCMoV) has been associated with sunflower chlorotic mottling and plant stunting symptoms and it has been reported in several provinces including Entre Ríos, Santa Fé, Buenos Aires and Córdoba. This virus has been fully characterized (Dujovny et al., 1998, 2000) and has recently been classified as a strain of *Potato virus Y* (Berger et al., 2005). Yield losses associated with this disease can be important if epidemics break up at an early stage of plant development (Lenardon et al., 2001).

Recently, a sunflower line tolerant to SuCMoV infection has been found and the resistance gene Rcmo-1 was mapped (Lenardon et al., 2005). Oxidative stress has been reported in compatible interactions between SuCMoV and a susceptible sunflower line (Arias et al., 2003) and sunflower lines with different responses to SuCMoV infection differed in chlorophyll loss, oxidative generation and antioxidant enzyme activity (Arias et al., 2005). The current work compares transcripts from infected plants of a SuCMoV tolerant-line and a susceptible one using oligonucleotide microarrays from *A. thaliana* in order to analyze the known biological pathways that are well developed for this species.

MATERIALS AND METHODS

Plant inoculations

SuCMoV was maintained on sunflower hybrid CF-7 and used as inoculum source for the whole experiment. The virus was mechanically inoculated (Dujovny et al., 1998) to sunflower SuCMoV-tolerant (B-133) and susceptible (20016) lines (Advanta Semillas S.A.I.C). The susceptible line showed severe systemic chlorotic mottling and yellow blotches 5-7 days after infection, whereas the tolerant line showed scant isolated chlorotic mottling 10-12 days after inoculation. Control plants of both lines were mock inoculated and did not show any type of symptom.

Sunflower total RNA isolation
Total RNA from infected sunflower leaves was obtained by a modified Schneitz-Lab protocol (www.unizh.ch/Cyto_website/Schneitzlab). Leaf tissue (0.5–1 g) was ground in liquid nitrogen and transferred into a 50 ml Falcon tube containing 10 ml of trizol reagent (Invitrogen Inc, CA, USA). After adding 1 ml 2M NaOAc pH 4.1, 10 ml phenol and 2 ml chloroform/isoamylalcohol (49:1) the tube was stirred for 10 sec and placed on ice for 15 min, centrifuged at 3,500 rpm at 4ºC for 20 min, then the aqueous phase was transferred into a new tube and 10 ml isopropanol were used to precipitate the RNA at -20ºC overnight. The RNA pellets were precipitated after centrifugation at 8,000 rpm during 30 min at 4ºC, washed with ice-cold ethanol 75% and air dried for 10 min. The pellet was dissolved in 1.5 ml sterile DEPC-water and total RNA was quantified using the Nanodrop (Nanodrop Techology, Inc). RNA from healthy sunflower leaves was isolated with the same procedure and used as control.

RNA amplification
Total RNA was amplified using BLR-PCR methodology (Balogh et al., 2006). The procedure involved three steps: 1. cDNA synthesis using the RT enzyme (Superscript from Invitrogen), 2. Amplification based on dT-T7 primer, and 3. *In vitro* transcription reaction of cDNA into RNA. Two rounds of amplifications were performed.

Fluorescent probe synthesis
The fluorescent probe synthesis for the aaRNA from BLR method used an indirect methodology. At the same time, a fluorescent probe from healthy sunflower leaves aRNA (control) was synthesized, in order to use it in the green channel to hybridize with tolerant or susceptible SuCMoV-infected sunflower leaves. For indirect probe-labeling, 5-10 µg of amplified RNA was used as starting material. For labeling, the total RNA and the aaRNA were combined with their respective primers and the mix was incubated at 70°C for 10 min, then chilled on ice for 10 min. Primer–RNA solution was added to the reverse transcriptase mix [5x first-strand buffer, 6 µl; 50x aa-dUTP/dNTPs (25 mM dATP, dGTP, and dCTP, 15 mM dTTP, and 10 mM aminoallyl-dUTP), 0.6 µl; DTT 0.1 M, 3 µl; Superscript II reverse transcriptase (Invitrogen/Life Technologies), 2 µl] and incubated at 42°C for 2 h. The reaction was terminated by adding EDTA (0.5 M, 10 µl) and the RNA was hydrolyzed with NaOH (1 M, 10 µl) at 65°C for 30 min. After cDNA precipitation with ethanol 100% in the presence of glycogen (20µg/µl) and NaOAc 3M (pH 4.5), it was centrifugated at 13,000rpm for 30 min and washed twice with ETOH 70%. The cDNA pellet was resuspended in 7. 5 µl of coupling buffer, adding 2.5 µl of prepared ester-Cy3 (for the control) or ester-Cy5 (for the virus treated-samples) fluorescent dyes (Amersham) and incubated for 1 h at RT in dark. After purification of the fluorescent probes with QIAquick PCR purification kit (Qiagen), they were quantified using the Nanodrop and 40 pmols of fluorescent cDNA was employed for each channel in each microarray.

Arabidopsis-oligonucleotide microarray hybridization
For microarray hybridization, Cy5-fluorescence dye was used for the SuCMoV treated plants (susceptible 20016 or tolerant B133 lines) and Cy3-fluorescence dye for the control non inoculated plants. 750 ng of Cy3-labeled control probe and 750 ng of Cy5-labeled sample probes were used to hybridize an Agilent 60-mer *Arabidopsis thaliana* oligo microarrays slide containing 22,000 features. Hybridization was performed in SureHyb chambers (Agilent Technlogies) at 60ºC for 18 hours and washed following the manufacturer's protocol. The arrays were scanned and analyzed with Feature extraction software (Agilent Technologies) to verify the hybridization quality. Oligonucleotide microarrays from *A. thaliana* were employed in order to analyze the known biological pathways that are well developed for this species.

Image Analysis
The data were analyzed considering at least three independent biological replicates. The acquired images were analyzed by ImaGene software (BioDiscovery Inc., El Segundo, CA), and statistical analysis was performed using Bioconductor softwares (Biodiscovery, Inc, CA), including Lowess normalization using local background correction. The FatiGo software (www.fatiGo.com) was used in order to establish the putative biological processes in which each gene product is involved.

Data Analysis and statistical interpretation
The Limma Package from Smyth (2005) software version was used for data analysis. All genes with p value below a threshold of 0.001 are selected as differentially expressed ones. The meaning of adjusted p-values is the expected proportion of false discoveries in the selected group should be less than the

threshold value, in this case less than 0.005. The B-statistic (odds or B) is the log-odds that the gene is differentially expressed. For example, if B = 1.5, then the odds of differential expression is exp (1.5)=4.48, i.e., about four and a half to one. The probability that the gene is differentially expressed is 4.48/(1+4.48)=0.82, i.e., about 82%. A B-statistic of zero corresponds to a 50-50 chance that the gene is differentially expressed. The B-statistic is automatically adjusted for multiple testing by assuming that 1% of the genes, or some other percentage specified by the user in the call to eBayes (Smyth, 2005; Smyth et al., 2005; Ritchie et al., 2006), are expected to be differentially expressed. The p-values and B-statistics will normally rank genes in the same order. In fact, if the data do not contain any missing values or quality weights, then the order will be precisely the same. As with all model-based methods, the p-values depend on normality and other mathematical assumptions, which are never exactly true for microarray data. It has been argued that the p-values are useful for ranking genes even in the presence of large deviations from the assumptions. The B-statistic probabilities depend on the same assumptions but require in addition a prior guess for the proportion of differentially expressed genes. The p-values may be preferred to the B-statistics because they do not require this prior knowledge. The eBayes function computes one more useful statistic. The moderated F-statistic (F) combines the t-statistics for all the contrasts into an overall test of significance for that gene (Benjamini and Hochberg, 1995).

RESULTS AND DISCUSSION

Genes whose expression significantly changed in inoculated vs. control plants are shown in Tables 1 and 2 for the SuCMoV-susceptible and the tolerant lines, respectively. Twenty two genes were differentially expressed in the susceptible line and 84 genes in the tolerant one.

Table 1. Genomic expression in susceptible-SuCMoV sunflower leaves[1]

Gene ID	Average-A	M	t	P.Value	adj.P.Val	B
A_84_P302790 expressed protein	7,4120	1,0950	9,8602	0,0000	0,0284	2,9315
A_84_P22706 protein kinase, putative contains protein kinase domain.	6,2267	0,8527	5,8979	0,0007	0,0481	0,1192
A_84_P87029 expressed protein contains Pfam profile PF04146	6,8442	0,7828	6,4191	0,0004	0,0320	0,5925
A_84_P20218 elongation factor P (EF-P) family protein similar to SP|P33398	6,9067	0,7319	5,9250	0,0007	0,0473	0,1448
A_84_P15683 myb family transcription factor	6,4775	0,7179	6,5879	0,0004	0,0293	0,7377
A_84_P186034 NLI interacting factor (NIF) family protein contains Pfam profile PF03031	7,2159	0,6916	6,8517	0,0003	0,0284	0,9574
A_84_P231939 glucose-6-phosphate/phosphate translocator	7,2044	0,6190	6,8201	0,0003	0,0284	0,9316
A_84_P15223 P-glycoprotein, putative similar to P-glycoprotein	6,8034	0,6128	10,9633	0,0000	0,0284	3,4647
A_84_P10874 dehydrin xero2 (XERO2) / low-temperature-induced protein LTI30 (LTI30) identical to dehydrin Xero 2	6,9251	0,6098	6,8311	0,0003	0,0284	0,9405
A_84_P222719 hypothetical protein [At3g09130.1]	6,8787	0,6087	6,5327	0,0004	0,0299	0,6907
A_84_P16415SSXT protein-related / transcription co-activator-related similar to SYT/SSX4 fusion protein	6,8820	0,5589	8,1311	0,0001	0,0284	1,9058
A_84_P22786 rhomboid family protein contains PFAM domain PF01694	6,7587	0,5292	6,0665	0,0006	0,0419	0,2765
A_84_P307770 KH domain-containing protein strong similarity to unknown protein	6,7846	0,5239	8,5221	0,0001	0,0284	2,1610
A_84_P179684 expressed protein [At5g09310.1]	6,3477	0,5042	7,3434	0,0002	0,0284	1,3438
A_84_P224379 expressed protein [At4g29590.1]	6,8426	0,5035	6,4543	0,0004	0,0313	0,6231
A_84_P15375 photosystem I reaction center subunit IV, chloroplast, putative / PSI-E, putative (PSAE2)	6,2624	0,4908	7,1903	0,0002	0,0284	1,2266
A_84_P17102 somatic embryogenesis receptor-like kinase 2 (SERK2)	6,8134	0,4804	5,9722	0,0007	0,0453	0,1891
A_84_P187084 peroxidase, putative similar to cationic peroxidase	5,9467	0,3793	6,1172	0,0006	0,0403	0,3230
A_84_P17469 expressed protein [At3g45830.1]	6,4204	0,3778	7,1371	0,0002	0,0284	1,1852
A_84_P10131640S ribosomal protein S15A (RPS15aD) cytoplasmic ribosomal protein S15a.	5,9350	0,3378	6,8354	0,0003	0,0284	0,9440
A_84_P13444452-phosphoglycerate kinase-related contains weak similarity to 2-phosphoglycerate kinase	6,3818	0,3014	6,1188	0,0006	0,0403	0,3244
A_84_P273760 expressed protein predicted proteins, Arabidopsis thaliana [At3g55600.1]	6,0882	0,2985	5,9758	0,0006	0,0453	0,1924

[1]The M-value (M) is the value of the contrast. This represents a log2-fold change between two or more experimental conditions although sometimes it represents a log2-expression level. The A-value (A) is the average log2-expression level for that gene across all the arrays and channels in the experiment. Column t is the moderated t-statistic. Column p-value is the associated p-value after adjustment for multiple testing. The B-statistic is the log-odds that the gene is differentially expressed.

Table 2. Genomic expression of SuCMoV-tolerant sunflower leaves.

Gene ID	Average-A	M	t	P.Value	adj.P.Val	B
A_84_P23884 arabinogalactan-protein (AGP17)	6,0295	-0,3572	-7,4609	0,0002	0,0284	1,4319
A_84_P20246 basic helix-loop-helix (bHLH) family protein contains	6,2236	-0,3772	-7,2189	0,0002	0,0284	1,2487
A_84_P20534ATP-dependent Clp protease proteolytic subunit	5,9659	-0,3795	-6,8156	0,0003	0,0284	0,9278
A_84_P16654 ankyrin repeat family protein / BTB/POZ domain-containing protein contains Pfam domain	6,0625	-0,3863	-6,3605	0,0005	0,0336	0,5411
A_84_P19128 WRKY family transcription factor contains Pfam profile	5,9804	-0,3927	-5,9904	0,0006	0,0450	0,2060
A_84_P10346 cysteine proteinase-related low similarity to cysteine proteinase	6,0577	-0,4107	-6,0972	0,0006	0,0408	0,3047
A_84_P198504 leucine carboxyl methyltransferase family protein contains	6,2713	-0,4120	-6,6227	0,0004	0,0291	0,7673
A_84_P10447 sugar transporter, putative similar to monosaccharide transporter PaMst-1	6,0660	-0,4131	-7,2179	0,0002	0,0284	1,2479
A_84_P185684 MADS-box family protein	6,1283	-0,4132	-6,2040	0,0005	0,0376	0,4018
A_84_P19906 alanine racemase family protein contains	6,0858	-0,4179	-6,4904	0,0004	0,0306	0,6543
A_84_P24164 OTU-like cysteine protease family protein	6,0449	-0,4194	-6,9337	0,0003	0,0284	1,0239
A_84_P20607 endomembrane protein 70, putative TM4 family	6,0823	-0,4235	-7,3464	0,0002	0,0284	1,3461
A_84_P15263 phosphoribulokinase/uridine kinase family protein	6,0928	-0,4236	-6,2281	0,0005	0,0369	0,4234
A_84_P18442 cation/hydrogen exchanger, putative (CHX28) monovalent cation:proton antiporter family 2 (CPA2)	6,1506	-0,4278	-7,7945	0,0001	0,0284	1,6738
A_84_P22466CBS domain-containing protein contains Pfam profile PF00571: CBS domain	6,2454	-0,4314	-5,8936	0,0007	0,0482	0,1152
A_84_P19135 beta-ketoacyl-CoA synthase family protein	6,0022	-0,4553	-6,4448	0,0004	0,0314	0,6149
A_84_P10794 glycoside hydrolase family 19 protein similar to class I chitinase	6,0612	-0,4602	-7,3277	0,0002	0,0284	1,3319
A_84_P175641 expressed protein	6,0996	-0,4631	-7,4331	0,0002	0,0284	1,4112
A_84_P15723NA	5,9461	-0,4677	-6,9532	0,0003	0,0284	1,0395
A_84_P20270NA	6,0177	-0,4742	-10,4829	0,0000	0,0284	3,2426
A_84_P290374 DNA-binding storekeeper protein-related contains Pfam profile	6,1028	-0,4810	-6,2562	0,0005	0,0363	0,4487
A_84_P11724 L-asparaginase, putative / L-asparagine amidohydrolase, putative similar to Swiss-Prot:P30364	6,0750	-0,4910	-6,1961	0,0005	0,0378	0,3947
A_84_P198874 myb family transcription factor contains Pfam profile	6,1290	-0,4922	-8,2374	0,0001	0,0284	1,9767
A_84_P19242NA	5,9641	-0,4974	-5,9192	0,0007	0,0473	0,1394
A_84_P10999 AP2 domain-containing transcription factor	5,9247	-0,5115	-6,5830	0,0004	0,0293	0,7336
A_84_P18950 glycine-rich protein	6,2429	-0,5159	-6,5620	0,0004	0,0296	0,7157
A_84_P23966 expressed protein	6,0259	-0,5217	-9,9400	0,0000	0,0284	2,9730
A_84_P17637 AP2 domain-containing transcription factor	6,0975	-0,5380	-9,1113	0,0001	0,0284	2,5188
A_84_P23205 mitochondrial ribosomal protein L51/S25/CI-B8 family protein	6,2225	-0,5382	-7,7502	0,0001	0,0284	1,6424
A_84_P18471 60S ribosomal protein-related contains weak similarity to 60S ribosomal protein L10A	6,0710	-0,5384	-7,3067	0,0002	0,0284	1,3160
A_84_P15363 F-box family protein contains F-box domain	5,9733	-0,5585	-10,3789	0,0000	0,0284	3,1925
A_84_P21494 pseudo-response regulator 7 (APRR7) identical to pseudo-response regulator 7	6,2043	-0,5588	-11,4663	0,0000	0,0284	3,6818
A_84_P13552 ribosomal protein S14 mitochondrial family protein identical to ribosomal protein S14	5,9936	-0,5659	-8,4420	0,0001	0,0284	2,1100
A_84_P10460 polyubiquitin, putative similar to polyubiquitin	6,0980	-0,5674	-6,2467	0,0005	0,0365	0,4401
A_84_P1684 armadillo/beta-catenin repeat family protein contains Pfam profile	6,1843	-0,5715	-6,8277	0,0003	0,0284	0,9378
A_84_P183904mitochondrial transcription termination factor-related / mTERF-related contains Pfam profile PF02536: mTERF [At5g54180.1]	6,0477	-0,5960	-6,1229	0,0006	0,0403	0,3282
A_84_P18169 polygalacturonase, putative / pectinase	6,0073	-0,6003	-5,8890	0,0007	0,0482	0,1108
A_84_P11898 KOW domain-containing protein contains Pfam PF00467	6,2659	-0,6143	-6,2367	0,0005	0,0367	0,4312
A_84_P22675FAD-binding domain-containing protein similar to SP|P30986 reticuline oxidase precursor (Berberine-bridge-forming enzyme)	6,0213	-0,6196	-9,7199	0,0000	0,0284	2,8576
A_84_P21736 expressed protein [At3g06895.1]	6,0732	-0,6251	-12,6469	0,0000	0,0249	4,1369
A_84_P108252S-locus protein kinase, putative similar to receptor protein kinase	6,2286	-0,6531	-9,0587	0,0001	0,0284	2,4882
A_84_P22508 expressed protein [At5g27730.1]	6,0478	-0,6557	-6,9455	0,0003	0,0284	1,0333
A_84_P19649 protein kinase-related contains protein kinase domain	6,1390	-0,6709	-8,7420	0,0001	0,0284	2,2982
A_84_P17183 phosphomannose isomerase, putative (DIN9) contains Pfam profile: PF01238 phosphomannose isomerase type I	6,1764	-0,6715	-6,9083	0,0003	0,0284	1,0034
A_84_P236543 phototropic-responsive NPH3 family protein contains NPH3 family domain	6,1433	-0,6807	-6,1101	0,0006	0,0405	0,3165
A_84_P10452 phenazine biosynthesis PhzC/PhzF family protein	6,1594	-0,6837	-8,6650	0,0001	0,0284	2,2507
A_84_P306780 expressed protein [At4g33690.1]	6,0027	-0,6965	-12,2907	0,0000	0,0249	4,0071
A_84_P22097 transducin family protein / WD-40 repeat family protein contains 2 WD-40 repeats	6,0636	-0,7002	-8,8670	0,0001	0,0284	2,3742
A_84_P12748 expressed protein [At1g48450.1]	6,1215	-0,7028	-7,7855	0,0001	0,0284	1,6674
A_84_P12828 multidrug resistance P-glycoprotein, putative similar to multidrug-resistant protein CjMDR1	6,1969	-0,7095	-8,4329	0,0001	0,0284	2,1041
A_84_P23698 gibberellin-responsive protein, putative similar to SP|P46688 Gibberellin-regulated protein 2 precursor	6,0641	-0,7243	-7,1993	0,0002	0,0284	1,2335
A_84_P258730 forkhead-associated domain-containing protein / FHA domain-containing protein	6,1882	-0,7355	-7,9378	0,0001	0,0284	1,7740
A_84_P181184 glycosyl hydrolase family 18 protein contains Pfam profile PF00704	6,1473	-0,7695	-6,4453	0,0004	0,0314	0,6153
A_84_P130796 expressed protein [At3g62580.1]	6,1998	-0,7836	-10,1474	0,0000	0,0284	3,0784
A_84_P17154Q9LY19 (Q9LY19) Pectin methyl-esterase-like protein, complete [TC263781]	6,0633	-0,7987	-7,3220	0,0002	0,0284	1,3276
A_84_P18903 Golgi SNARE 11 protein identical to Golgi SNARE 11 protein	6,3686	-0,8001	-8,3804	0,0001	0,0284	2,0702
A_84_P88749 expressed protein contains Pfam profile PF03080	6,1768	-0,8091	-8,7249	0,0001	0,0284	2,2877
A_84_P107132 F-box family protein contains F-box domain Pfam:PF00646 [At1g32140.1]	6,2277	-0,8256	-9,9183	0,0000	0,0284	2,9618
A_84_P91239 expressed protein [At2g16760.1]	6,1667	-0,8421	-6,8491	0,0003	0,0284	0,9553
A_84_P21687 glyceraldehyde-3-phosphate dehydrogenase B, chloroplast subunit B (GAPB) / NADP-dependent glyceraldehydephosphate dehydrogenase	7,2667	-0,8634	-8,7954	0,0001	0,0284	2,3309
A_84_P216998 expressed protein strong similarity to unknown protein	6,2842	-0,8736	-6,3126	0,0005	0,0349	0,4989
A_84_P154245 expressed protein [At4g33480.1]	6,1892	-0,8785	-6,6138	0,0004	0,0292	0,7597
A_84_P117052 expressed protein contains Pfam profile PF04759	6,3999	-0,8922	-7,9861	0,0001	0,0284	1,8073
A_84_P13513 protein kinase, putative contains protein kinase domain	6,0580	-0,9007	-12,2489	0,0000	0,0249	3,9915
A_84_P13102 transferase family protein similar to anthranilate N-hydroxycinnamoyl/benzoyltransferase	6,6334	-0,9245	-6,4834	0,0004	0,0307	0,6483
A_84_P84909Q9LSN6 (Q9LSN6) Arabidopsis thaliana genomic DNA, chromosome 3	6,2436	-0,9249	-11,7643	0,0000	0,0280	3,8036
A_84_P13924 homeodomain protein (BELLRINGER) several homeodomain proteins	6,0814	-0,9299	-10,9980	0,0000	0,0284	3,4801
A_84_P13746 serine/threonine protein kinase, putative similar to Pto kinase interactor 1 (Pti1)	6,1311	-0,9605	-8,9603	0,0001	0,0284	2,4301
A_84_P10718 two-component responsive regulator-related / response regulator protein-related	6,2384	-0,9667	-6,2855	0,0005	0,0355	0,4748
A_84_P14575 halotolerance protein (HAL3A) identical to GB:AAD51616 from	6,1256	-0,9865	-16,7928	0,0000	0,0220	5,2825
A_84_P19632 arabinogalactan-protein (AGP15)	6,3536	-0,9896	-7,2109	0,0002	0,0284	1,2425
A_84_P1658840S ribosomal protein S13 (RPS13A) AtRPS13A mRNA	6,3063	-1,0191	-10,0159	0,0000	0,0284	3,0119
A_84_P18451RNA polymerase sigma subunit SigC (sigC) / sigma factor 3 (SIG3)	6,2597	-1,1044	-8,3487	0,0001	0,0284	2,0496
A_84_P244595 expressed protein [At5g67620.1]	7,6589	-1,1428	-6,2554	0,0005	0,0363	0,4479
A_84_P19563 L-ascorbate peroxidase, putative similar to ascorbate peroxidase [Gossypium hirsutum]	6,2166	-1,2228	-7,2824	0,0002	0,0284	1,2974
A_84_P21499 aspartyl aminopeptidase, putative similar to SP|Q9ULA0 Aspartyl aminopeptidase	6,3542	-1,2562	-6,4943	0,0004	0,0306	0,6577
A_84_P155285 calmodulin-binding protein-related contains	7,0546	-1,3937	-8,4296	0,0001	0,0284	2,1020
A_84_P307190 integral membrane protein, putative contains 4 transmembrane domains	6,3567	-1,4500	-7,6461	0,0001	0,0284	1,5677
A_84_P18413 lectin protein kinase family protein contains Pfam domains PF00138	6,4838	-1,4833	-7,4521	0,0002	0,0284	1,4254
A_84_P13868 stigma-specific Stig1 family protein low similarity to stigma-specific protein STIG1	6,8161	-1,5079	-8,7475	0,0001	0,0284	2,3016
A_84_P270250 expressed protein [At3g45750.1]	6,5488	-1,5180	-7,9357	0,0001	0,0284	1,7726
A_84_P21185 leucine-rich repeat transmembrane protein kinase	6,6231	-1,5840	-8,7576	0,0001	0,0284	2,3078
A_84_P23790RWP-RK domain-containing protein similar to nodule inception protein	6,3086	-1,6702	-6,7172	0,0003	0,0286	0,8465
A_84_P833592,4-dienoyl-CoA reductase-related low similarity to peroxisomal 2,4-dienoyl-CoA reductase	6,2780	-1,6970	-8,0776	0,0001	0,0284	1,8697
A_84_P11251UDP-galactose/UDP-glucose transporter-related	7,0793	-1,8752	-7,9786	0,0001	0,0284	1,8021
A_84_P15770 methylcrotonyl-CoA carboxylase beta chain, mitochondrial / 3-methylcrotonyl-CoA carboxylase 2 (MCCB)	6,7478	-1,8988	-13,4946	0,0000	0,0249	4,4224
A_84_P159445 invertase/pectin methylesterase inhibitor family protein	6,4597	-2,0236	-7,7064	0,0001	0,0284	1,6111
A_84_P278320 expressed protein [At4g38060.2]	6,4566	-2,0826	-12,2350	0,0000	0,0249	3,9863
A_84_P13029 lipase class 3 family protein low similarity to Triacylglycerol Acylhydrolase	6,4270	-2,1386	-7,5034	0,0002	0,0284	1,4634
A_84_P19134 60S ribosomal protein L23 (RPL23B)	6,5888	-2,8132	-12,9919	0,0000	0,0249	4,2569
A_84_P22874 GDSL-motif lipase/hydrolase family protein similar to myrosinase-associated proteins	7,2955	-3,9183	-5,9822	0,0006	0,0452	0,1984

After obtaining the number of genes differentially expressed in the susceptible and tolerant sunflower lines, the biological functions related to those genes were studied. 16.7% of the expressed genes in the tolerant line and only 0 to 2.9% in the susceptible line were related to responses to inorganic substances, osmotic stress, jasmonic acid stimulus, salicylic acid stimulus, water and hormone stimulus. 5.8% gene expression belonged to the cellular catabolic process in the susceptible leaves (Fig. 1).

Fig. 1. Percentage of genes related to different biological processes in virus susceptible (upper bars) and tolerant (lower bars) sunflower plants, as expressed in leaf tissue.

The results indicate that the defense mechanisms involving the molecular response were up-modulated in the SuCMoV-tolerant sunflower. Other biological functions did not show significant differences between either group. In the SuCMoV-susceptible sunflower plants we also observed some expression of defense-related genes, but less importantly than in the tolerant ones.

The data suggest that sunflower plants activate different defense mechanisms, from changes in cell wall composition (mechanical protection) to the activation of defense pathways. The energy suppression in the infected-plants, and the increased lignification of the tolerant plants could be other responses to the

pathogen. Defense mechanisms in the sunflower were: activation of SAR, oxidative pathway induced by oxigene, response to salicylic acid (SA) stimulation, gene expression involving specific molecular pathway, activation of local resistant LAR and response to jasmonic acid (JA) stimulation.

These results have been validated by real time-RT-PCR, and by experiments aimed to establish whether the lines differ in SA concentration and if this is related to the different tolerance to SuCMoV. Likewise, it would be interesting to determine whether responses to SA and JA are antagonic.

ACKNOWLEDGEMENTS

Dr. Peter Morrison, Department of Biostatistics, Biodiscovery, Inc, San Diego, California, USA for the biostatistics analysis, Dr. Dough Postl, Department of Technology, Agilent Technology, San Diego, California, USA for technical advises and Dr. Edith Taleisnik, CONICET, for reviewing and critical comments.

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Molecular mapping of a new induced gene for nuclear male sterility in sunflower (*Helianthus annuus* L.)

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ABSTRACT

Nuclear male sterility (NMS) is a valuable breeding tool for producing sunflower hybrids without any tedious emasculation. A new NMS line, NMS HA89-872, induced by mitomycin C and streptomycin carries a single recessive male-sterile gene ms_6. The objectives of this study were to identify molecular markers linked to the male sterility gene, and to map the *ms* locus on the sunflower genome. An F_2 population of 88 plants was obtained from a cross between the nuclear male-sterile mutant NMS HA89-872 (*msms*) and the male-fertile line RHA271 (*MsMs*). More than 230 pairs of primers, including 225 SSR primers and 9 STS primers were assayed. Nine SSR and three STS markers were polymorphic between male-fertile and male-sterile bulks, and were used to screen the mapping population. Seven SSR (ORS31, ORS294, ORS495, ORS519, ORS885, ORS807, ORS996) and two RFLP-derived STS (STS5C1 and STS11D3) markers were identified to be linked with ms_6. The ms_6 locus was flanked by ORS 807 and ORS996 at a distance of 7.2 and 18.5 cM, respectively, on linkage group 16 of the sunflower SSR genetic map.

Key Words: nuclear male sterility – Sequence Tagged Site (STS) – Simple Sequence Repeat (SSR) – sunflower.

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the most important worldwide oil crops based on the utilization of the heterosis phenomenon. The male sterility represents the malfunction of plants to produce functional anthers, pollen, or male gametes (Kaul, 1988), while the gynoecium is functional. The male sterility can appear spontaneously or chemically induced via mutations in nuclear and/or cytoplasmic genes. As a result, it leads to the production of nuclear or genic male sterility (NMS or GMS) and cytoplasmic male sterility (CMS), respectively. Genetic control of NMS can be determined by several types of gene actions (Chaudhury, 1993; Horner and Palmer, 1995). However, the most common genetic control of nuclear male sterility is monogenic (Gundaev, 1971; Leclercq, 1966). About 10 Romanian nuclear male sterility lines were established which were under the control of five NMS genes, designated ms_1-ms_5 (Vrânceanu, 1970).

Four additional NMS lines were obtained from HA89 treated with mitomycin C and streptomycin. The resulting NMS lines were designed as ms_6-ms_9 (Jan and Rutger, 1988; Jan, 1992). The ms_9 gene was mapped to linkage group 10 of the sunflower SSR genetic map by Chen et al. (2006). NMS genes in lines B11A3 and P21 were designated ms_{10} and ms_{11} (Jan, 1992), and mapped to linkage group 11 and 8, respectively, by Pérez-Vich et al. (2005). However, other NMS genes have not been mapped. The objectives of this study were to identify molecular markers linked to ms_6 in NMS HA89-872, and to map the ms_6 locus onto the sunflower linkage group.

MATERIALS AND METHODS

Plant material

The line NMS HA89-872 induced by chemical mutagenesis (Jan and Rutger, 1988) and RHA271 were used as parents in this study. NMS HA89-872 is homozygous for the recessive alleles ms_6ms_6 controlling nuclear male sterility (Jan, 1992). RHA271 is a fertility restorer line for the cmsPET1 cytoplasm. An F_2 mapping population of 88 plants was developed from crossing NMS HA89-872 and RHA271. F_3 families were produced by self-pollination of male-fertile F_2 plants, and scored as male-fertile or male-sterile to

distinguish hetero- and homozygous F_2 progeny. The segregation of male-fertile to male-sterile genotypes of F_2 progeny was tested by Chi-square goodness of fit.

DNA isolation
Genomic DNA was isolated from lyophilized leaf powders of each individual. DNA extraction was performed in CTAB buffer, following standard steps of DNA purification. Bulk segregant analysis (Michelmore et al., 1991) was performed by pooling an equal quantity of DNA from 10 homozygous male-fertile and 10 homozygous male-sterile F_2 plants.

Primer design
In total**,** 225 SSR primers selected from the Compositae species database (compositdb.ucdavis.edu) were used for the *ms*$_6$ gene screening. Sequence Tagged Site (STS) primers were designed based on the associated RFLP sequences (Jan et al., 1998), using Primer3 software (http://frodo.wi.mit.edu). The sequences of two STS primers which produced polymorphic markers are listed in Table 1.

Table 1. STS primers used in genetic mapping of *ms*$_6$ locus.

Locus	Primer	
	Forward (5'→3')	Reverse(5'→3')
STS5C1	GGATTTCCGAAAACAGTACA	TTGTTGTAAGCCTGGAGAGT
STS11D3	AAAAACATTTGTCCCATTTG	CAAAAGGACATGTGAAAAGC

PCR amplification and data analysis
For SSR analysis, about 20 ng genomic DNA was used as a template in a 15 µl reaction volume per PCR reaction and the products were amplified following the Touchdown PCR profile (Tang et al., 2002) with slight modifications. Electrophoresis was performed in 6.5% polyacrylamide gel at 60 w for 2.2 h. The images were obtained using a Typhoon 9410 variable mode imager (Molecular Dynamics Inc., CA, USA) and ImageQuant software (GE Healthcare). The scoring codes were 1 for present, 0 for absent, and a dash "-" for missing.

A genetic linkage map was constructed using the Kosambi function (Kosambi, 1944) of Mapmaker/Exp version 3.0b (Lander et al., 1987). Map distances in centimorgans (cM) were evaluated and the linkage map was drawn with MapChart 2.0 (Voorips, 2002).

RESULTS

Inheritance of the ms$_6$ *gene*
The analysis of F_2 progeny phenotype allowed scoring the male-sterile/male-fertile plants, with 65 classified as male-fertile and 23 male-sterile. Chi-square analysis of F_2 population revealed a good fit to 3 fertile: 1 sterile ratio (χ^2 =0.06, 0.80<P<0.90), indicating that male sterility was conditioned by a single recessive gene. The $F_{2:3}$ progeny test separated the F_2 progeny into 23 homozygous male-fertile plants, 42 heterozygous male-fertile plants, and 23 male-sterile plants, which correspond to the segregation ratio of 1:2:1 (χ^2 = 0.18, 0.90 < P<0.95).

Molecular mapping of the ms$_6$ *gene*
Two hundred and twenty-five SSR primers randomly chosen from 17 linkage groups of the public SSR genetic map (Tang et al., 2002) were used to screen the male-sterile and male-fertile bulks. Nine SSR markers were shown to be polymorphic between the two bulks (Table 2), and seven of them, ORS31, ORS294, ORS495, ORS519, ORS807, ORS885, ORS996, were linked to male-sterile allele *ms*$_6$ on linkage group 16. The segregation ratios of these markers fit a 3:1 ratio, with small deviations (χ^2 =0.06-2.18, 0.1< P <0.8). (Table 3)

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Table 2. Genetic polymorphism of molecular markers between male-fertile and male-sterile bulks.

	SSR markers	STS markers
No. of primers	225	9
No. of Polymorphic primers	9	3
No. of Polymorphic primers linked to *ms*$_6$	7	2
Percentage of polymorphic primers (%)	4.1	33.3

Table 3. Chi-square test for the segregation ratio of the male sterility/fertility trait, SSR markers, and STS markers in the F_2 population of NMS HA89-872 × RHA271.

	Number of plants	Type of inheritance[1]					Ratio fit	χ^2-value	Probability ($>\chi^2$)
		AA	HH	BB	CC	DD			
ms$_6$	88	23	42	23			1:2:1	0.18	0.91
STS5C1	88	17	46	25			1:2:1	1.64	0.44
ORS996	88	24			64		3:1	0.24	0.62
STS11D3	88	28			60		3:1	2.18	0.14
ORS31	74	22			52		3:1	0.88	0.35
ORS294	88	28			60		3:1	2.18	0.14
ORS495	87	27			60		3:1	1.69	0.19
ORS885	88	21			67		3:1	0.06	0.81
ORS519	88	29			59		3:1	2.97	0.08
ORS807	84			25		59	3:1	1.02	0.31

[1]Genotypes: AA = NMS 89-872 (*msms*); HH = heterozygous (*Msms*); BB = RHA271 (*MsMs*); CC = not AA (*MsMs* or *Msms*); DD = not BB (*Msms* or *msms*).

After confirming the location of *ms*$_6$ to linkage group 16, nine STS primers were designed based on the sequences of RFLP markers from linkage group 3 (Jan et al., 1998), which cross-referenced with linkage group 16 of the SSR genetic map (Yu et al., 2003), to screen the F_2 population. Two STS primers, STS5C1 and STS11D3, were polymorphic and linked to *ms*$_6$ male sterility gene (Table 3). STS5C1 was represented by 25 homozygous male-fertile, 46 heterozygous male-fertile plants, 17 homozygous male-sterile plants (Fig. 1), with a ratio of 1:2:1 (χ^2 =1.64, 0.4< P <0.5). Segregation of STS11D3 fit to 3:1 ratio, indicating a recessive male sterile inheritance. Based on 8 SSR markers and 2 STS markers linked with *ms*$_6$ gene, the map of the *ms*$_6$ gene spanned within a region of 69.2 cM, and the *ms*$_6$ locus was flanked by ORS 807 and ORS996 at the distance of 7.2 and 18.5 cM, respectively (Fig. 2).

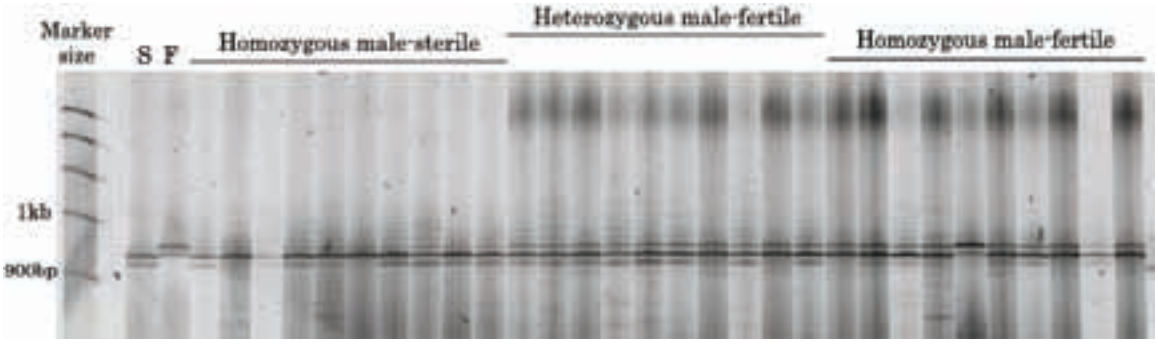

Fig. 1. The banding pattern of the marker STS5C1 for two bulks and homozygous male-sterile, heterozygous male-fertile and homozygous male-fertile plants in the F_2 generation. F=male-fertile bulk; S=male-sterile bulk.

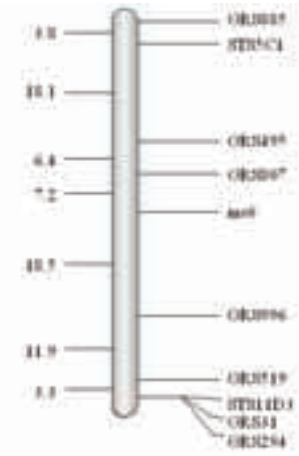

Fig. 2. Mapping of the *ms*$_6$ gene on LG 16. Distances are shown in centiMorgans (cM).

DISCUSSION

Pollen development in higher plants is a complex process (McCormick, 1993). The abortion of anther development results in the appearance of male-sterile plants. Molecular markers linked with *ms* locus can offer the quick and precise detection of genotype bearing a male-sterility allele.

In this study, different marker systems, including SSR and RFLP-derived STS, were used for mapping the *ms* locus in sunflower. Of 234 markers, nine proved to be associated with the male- sterility gene. The ms_6 gene was mapped to linkage group 16 and it was flanked by SSR markers ORS807 and ORS996. A total of two STS markers based on RFLP sequences were effectively mapped and integrated into the previously described linkage maps (Jan et al., 1998; Yu et al., 2003). The locus order for the SSR and STS markers were similar to the reference maps (Jan et al., 1998; Yu et al., 2003). These SSR and STS markers with co-dominant and dominant gene actions would be useful for marker-assisted selection in breeding programs.

ACKNOWLEDGEMENTS

The authors thank Bing Yue and Caifeng Li for technical assistance. We would also like to thank Gerald J. Seiler and Steve S. Xu for their critical review of this manuscript. The paper was realized by support of MRDA/CRDF, travel project MTFP-1017A.

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Fine mapping of the downy mildew resistance locus Pl_{ARG} in sunflower

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ABSTRACT

The oomycete *Plasmopara halstedii* is the causal agent of downy mildew and can cause high yield losses in sunflower (*Helianthus annuus* L.). Several *Pl* loci from different sources were introduced into breeding material to control the pathogen. One of it is Pl_{ARG} which originates from the wild sunflower species *Helianthus argophyllus* and protects sunflower against all known races of *P. halstedii*. The aim of our project was to investigate the fine structure of the Pl_{ARG} locus to answer the question, whether the Pl_{ARG} locus is a single resistance gene or a cluster of resistance genes effective against different races of *P. halstedii*. Pl_{ARG} was mapped on linkage group (LG) 1 in a population from cross cmsHA342 x ARG1575-2 comprising more than 1,000 F_2 plants. In comparison with the intraspecific *H. annuus* reference map, recombination on LG 1 of our cross was suppressed. Several flanking markers and a few which completely cosegregate with Pl_{ARG} could be identified. ARG1575-2, a backcross-derived resistant inbred line, carries *H. argophyllus* (donor) alleles throughout LG 1 and no alleles from the recurrent parent (HA89); thus, LG 1 in ARG1575-2 originated from *H. argophyllus*.

Key words: fine mapping – *H. annuus* – *H. argophyllus* – *Plasmopara halstedii* – suppressed recombination.

INTRODUCTION

Plasmopara halstedii (Farl.) Berl and de Toni, a seed-, soil- and wind-born pathogen, is one of the major diseases in cultivated sunflower (*Helianthus annuus* L.) and can cause yield losses of up to 95%. The disease can be controlled with fungicides (e.g. metalaxyl) and resistant hybrids. Pl_1 was the first known resistance locus against downy mildew, pathotype 100, discovered by Vrânceanu and Stoenescu (1970). Later, additional *Pl* genes, originating from different species of *Helianthus*, were described and conferred resistance against one or more pathotypes (Zimmer and Kinman, 1972; Miller and Gulya, 1991; Rahim et al., 2002). Mapping of *Pl* genes and studies on the inheritance of resistance to different races of *P. halstedii* showed that some of the *Pl* genes are clustered and are not single genes as initially reported (Mouzeyar et al., 1995; Roeckel-Drevet et al., 1996; Vear et al., 1997; Bert et al., 2001; Bouzidi et al., 2002; Radwan et al., 2003).

The question remains unsolved whether the Pl_{ARG} locus is a single gene which can cause a non-race-specific complete resistance against several pathotypes or whether this is a complex locus containing several closely linked *Pl* genes, each of which provides resistance to one single pathotype. Clusters of resistance genes have been identified in different plant species including lettuce, a member of the *Compositae*, where resistance genes against the downy mildew fungus *Bremia lactucae* exist and are organized in multigene families (Meyers et al., 1998).

The aim of this project is to explore the structure of the Pl_{ARG} locus, which was introgressed from the wild species *H. argophyllus* (Seiler et al., 1991). Pl_{ARG} confers resistance to all known races of *P. halstedii* (Seiler et al., 1991; G.J. Seiler, personal communication). The Pl_{ARG} locus is unlinked to other known downy mildew resistance genes in sunflower and has been mapped to LG 1 (Dußle et al., 2004).

MATERIALS AND METHODS

Sunflower genotypes

The sunflower line ARG1575-2 carries the Pl_{ARG} locus (Seiler et al., 1991) and is resistant to all known races of *P. halstedii*. ARG1575-2 arose by backcrossing the wild species *H. argophyllus* (Acc. 1575) three times with cmsHA89 followed by five selfing generations (Seiler et al., 1991). The isogenic

sunflower lines cmsHA342 and HA342 are susceptible to all known races of *P. halstedii*. ARG1575-2 was crossed to each of the susceptible lines and several flower heads were used for generating two populations for fine mapping of the Pl_{ARG} locus. Population 1 (cmsHA342 x ARG1575-2) contains 1063 F_2 individuals and population 2 (HA342 x ARG1575-2) includes 1,084 F_2 individuals.

Resistance tests

Phenotypic resistance evaluation was conducted in a subset of 185 $F_{2:3}$ families of population 1 (cmsHA342xARG1575-2), using the whole seedling immersion test (Gulya, 1996) with a suspension of *P. halstedii* spores of race 730. The resistance of F_2 plants was investigated by testing 16-40 F_3 seedlings per F_2 individual. Seedlings were scored as susceptible if a high fungal sporulation was evident on cotyledons. Seedlings were scored to be resistant if no or only spurious sporulation was observed on cotyledons. Progenies with unclear evaluation were re-tested and 4-10 $F_{3:4}$ families were tested additionally. According to the segregation in the corresponding F_3 or F_4 families, F_2 plants were then classified as homozygous susceptible, homozygous resistant or heterozygous.

Marker analysis

Around 180 simple sequence repeat (SSR) markers (Tang et al., 2002) were screened for polymorphisms between cmsHA342 and ARG1575-2. SSRs showing polymorphisms between the resistant and the susceptible parents were analysed with bulked segregant analysis (BSA) (Michelmore et al., 1991). Each phenotypic bulk contained 15 homozygous susceptible or 15 homozygous resistant F_2 individuals, respectively. Codominant SSRs exhibiting differences between the resistant and the susceptible bulks were mapped. Further markers like single nucleotide polymorphism (SNP) markers (Lai et al., 2005a) and resistance gene candidate (RGC) markers were screened with BSA using the single strand conformation polymorphism (SSCP) method or analysed as CAPS (cleaved amplified polymorphic sequences) markers.

Statistical analysis

Goodness-of-fit test and maps were constructed with JOINMAP 3.0 (Stam 1993) using a LOD threshold of 4.0 and the mapping function of Kosambi (1944).

RESULTS

Resistance tests with race 730 were carried out on the F_3 progenies derived from a subset of 185 F_2 individuals from population cmsHA342 x ARG1575-2. The distribution of the F_2 subset was 26 homozygous resistant, 114 heterozygous-resistant and 45 homozygous-susceptible. We observed a significant distortion from the expected 1:2:1 segregation ratio for all markers and for Pl_{ARG} (χ^2=13.9, DF=2, p=0.001).

Sixteen codominant SSR markers all mapping to LG 1 were found with BSA. Thus, marker analysis focused on this linkage group. Eleven SNPs (Lai et al., 2005a) and three RGCs from LG 1 were screened with BSA. For SNP marker HT211 and one RGC cleaved amplified polymorphic sequences (CAPS) makers were developed and were mapped into the target interval.

A cluster of markers including ORS610, ORS543, ORS1128, CRT272, ORS1039, ORS1182 and ORS710 cosegregated at the distal end of LG 1. Between these markers and HT211, ORS662 which completely cosegregated with Pl_{ARG} in population 1, we found only a few recombinations. Most recombinations were proximal to Pl_{ARG} where we mapped markers ORS053, ORS959 and ORS371. In comparison with the intraspecific *H. annuus* map of LG 1 of Tang et al. (2002) our map is ten times shorter for the interval ORS610-ORS959. Therefore we screened a second F_2 population from the same cross to find more recombinants. In the second population the susceptible parent HA342 does not carry the CMS plasma. Here, we found the expected segregation ratio of 1:2:1 for all markers. Marker order and map length are the same as in population 2. Phenotyping is still in progress and thus Pl_{ARG} has not been mapped in population 2. In both populations, the order of the markers was the same as in the reference map of Tang et al. (2002).

The comparison of the marker alleles of 13 markers evenly distributed along LG 1 showed that the resistant parent ARG1575-2 carries only alleles from the wild species *H. argophyllus* and no allele from the backcrossing parent cmsHA89. This indicates that no recombination occurred on LG 1 during the backcrossing process.

DISCUSSION

Several reports indicate the wide spread of existing pathotypes and the discovery of new downy mildew races (Gulya et al., 1991; Molinero-Ruiz et al., 2002). Some races developed tolerance to the fungicide metalaxyl (Albourie et al., 1998). Therefore, it is necessary to search for new resistance sources against *P. halstedii* and to investigate the structure and functionality of the *Pl* loci in order to use them effectively and durably in plant breeding.

Wild species are often used as a source for resistance genes in different cultivars, because they represent a rich source of untapped R genes (Slabaugh et al., 2003). For example, sunflower rust resistance genes were introduced from wild *Helianthus* species into the cultivated sunflower (Quresh et al., 1993). Pl_{ARG}, introduced from *H. argophyllus*, is an outstanding source of resistance because of its wide range of efficacy against all known races of *P. halstedii*. However, the genetic structure of the locus is still unclear. Vear et al. (1997) showed that Pl_6 is not a single gene, conferring resistance to all downy mildew races, but rather a cluster of genes, each providing resistance to one or few downy mildew races. Slabaugh et al. (2003) gave the physical evidence for a large cluster of resistance genes in the Pl_1-Pl_2-Pl_6 region. Therefore, it is an obvious assumption that Pl_{ARG} could be a cluster of resistance genes too and thus the goal of our project is to investigate the fine structure of the Pl_{ARG} locus. First Pl_{ARG} was mapped in a subset of lines to identify flanking markers for the Pl_{ARG} locus. Only the flanking markers were used to screen the whole population (cmsHA342 x ARG1575-2) to identify F_2 individuals with recombination events in the target region. This strategy allows to restrict phenotyping work with different races of *P. halstedii* to genetically informative lines (Bauer and Graner, 1995).

On LG 1 recombination was suppressed as concluded from comparing the map distances with published intraspecific maps. Several flanking markers and a few which completely cosegregated with Pl_{ARG} could be detected. To investigate the reason for the suppressed recombination, the marker alleles of LG 1 were compared between cmsHA342, ARG1575-2 and cmsHA89, the recurrent backcrossing parent. Marker alleles of ARG1575-2 and cmsHA89 differed for all markers tested along LG 1. It can be concluded that LG 1 was derived from the original *H. argophyllus* accession used as the donor and that during backcrossing no recombination occurred between linkage group 1 in *H. annuus* and the homologous *H. argophyllus* chromosome. Thus, with respect to LG 1, HA342 x ARG1575-2 can be regarded as a cross between the wild species *H. argophyllus* and cultivated sunflower.

Suppressed recombination often occurs in populations with gene material from wild species and sometimes is accompanied by negative side-effects such as undesirable linkage drag. The root-knot nematode resistance gene (*Mi*) was introgressed into the cultivated tomato from the wild species, *Lycopersicum peruvianum*. In crosses containing the *Mi* gene, suppressed recombination occurred and the map was five times shorter than in crosses without the *Mi* gene (Messeguer et al., 1991). The authors assumed that the suppressed recombination may be due to reduced homology between the *L. esculentum* DNA and the *L. peruvianum* DNA containing the *Mi* gene.

In sunflower, comparative genetic linkage maps of *H. annuus*, *H. petiolaris*, *H. anomalus*, *H. deserticola* and *H. paradoxus* were established to study karyotypic evolution. It became apparent that chromosomal rearrangements had the highest rate reported for any taxonomic group (Burke et al., 2004; Lai et al., 2005b). Heesacker et al. (2006) extended comparative mapping to *H. argophyllus*, because silver-leaf sunflower is a source of novel alleles for the improvement of common sunflower. They showed that nine *H. argophyllus* and *H. annuus* linkage groups are collinear and the other *H. argophyllus* linkage groups carry translocations, inversions, or both when compared to *H. annuus* linkage groups. LG 1 is one of the collinear linkage groups compared with *H. annuus*, and, therefore, chromosomal rearrangements are not a likely reason for suppressed recombination. Recombination on LG 1 could be suppressed, because of reduced homology between *H. annuus* and the wild *H. argophyllus*. In the intraspecific cross of *H. argophyllus* recombination was suppressed per se in the region distal to Pl_{ARG} (A. Heesacker, personal communication) which could be an alternative explanation for our findings or it could be an interplay of both mechanisms.

Further work is in progress to investigate the fine structure of Pl_{ARG}. A large population which does not segregate for Pl_{ARG} is being developed to map additional markers with a higher genetic resolution and to explore the relationship between genetic and physical distances in the target region. With cosegregating markers, a BAC library will be screened and BAC-ends will be sequenced and remapped into the mapping populations. cDNA-AFLPs are used to identify differentially expressed transcripts between the susceptible and resistant parent, each non inoculated and upon inoculation with different races of *Plasmopara halstedii*. Interesting fragments will be sequenced and remapped to the segregating populations to further enrich the target region with potential resistance candidate genes.

ACKNOWLEDGEMENTS

Sunflower seeds of cross cmsHA342 x ARG1575-2 were kindly provided by W. Friedt, Justus-Liebig University, Gießen, Germany. We thank A. Heesacker and O. Radwan for sharing unpublished results. This study is supported by a grant from the Deutsche Forschungsgemeinschaft, Grant no. BA 2073/2-3.

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Identification of molecular markers linked to a new nuclear male-sterility gene *ms$_7$* in sunflower (*Helianthus annuus* L.)

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ABSTRACT

Nuclear male sterility (NMS) is an important alternative system to the cytoplasm male sterility (CMS) in hybrid sunflower breeding programs because of its stable male sterility and the abundance of available restorers. NMS HA89-552 (*Helianthus annuus* L.) is a nuclear male-sterile mutant induced by mitomycin-C and streptomycin from inbred maintainer line HA89, which possesses a single recessive gene, *ms$_7$*, controlling male sterility. Molecular markers linked to the *ms$_7$* gene were identified using a 93-plant F_2 population derived from the cross of NMS HA89-552 × RHA271 with simple sequence repeat (SSR), RFLP-derived sequence tagged site (STS), and target region amplification polymorphism (TRAP) markers. The *ms$_7$* gene was mapped to linkage group 6 of the public SSR genetic map. Four SSR markers (ORS349, ORS608, ORS1229 and ORS483), two RFLP-STS markers (STS8C4, STS9C1) and two TRAP markers (Tg3r165a-220, Tg3r165a-185) were located near the *ms$_7$* locus. The total genetic distance covered by the markers was 53.4 cM. SSR marker ORS608 and TRAP marker Tg3r165a-185 flanked the *ms$_7$* gene, at distances of 2.6 cM and 4.7 cM, respectively. The markers linked with the *ms$_7$* locus could be used to accelerate the breeding of the male-sterile line through marker-assisted selection.

Key words: molecular mapping – nuclear male sterility – sunflower

INTRODUCTION

Male sterility, including cytoplasmic male sterility (CMS) and nuclear male sterility (NMS), provides valuable tools for hybrid breeding programs. Four non-allelic NMS genes, designated *ms$_6$*, *ms$_7$*, *ms$_8$* and *ms$_9$*, were identified from mutant HA89 after mitomycin-C and streptomycin treatment (Jan and Rutger, 1988; Jan, 1992a). The *ms$_9$* gene of NMS HA89-360 has been mapped to linkage group 10 (Chen et al., 2006) of the public SSR genetic map (Tang et al., 2002; Yu et al., 2003), but *ms$_7$* remains unmapped since the release of NMS HA89-552 (Jan, 1992b). The objective of the present research was to identify molecular markers linked to the *ms$_7$* gene in NMS HA89-552.

MATERIALS AND METHODS

Ninety-three F_2 progenies from NMS HA89-552 × RHA271 were obtained by selfing individual F_1 plants. F_3 families from selfed male-fertile F_2 plants were grown in the field to differentiate heterozygous plants from homozygous F_2 plants. Male fertility for F_2 and F_3 individuals was visually scored at flowering. The Chi-square test was used to determine the segregation ratio of the male-fertile and male-sterile phenotype of the F_2 and F_3 progenies.

Genomic DNA was extracted from lyophilized leaf powder of the two parents and the F_1 and F_2 plants with the procedure reported by Rogers and Bendich (1985). Equal amounts of DNA from 12 male-sterile homozygous and 12 male-fertile homozygous F_2 individuals were pooled to form male-sterile and male-fertile bulks.

Two hundred and sixty-five ORS primer pairs were randomly chosen from 17 linkage groups of the public SSR genetic map (Tang et al., 2002; Yu et al., 2003) for screening the two bulks. After confirming that the *ms7* gene was located on linkage group 6 of the SSR genetic map, which cross-references to linkage group 15 of the RFLP map developed from a cDNA library (Jan et al., 1998), 15 single- or low-copy RFLP markers from linkage group 15 were chosen to design STS primers. Using expressed sequence tag (EST) database information, 224 TRAP markers were also generated to screen the male-

sterile and male-fertile bulks (Hu and Vick, 2003). The sequences of RFLP-STS and TRAP primers that produced polymorphic markers in the F_2 population are listed in Table 1.

PCR amplification for SSR and STS primers was performed according to Tang et al. (2002). The amplification products were separated on a 6.5% denaturing polyacrylamide gel at 60W for 2.0 h. Gel images were collected with a Typhoon 9410 variable mode imager (Molecular Dynamics Inc., CA, USA). TRAP analysis was conducted following the procedure of Chen et al. (2006).

Table 1. The sequences of TRAP and RFLP-STS primers producing polymorphic bands.

TRAP primers		Sequences (5'- 3')	STS primers	Sequences (5'- 3')
Fixed primer	Mir165a	GATCCGTCTATGCTTTT	STS8C4	GGGGATCATGAACAGTTTTA
				CCTTGGTTCCTTCAGACAC
Arbitrary primer	Ga3-800	TCATCTCAAACCATCTACAC	STS9C1	TGGCTTCACGTTTTAAAGTT
				GAATCGGACAAAACAAAAAC)

Linkage analysis was performed using MAPMAKER/EXP version 3.0b (Lander et al., 1987). Marker order was determined with a LOD threshold of 3.0, and map distances were estimated by the Kosambi function (Kosambi, 1944). The linkage map was produced using MapChart 2.0 (Voorips, 2002).

RESULTS

Inheritance of the ms7 gene in the F_2 population

The segregation of 78 male-fertile to 15 male-sterile plants in the F_2 population fit a 3:1 ratio (χ^2=3.89, 0.05<P<0.01), indicating a single recessive gene control of male-sterility. The F_3 progeny tests classified the 78 male-fertile F_2 plants into 23 homozygous male-fertile and 55 heterozygous male-fertile plants, further confirming that the segregation ratio best fit the 1:2:1 ratio for a single recessive gene control of male-sterility (χ^2=4.48, 0.2<P<0.1) (Table 2).

Table 2. Segregation of the *ms_7* male-sterility locus and eight markers in the F_2 population.

Traits or markers	Number of plants[1]	Observed number[2]				χ^2-value	
		AA	HH	BB	DD	1:3	1:2:1
ms_7	93	15	55	23			4.48
ORS1229	87			24	63	0.31	
ORS608	92			25	67	0.23	
ORS349	83	16	40	27			3.02
STS9C1	88	14	55	19			6.06*
STS8C4	92			24	68	0.06	
ORS483	92			28	64	1.45	
Tg3r165a-185	93			24	69	0.03	
Tg3r165a-220	93			21	72	0.29	

[1]For ORS1229, six plants were not scorable; for ORS608, STS8C4 and ORS483 one plant each was not scorable; for ORS349, ten plants were not scorable. For STS9C1, five plants were not scorable.
[2]Genotypes: AA, NMS HA89-552 (*msms*); HH, heterozygous (*Msms*); BB, RHA271 (*MsMs*); DD, not BB (*Msms*,*msms*).
*Significant at the 0.05 level of probability.

Molecular mapping of the ms_7 locus

The 265 pairs of SSR primers randomly chosen from 17 linkage groups (LG) of the public SSR genetic map (Tang et al., 2002; Yu et al., 2003) were screened for polymorphisms between the male-fertile and male-sterile bulks. Fourteen primers presented polymorphism between the two bulks. One SSR primer ORS349 on LG 6 showed a linkage with the *ms_7* gene in the F_2 population. Subsequently, seven ORS primers around ORS349 on LG 6 and 15 RFLP-derived STS primers from linkage groups 15 of the RFLP genetic map (Jan et al., 1998) were chosen to screen the F_2 population. Four ORS primers, ORS349, ORS608, ORS1229, ORS483, and two RFLP-STS primers, STS8C4 and STS9C1, were linked with the *ms_7* gene. ORS608 was present in all 15 homozygous male-sterile plants and 52 heterozygous male-fertile plants, but was absent in all 23 homozygous male-fertile plants and two heterozygous male-fertile plants (Fig. 1).

Fig. 1. PCR amplification of ORS608. The marker was present in all homozygous male-sterile and heterozygous male-fertile plants, and absent in all homozygous male-fertile plants. M, 100 bp DNA ladder (bp); F= bulk of male-fertile F_2 plants; S=bulk of male-sterile F_2 plants; F_1= F_1 hybrid.

To identify more markers linked to the *ms*$_7$ locus, 224 TRAP markers were used to screen the male-sterile and male-fertile bulks. Six polymorphic fragments from the primer combinations distinguished the two bulks, and two fragments (Tg3r165a-185 and Tg3r165a-220) were confirmed to link to male-sterile phenotypes. The segregation ratio of the two markers fit a 3:1 ratio, depending on their relationship with the *ms*$_7$ gene. Two polymorphic fragments were considered as candidate markers for the *ms*$_7$ gene.

Linkage analysis and map construction

A linkage map of the *ms*$_7$ gene region was constructed using MAPMAKER/EXP 3.0b with LOD>3.0. All markers could be placed on linkage group 6 around the *ms*$_7$ locus (Fig. 2). The total genetic distance covered by those markers was 53.4 cM. The NMS *ms*$_7$ gene was flanked by SSR marker ORS608 and TRAP markers Tg3r165a-185 at distances of 2.6, and 4.7 cM, respectively. Two RFLP-STS markers, STS9C1 and STS8C4, were 7.3 and 19.3 cM proximal, respectively, of the *ms*$_7$ locus. The locus order for the public SSR markers and the reference linkage maps (Tang et al., 2002; Yu et al., 2003) was identical. Therefore, it is concluded that the *ms*$_7$ gene is located on LG 6 of the public sunflower SSR genetic map.

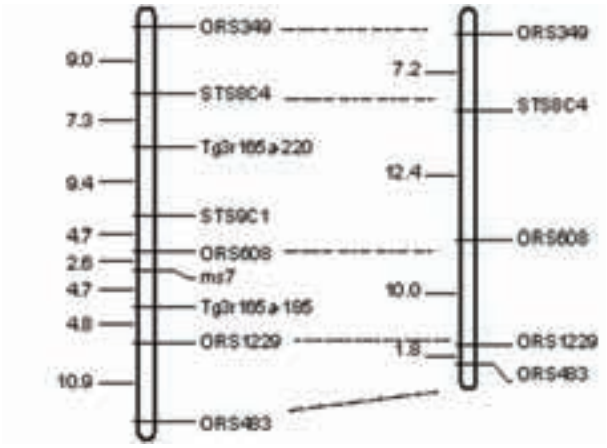

Fig. 2. A: The map position of the *ms*$_7$ gene on linkage group 6 in relation to four SSR markers, two STS markers, and two TRAP markers. B: A corresponding partial linkage map of the region surrounding ORS608 on linkage group 6 of the public sunflower SSR linkage map (Tang et al., 2002; Yu et al., 2003). Distances are shown in centiMorgan (cM).

DISCUSSION

The *ms*$_7$ gene was mapped to LG 6 on the public sunflower SSR genetic map, and was flanked by four SSR markers (ORS608, ORS1229, ORS349, ORS483), two RFLP-STS markers (STS9C1, STS8C4) and two TRAP markers (Tg3r165a-185, Tg3r165a-220). SSR marker ORS608 was the nearest to the *ms*$_7$ gene in LG 6. Two STS markers, STS9C1 and STS8C4, derived from the associated RFLP markers (Jan et al., 1998), were linked to the *ms*$_7$ gene in this study, indicating that it is feasible to convert RFLP markers into STS markers. The markers linked to the *ms*$_7$ gene provide a useful tool for easy identification of lines carrying the male-sterility allele when applying a marker-assisted selection technique.

The molecular mapping of NMS genes in sunflower was first reported by Pérez-Vich et al. (2005) for *ms*$_{10}$ and *ms*$_{11}$, which mapped to LG11 and LG8, respectively, on the public sunflower SSR linkage map. The *ms*$_9$ gene of NMS HA89-360 was mapped to LG 10 (Chen et al., 2006). Though NMS HA89-360 and NMS HA89-552 were derived from streptomycin-treated HA89 seed, the loci of the two NMS genes were different: the *ms*$_7$ gene of NMS89-552 was mapped to LG 6.

ACKNOWLEDGEMENTS

The authors thank Jinguo Hu and Bing Yue for their valuable advice and suggestions. We also would like to thank G.J. Seiler for critical review of the manuscript.

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Construction of a linkage map with TRAP markers and identification of QTL for four morphological traits in sunflower (*Helianthus annuus* L.)

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ABSTRACT

A linkage map containing 202 TRAP (target region amplification polymorphism) and 24 SSR markers was constructed in an F_2 population derived from a cross between two sunflower breeding lines. This map contains 17 linkage groups spanning a total distance of 1597.5 cM. The QTL for plant height, leaf color, leaf shape and head shape were identified in the F_2 and F_3 generations. Totally 18 QTL were detected for these traits with individual QTL explaining 6.7-49.5% of phenotypic variation, suggesting the multiple gene status for these traits. Two QTL for plant height and two QTL for chlorophyll content were identified in both F_2 and F_3 generations, and one of them each explained more than 27.2% of the phenotypic variation. These QTL will be useful in molecular breeding.

Key words: chlorophyll content – head shape – leaf shape – plant height – QTL mapping – TRAP

INTRODUCTION

The cultivated sunflower (*Helianthus annuus* L.) is one of the most important oilseed crops of the world. Sunflower oil accounts for approximately 10% of the total world consumption of plant-derived edible oil (Jan and Seiler, 2007). The advent and development of molecular markers and genetic maps assists in understanding the genetic basis of economically important traits and facilitates plant breeding via marker-assisted selection. To date, about a dozen linkage maps have been constructed using different molecular markers, including RFLP, RAPD, and SSR in sunflower, and most of the maps had 17 linkage groups (Berry et al., 1995; Gentzbittel et al., 1995; Gedil et al., 2001; Tang et al., 2002).

The TRAP marker technique, developed by Hu and Vick (2003), takes advantage of the annotated EST information to generate PCR-based markers near the target sequence. This molecular marker technique has demonstrated great potential in exploiting genome polymorphisms and molecular mapping (Hu et al., 2004). It has been successfully used in defining the linkage group ends (Hu, 2006), in mapping a nuclear male-sterile gene (Chen et al., 2006), and in mapping an apical branching gene (Rojas-Barros et al., personal communication) in sunflower. This marker technique has also been used for molecular mapping in other crops, such as mapping of disease resistance QTL in common bean (Miklas et al., 2006) and wheat (Liu et al., 2005).

Morphological traits like plant height, leaf color, leaf and head shape etc. are important traits in sunflower breeding. However, genetic studies on only one or two of these traits have been conducted in previous reports (Hervé et al., 2001; Mokrani et al., 2002; Burke et al., 2002; Bert et al., 2003; Al-Chaarani et al., 2004). Here we report the construction of a linkage map comprised primarily of TRAP markers and QTL mapping for the four morphological traits mentioned above in sunflower.

MATERIALS AND METHODS

Two sunflower inbred lines with significant differences in some morphological traits of interest, Lgl (light green leaf) and HA379, were selected for developing an F_2 population in this study. Lgl was introduced from Australia with light green leaf color, HA379 is a male maintainer line with reduced-height released by USDA-ARS (PI 561919) (Miller, 1993). One hundred and twenty F_2 individuals and their parents were planted in one-gallon plastic pots in the greenhouse in the winter of 2006, one plant per pot. Ninety-five $F_{2:3}$ families and their parents were planted in the experimental field in Fargo, ND, USA, during the growing season of 2007 following a field design of randomized complete block with two replicates, with 15 to 20 plants in a one-row plot.

Four traits, including leaf color (chlorophyll content or greenness degree), plant height, leaf shape, and head shape were investigated in the F_2 and F_3 generations. At the flowering stage, leaves from each plant of the F_2 population were sampled for measuring chlorophyll content following the procedures described by Chory et al. (1989). The sampling and measurements for each individual were conducted

twice and the average values were used for analysis. In the F_3 generation, the plants within a family segregated for chlorophyll content. Therefore, the greenness degree, a parameter highly related to leaf chlorophyll content, was measured for each plant with a handheld chlorophyll meter (SPAD-502, Minolta Camera Co. LTD, Japan) following the manufacturer's instructions. Measurements were performed on the fully expanded uppermost leaves with a minimum of three measurements taken per leaf, about 2 cm away from the leaf edge.

Plant height (cm) was measured from the soil surface to the head at the mature stage for all the plants in both generations. Leaf shape and head shape were visually scored on a scale of 1 (triangle leaf shape, flat head shape) through 4 (round leaf shape, most convex and misshapen head) for all the plants at the mature stage. The means of the data collected from individual plants of each $F_{2:3}$ family were used in the analysis. Total genomic DNA was isolated from about 50 mg (fresh weight) leaf tissue sampled from individual plants of the parental lines and the F_2 population using the Qiagen DNeasy® 96 Plant Kit (Qiagen, Valencia, CA), following the manufacturer's instructions. The TRAP assays followed the updated procedures described by Hu (2006). Totally 27 fixed primers were designed against ESTs involved in chlorophyll synthesis, gibberellin synthesis, microRNA sequences and disease resistance, as well as seven arbitrary primers labeled with either IR (infrared) 700 or IR 800 dye (Hu and Vick 2003), and were used to generate TRAP markers for map construction (Table 1). The TRAP markers were designated by the combination of the code of the fixed primer involved, the code of the labeled arbitrary primer, and the fragment size in base pairs.

Table 1. ESTs used for fixed primer design and sequences of fixed and arbitrary primers.

Code	EST accession no.	Sequences 5'—3'
Fixed primer		
T99	QHB26P17.yg.ab1	GTT TTC CGT CAT ACT CGT TA
T100	QHB33I23.yg.ab1	GAA GGG GTC AAA AAT TTA AC
T101	QHB34F17.yg.ab1	TCC ACA CTT TTG AAG TCA TT
T102	QHG18P13.yg.ab1	AAG AGT TTG ACC AAT GTC AA
T103	QHK1O04.yg.ab1	GAT ACA GGT TAT GGC AGA AA
T104	QHK7L05.yg.ab1	TTA TGT CTA TGG CAC CAA CA
T105	QHL12I06.yg.ab1	GCT TAC CGT CAT CAA GAA AC
T109	EB700927	GTA TCC AAA CGA CAC GAG TT
T110	CV987281	CAT ACA AGG TGG TCG AAA TT
T111	EC683354	AGG AAA TGT CTA TTT GGC AA
T112	QHJ12G10.yg.ab1	ACC ACA CAA TCA TGA CTA GG
T114	QHJ4A19.yg.ab1	TAA TAG CAA AAG CTC CAA TG
T115	QHB12C18.yg.ab1	ATT CAC TAT ATC ACG AGC CA
T116	QHB29B22.yg.ab1	GCA TTA TAC TTT GGT GGA GA
T117	QHM10I05.yg.ab1	ATT TGT TTG TTT GTT TTT GG
T02	QHA10B18b.yg.ab1	GTT TGC CTT TAA GAA CCG
T05	QHA11D14F1.yg.ab1	ATA CCC ACC CGT CAC TAC
T07	QHA11I24a.yg.ab1	AGG CTT GGA TGT TGA TGC
T10	QHA12P24b.yg.ab1	CTC CAG TCT GAC CCG TTG
T30	QHB14G14b.yg.ab1	AAT CTC AAG GAC AAA AGG
T37	QHB22D05b.yg.ab1	GAAGCTTCACAGGGAGTT
T131	miR157b	GATCATTGTCCAGATTC
T134	miR159a	GATCCTTGGTTCTTTGG
T137	miR165a	GATCCGTCTATGCTTTT
T141	miR166f	GATCACCTAATTCTCTA
T146	miR170	GATCGGATGCTCCTTTC
T152	miR394a	GATCAAGGAATAGGTGA
Arbitrary primer		
R03	TRAP03(IR-700)	CGTAGCGCGTCAATTATG
R19	SA12(IR-700)	TTCTAGGTAATCCAACAACA
R20	SA14(IR-700)	TTACCTTGGTCATACAACATT
R21	SA4(IR-700)	TTCTTCTTCCCTGGACACAAA
R13	TRAP013(IR-800)	GCGCGATGATAAATTATC
R22	GA3(IR-800)	TCATCTCAAACCATCTACAC
R23	GA5(IR-800)	GGAACCAAACACATGAAGA

We selected a total of 223 mapped SSR markers from each of the 17 linkage groups (Yu et al., 2003) in the initial screening for polymorphisms between the two parents. Twenty-four polymorphic SSRs were used to genotype the whole F_2 population to align the linkage groups constructed in this study with the published sunflower SSR map. SSR assays were carried out following the procedures described by Tang et al. (2002).

The linkage maps were constructed using the computer program of Mapmaker/EXP 3.0 (Lander et al., 1987) (LOD>4.5). Interval QTL mapping was performed with both F_2 and F_3 data employing the software of Mapmaker/QTL1.1 (Lander and Botstein, 1989; Lincoln et al., 1993).

RESULTS

The phenotypic differences between the parents, as well as the variation in the populations are summarized in Table 2. Transgressive segregation was observed in one or both directions for all the traits investigated. The values of skewness and kurtosis for these traits are less than or close to 1.0 except for chl*a*/*b*, indicating that these traits (with the exception of chl*a*/*b*) fit a normal distribution (Table 2). HA379 had deep green leaf color with significantly higher values of chl*a*, chl*b*, chl*t*, and greenness degree than Lgl in both generations. On the other hand, the values of chl*a*/*b*, as well as leaf shape, head shape, and plant height in Lgl, were significantly higher than that of HA 379. The mean for plant height in the F_3 generation was much higher than that in the F_2 generation, and this was the case for the parents in both generations (Table 2). This could be explained by the differences in their growing conditions, i.e. the F_2 generation in the greenhouse and the F_3 generation in the field.

Table 2. The measurements of the traits in the F_2, $F_{2:3}$ populations and their parents.

Traits [1]	HA379[2]	Lgl [2]	Mean	$F_2/F_{2:3}$ Range	Skewness	Kurtosis
Leaf shape	0.0/0.3	$3.0^{**}/3.0^{**}$	2.0/2.2	(0.0-4.0)/(0.5-3.8)	0.3/-0.1	-1.0/-0.4
Head shape	0.0/0.0	$3.0^{**}/3.0^{**}$	2.5/2.0	(0.0-4.0)/(0.0-3.9)	-0.5/0.3	0.1/-0.8
Plant height	38.1/63.9	$102.2^{**}/161.1^{**}$	71.9/108.8	(13.3-162.6)/(58.3-183.7)	0.6/0.3	-0.1/-0.7
Chl*a*	1.8^{**}/-	0.5/-	1.4/-	(0.4-2.3)/-	-0.4/	-0.6/-
Chl*b*	0.5^{**}/-	0.1/-	0.4/-	(0.1-0.6)/-	-0.7/-	-0.6/-
Chl*t*	2.3^{**}/-	0.6/-	1.8/-	(0.5-2.7)/-	-0.5/	-0.7/-
Chl*a*/*b*	3.6/-	4.8^{**}/-	3.9/-	(3.3-6.5)/-	1.6/	2.8/-
GD	-/42.9^{**}	-/20.1	-/37.7	-/(26.0-46.7)	-/-0.3	-/-0.4

[1]GD is greenness degree, the values on the left side of the sign "/" are the data collected in the F_2 generation, and those on the right side were collected in the F_3 generation.
[2]**, the difference is significant at the 0.01 level between the two parents.

Four traits related to leaf color (chl*a*, chl*b*, chl*t* and greenness degree) were highly intercorrelated ($0.6<r<1.0$), especially for chl*a* and chl*t* ($r=1.0$). Head shape in the F_3 generation was positively and significantly correlated to leaf shape, chl*a*, chl*b*, and chl*t* in the F_2 generation ($0.21<r<0.25$) and to greenness degree in the F_3 generation (0.26). However, chl*a*/*b* was negatively correlated to chl*a*, chl*b*, chl*t*, and leaf shape in the F_2 generation ($-0.64<r<-0.27$), and to greenness degree in the F_3 generation ($r=-0.55$). Significant negative correlations were also identified between plant height and greenness degree ($-0.40<r<-0.36$). Moreover, correlations between the F_2 and F_3 generations for these traits were strong, ranging from 0.42 for leaf shape to 0.91 for plant height.*A total of 322 polymorphic bands/markers were generated from 54 pairs of TRAP primer combinations (one fixed primer + one arbitrary primer labeled by IR700 or IR800). Each primer combination amplified 1 to 16 markers with an average of 6.0 markers per combination. Of the 223 SSRs screened, only 22 (9.9%) were polymorphic between the two parents and resulted in the generation of 24 SSR markers.*

After a preliminary mapping test, 202 TRAP markers that were evenly distributed across the sunflower genome and 24 SSR markers were selected to construct the linkage map for QTL analysis. The linkage map had a total length of 1597.5 cM, and the average distance between adjacent markers was 7.1 cM (Fig. 1). Integration of the 24 previously mapped SSR markers to the TRAP map allowed us to align 12 of the linkage groups to the previously published SSR maps (Yu et al., 2003), with each linkage group containing 1 to 4 SSR markers. One TRAP marker (T10R21-280) had already been assigned to linkage group 12 according to another sunflower map (data unpublished). The 13 linkage groups identified in this study corresponded to linkage groups 1, 2, 3, 5, 7, 8, 10, 11, 12, 13, 14, 16 and 17, respectively, on the Yu et al. (2003) map.

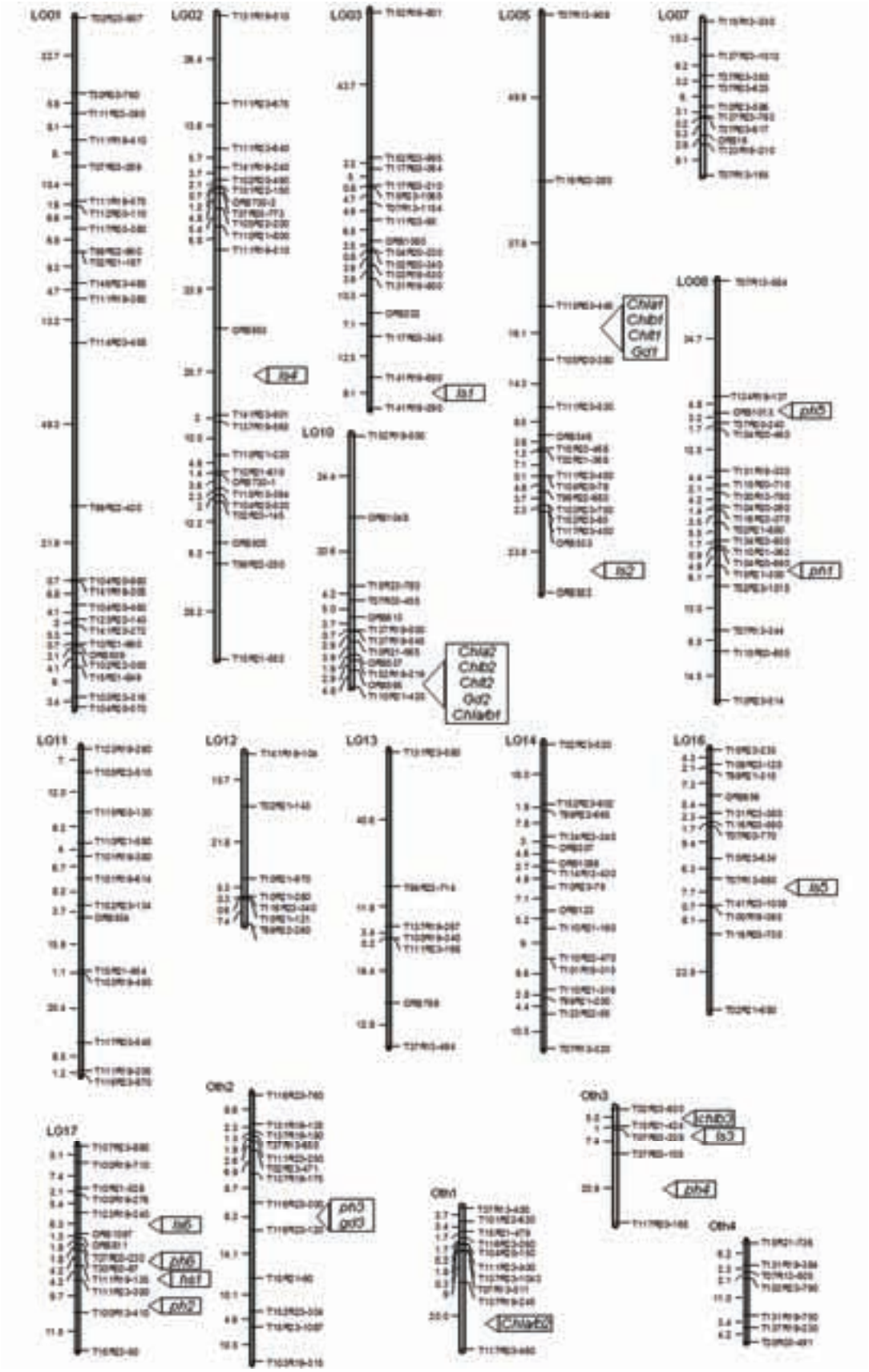

Fig. 1. The genetic linkage map and locations of the QTL detected in both generations. Designations of markers are on the right and genetic distances (cM) are on the left.

A total of six QTL for plant height were resolved in the two generations, including two detected in both generations. Individual QTL explained 6.7%-36.9% of phenotypic variation (Fig. 1). Among them, *ph3*, a major QTL for plant height, alone explained more than 30% of phenotypic variation in both generations. Six QTL were identified for leaf shape, which explained 7.8%-14.5% of phenotypic variation. None of them were detected in both generations. Only one QTL was detected for head shape in the F_3 generation, which explained 12.2% of phenotypic variation. Alleles from Lgl at nine of the QTL for these three traits had positive effects that coincided with the performance of this parent for these traits. For leaf-color-related traits, two, three, two, and two QTL were detected for chl*a*, chl*b*, chl*a*/*b* and chl*t* in the F_2 generation, respectively. Individual QTL explained 8.8%-49.5% of phenotypic variation. Three QTL for greenness degree were identified in the F_3 generation. Each explained 10.8%-27.2% of phenotypic variation.

Two chromosomal regions were identified that harbored more than three QTL for specific traits, and one region was identified that harbored two QTL (Fig. 1). The chromosomal interval *T115R03-446 – T105R20-380* on linkage group 5 contained the QTL for the four chlorophyll-content-related traits, *chla1*, *chalb1*, *chalt1* and *gd1*. The interval *ORS595 – T110R21-420* on linkage group 10 contained the QTL for all of the five chlorophyll-related-traits. The alleles from HA 379 at these loci increased chlorophyll content, but reduced the ratio of chlorophyll *a* to chlorophyll *b*. Two QTL, *ph3* and *gd3*, clustered in the region *T116R23-300 – T116R23-120* on linkage group Oth2, while the alleles from different parents had positive effects on the two traits, respectively. These results were consistent with the correlations observed among these traits.

DISCUSSION

TRAP markers, in combination with SSR markers, have been used to construct linkage maps in wheat, sunflower, and common bean (Liu et al., 2005; Miklas et al., 2006; Hu, 2006). The successful construction of a sunflower TRAP map and application of the map for QTL analysis in this study also indicates that TRAP is an efficient PCR-based marker technique for molecular mapping. For instance, each TRAP PCR reaction generated 12 polymorphic markers in the mapping populations of this study, whereas the SSR marker technique detected only 9.9% polymorphisms between the two parents. Moreover, TRAP takes advantage of the annotated EST information to generate markers at and near the target sequence (Hu and Vick 2003). The ESTs identified at the gene loci flanking the QTL of interest may provide useful information for the cloning of the QTL. In the present study, the TRAP markers flanking the two major QTL, *chla2* and *ph3*, were generated by the fixed primers designed against chlorophyll synthesis (CV987281) and gibberellin synthesis (QHB29B22.yg.ab1) related ESTs, respectively

The genetic basis of the four morphological traits is very complex, and it has been reported that some of the traits are under the control of multiple genes in sunflower (Hervé et al., 2001; Burke et al., 2002; Bert et al., 2003; Al-Chaarani et al., 2004). The results in this study also support this. It is difficult to compare the QTL in these studies with previous reports due to the unrelated markers used for map construction. However, near SSR marker *ORS 811*, a QTL for plant height, *ph6*, on LG17 detected in this study shared the same chromosomal region with a QTL for plant height reported by Burke et al. (2002).

In this study, one chromosomal region (ORS595 - T110R21-420) was identified to be involved in conditioning all the five leaf-color-related traits and explained more than 27.2% of the phenotypic variation (Fig. 1). A major QTL for chlorophyll content was also identified and positioned to the same chromosomal region in another sunflower mapping population (unpublished data). The identification of a major QTL controlling chlorophyll content in sunflower offers the opportunity to achieve a higher photosynthesis rate and to increase biomass and grain yield through genetic manipulation in the future. Moreover, the QTL for plant height, *ph3*, detected in two generations and which explained more than 30% of phenotypic variation in this study, will be also useful in sunflower plant breeding.

ACKNOWLEDGEMENTS

The authors greatly appreciate the technical assistance from Wenge Yuan, Ping Wang, and Angelia Hogness. This work was partially supported by USDA-ARS CRIS project 5442-21000-027-00D and a competitive grant from the National Sclerotinia Initiative to JH.

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Candidate gene analysis and identification of TRAP and SSR markers linked to the *Or5* gene, which confers sunflower resistance to race E of broomrape (*Orobanche cumana* Wallr.)

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ABSTRACT

Sunflower broomrape (*Orobanche cumana* Wallr.) is a root holoparasitic angiosperm considered as being one of the major constraints for sunflower production in Mediterranean areas. Breeding for resistance has been crucial for protecting sunflowers from broomrape damage. The *Or5* gene, which confers resistance to race E of broomrape, has been efficiently used for years in sunflower commercial programs to control broomrape. Despite its importance in sunflower breeding, little is known about the nature of the *Or5* gene. The objective of this study was to explore different strategies to determine the nature of this gene. These include a map-based cloning strategy and a candidate gene approach.

Key words: candidate genes – *Orobanche cumana* – race E – SSR– sunflower resistance – TRAP.

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the most important annual oilseed crops in the world. *Orobanche cumana* Wallr. (sunflower broomrape), a holoparasitic angiosperm that infects sunflower roots, is regarded as one of the most important constrains in sunflower production in Spain and most countries of southern and eastern Europe and the Middle East (Parker, 1994). Control of this parasite remains extremely difficult, as thousands of tiny seeds produced by one single broomrape plant can be easily dispersed by wind, water, animals, humans, machinery or attached to sunflower seeds. Although the use of herbicides is being considered as a promising control measure, at present genetic resistance offers the most effective and feasible control against *O. cumana*. However, the introduction of new resistance sources has been frequently followed by the appearance of new pathogenic races overcoming the resistance. In a classic study in the late 1970s, Vrânceanu et al. (1980) identified five pathogenic races in Romania, named A through E, with a set of sunflower differentials carrying the dominant resistance genes *Or1* through *Or5*, which provided an accumulative resistance to the five successive races. Races B and C were identified at that time in Spain in addition to populations of *O. cumana* of higher virulence than those reported in Romania (Melero-Vara et al., 2000). Besides, race F which overcomes the resistance gene *Or5*, was identified in the mid 1990s in Spain (Alonso et al., 1996). Resistance to this new race was found in germplasm of both cultivated and wild sunflower (Sukno et al., 1999; Fernández-Martínez et al., 2000).

The host-parasite system of sunflower-*O. cumana* described for races A to E appears to follow the gene-for-gene model. The previously mentioned study of Vrânceanu et al. (1980) already established the monogenic and dominant inheritance of resistance to sunflower broomrape races A to E, which was later confirmed by other authors (Ish-Shalom-Gordon et al., 1993; Sukno et al., 1999). However, the nature of the *Or1* to *Or5* genes involved in this interaction is not known. Molecular mapping studies have revealed that the *Or5* gene conferring resistance to race E of broomrape is located on a telomeric region of linkage group (LG) 3 of the sunflower genetic map (Lu et al., 2000; Tang et al., 2003a; Pérez-Vich et al., 2004). Recently, Letousey et al. (2007) have identified different genes differentially expressed in resistant genotypes when infected with race E of broomrape. Among them, a sunflower defensin gene may play a major role in *Orobanche* cell death (de Zélicourt et al., 2007) during the incompatible reaction.

The aim of this study was to investigate the nature of the *Or5* gene through a candidate gene approach, and to identify markers close to the gene in order to facilitate map based cloning strategies.

MATERIALS AND METHODS

Plant material, phenotypic evaluation for race E resistance and RFLP linkage map construction

The sunflower lines used to generate the F_2 mapping population were P-96, an inbred line resistant to races E and F of broomrape developed from cultivated sunflower of Yugoslavian origin, and P-21, a Genetic Male Sterile (GMS) line of sunflower, which is highly susceptible to broomrape. The development of the F_2 and F_3 populations from the cross P-21 x P-96 and the F_3 phenotypic evaluation for disease reactions to race E of broomrape are described in Pérez-Vich et al. (2004). From the P21 x P-96 population, an RFLP linkage map comprising 17 LGs was constructed, in which the *Or5* gene was mapped to LG 3 (Pérez-Vich et al., 2004).

SSR marker analysis

Genomic DNA from four samples of the F_2 population P21 x P-96 and the parental lines were screened with LG 3 previously mapped simple sequence repeats (SSRs) (Tang et al., 2003b; Yu et al., 2003), identified by ORS and CRT prefixes, in order to find polymorphic SSR markers and map them in the P-21 x P-96 population. PCRs were performed as described by Tang et al. (2002), and the amplification products were resolved by electrophoresis on 3% Metaphor® (BMA, Rockland, ME, USA) agarose gels in 1x TBE buffer with ethidium bromide incorporated in the gel.

TRAP analysis

The TRAP analysis focused essentially on telomere-associated TRAP markers, especially those identified on LG 3 (Hu, 2006). Assays followed the updated procedures described by Hu (2006). Totally 3 fixed primers (TeloRCN, TeloR, and QGA7H07L) designed against *Arabidopsis*-type telomere repeat sequences and EST homologous to *Arabidopsis* homeobox genes (Hu et al., 2005; Hu, 2006) as well as six arbitrary primers (Ga3-800, Ga5-800, Trap13-800, Sa4-700, Sa12-700, and Trap03-700) labeled with either IR (infrared) 700 or IR 800 dyes were used to generate TRAP markers. The TRAP markers were designated by the combination of the code of the fixed primer involved, the code of the labeled arbitrary primer, and the fragment size in base pairs.

Candidate gene analysis

Twelve defense-related genes known to be involved in different metabolic pathways (phenylpropanoids, jasmonate, ethylene) and/or resistance mechanisms against microorganisms (Table 1) were used for the candidate gene approach. Primers from these genes were designed by Letousey et al. (2007) and screened for polymorphisms in the P-21 x P-96 population. PCRs were performed as described by Yu et al. (2003) for INDEL markers, using the annealing temperatures described in Letousey et al. (2007) for each specific primer pair. The amplification products were resolved by electrophoresis on 3% Metaphor® (BMA, Rockland, ME, USA) agarose gels in 1x TBE buffer with ethidium bromide incorporated in the gel.

Molecular mapping of polymorphic loci and linkage map construction

Polymorphic SSR, TRAP, and candidate gene markers were genotyped in the F_2 population P-21 x P-96 developed by Pérez-Vich et al. (2004). The RFLP map of Pérez-Vich et al. (2004), comprising 17 LGs and spanning a 1,144.4 cM distance, was used to map the SSR, TRAP, and candidate gene loci. Linkage maps were constructed using the software MAPMAKER/EXP version 3.0b (Whitehead Institute, Cambridge, MA, USA) (Lander et al., 1987). The LG nomenclature follows that of the reference map of Tang et al. (2002). Linkage group maps were drawn using the MapChart software (Voorrips, 2002).

Table 1. Primer pairs used for the candidate gene analysis (Letousey et al., 2007)

Gene	Product	Function	PCR primers
ef1-α	Elongation factor 1α (control gene)	Translation	5' GTCAGCACGCTCTTCTCGCT 5' GAGACTCGTGGTGCATCTCA
ubq	Ubiquitin	Proteolytic cofactor	5' TGTGAAGACGTTGACTGGAA 5' CGCAGACGAAGAACAAGGTG
chit.	Chitinase (PR3 protein)	Antifungus	5' CTGCAGTGTCAGCAGCTGAT 5' CTGCACCAGATGGGCGATTT
pal	Phenylalanine-ammonia-lyase	Phenylpropanoid synthesis	5' GTTATGGTGCACTACTGGAT 5' TCCGACAACAATGCAAGTACA
c4h	Cinnamate-4-hydroxylase	Phenylpropanoid synthesis	5' ATGGGTCAGCGTAACCTAGT 5' AAGCTCTGAGCTAATCGACT
lox	Lipoxygenase	JA synthesis	5' GTAGGCGTTGGTGGTATTGTT 5' AATCTTCATCCGCAGGTACAT
HACS.1	ACC synthase	Ethylene synthesis	5' ACCGGATTGTTATGAGCGGT 5' TACGAGCGATGCTCACAACA
ACCO1	ACC oxydase	Ehylene synthesis	5' GATCATTACAAGAAGTGTATGG 5' AGAAGCTGTAGACCGCTAAC
def.	Defensin	Antifungus, marker gene of SA pathway	5' GTGAGAAGGCAAGCCAGACA 5' TCAAGGTTTGGCTGTCGCCT
PR5-1	PR5 protein	Antifungus, marker gene of JA pathway	5' CACAGCAGGAGCCCGTATAT 5' TATGCATCAGGACATCTGGTC
sco	Carbohydrate oxidase	H_2O_2 production, antifungus	5' GTCATGCTTGATCTCGTCAA 5' ACTCGGTGTTAAGCTGAACT
HaAC1	Aldoketo reductase	Polyol synthesis, marker gene of SA pathway	5' CTCGCTACCAAATATGGGAT 5' TCTTCGAGCTTCTCCATCAT

RESULTS

SSR marker analysis

Nine SSR markers mapping to LG 3 (ORS10, ORS202, ORS665, ORS949, ORS1036, ORS1114, ORS1222, CRT392, and CRT314) were screened for polymorphisms in four F_2 individuals and the parental lines P-21, and P-96. Three of them (ORS949, CRT392, and CRT314) were polymorphic (Fig. 1). These polymorphic loci were genotyped in the F_2. ORS949 and CRT314 mapped to LG 3 (LOD score of 7 and maximum recombination frequency of 0.30) (Fig. 2). CRT314 and ORS949 mapped 9.9 cM and 57.1 cM, respectively, downstream from *Or5*. Two polymorphic loci from CRT392 (CRT392a-100bp, and CRT392b-250bp) mapped to LG 9 (LOD score of 7 and maximum recombination frequency of 0.30). These two CRT392 loci on LG 9 have also been reported by Yu et al. (2003). A third CRT392 locus (CRT392c) segregated in the P-21 x P-96 population, but it was not possible to map it in high resolution agarose gels. This locus may be the one mapped close to *Or5* by Tang et al. (2003a).

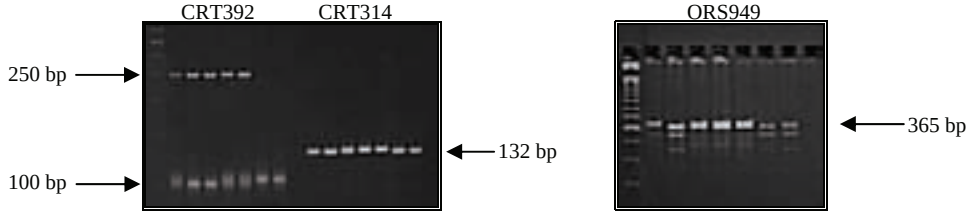

Fig. 1. Amplification profiles of SSR markers CRT392, CRT314, and ORS949 in samples of four F_2 P-21 x P-96 individuals, one P-21 individual, and two P-96 individuals. Mapped polymorphic loci are indicated by arrows. *Lane 1*, 50 bp DNA ladder; *lanes 2-5*, F_2 individuals; *lane 6*, P-21 parental line; *lanes 7-8*, P-96 parental line.

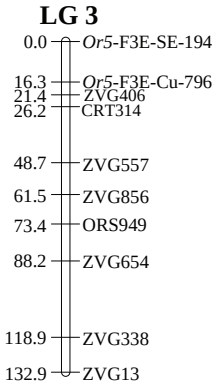

Fig. 2. Linkage map of LG 3 from the P-21 x P-96 population containing the *Or5* gene for resistance to race E of broomrape, as scored in two different phenotypic experiments [F3-race E (SE-194), and F3-race E (CU-796); Pérez-Vich et al., 2004]

TRAP analysis

Six F_2 individuals from the P-21 x P-96 population phenotyped as susceptible to race E of broomrape, and six F_2 individuals from the same population phenotyped as resistant to race E, and selected as homozygous based on the genotype of the closest RFLP marker (ZVG406), were assayed. A fragment of 133 bp in length generated by the primer combination TeloRCN+Sa4-700 (TRAP marker TRC27133) was polymorphic between the resistant and the susceptible groups, and was associated with the resistant phenotype (Fig. 3). The TRC27133 TRAP marker was mapped in previous studies to the upper end of LG 3 (Hu, 2006).

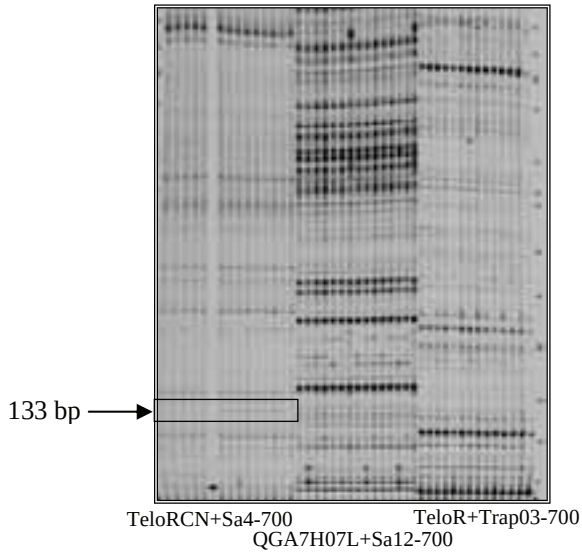

Fig. 3. Gel image generated by primer combinations TeloRCN+Sa4-700 (*left*), QGA7H07L+Sa12-700 (*center*), and TeloR+Trap03-700 (*right*). The TRAP marker TRC27133 polymorphic between susceptible and resistant groups is a fragment of 133 bp of primer combination TeloRCN+Sa4-700, and it is highlighted in a box. *Lanes 1-6*, six susceptible F_2 individuals; *lane 7*, susceptible parental line P-21; *lane 8-13*, six resistant F_2 individuals; *lane 14*, resistant parental line P-96; *lane 15*, susceptible parental line P-21.

Candidate gene analysis

From the twelve candidate gene-markers assayed, four of them generated polymorphic loci among F_2 individuals from the P-21 x P-96 population. These corresponded to the candidate genes *ef1-α* (elongation factor 1-α), *chit.* (chitinase, PR3 protein), *c4h* (cinnamate-4-hydroxylase), and *HaAC1* (aldoketo reductase). Dominant polymorphic *ef1-α* (elongation factor 1-α), *chit.* (chitinase, PR3 protein), and

HaAC1 (aldoketo reductase) loci were mapped to LGs 7, 9, and 17, respectively (LOD score of 5 and maximum recombination frequency of 0.30). None of these loci were located on LG 3, where the *Or5* for race E resistance has been mapped. In addition, several QTL for race F resistance have been described in the P-21 x P-96 population (Pérez-Vich et al., 2004), but none of the candidate gene loci mapped in this study co-located with any of them. Finally, a dominant *c4h* (cinnamate-4-hydroxylase) locus was not associated with any of the linkage groups of the P-21 x P-96 map (Pérez-Vich et al., 2004).

DISCUSSION

Different mechanisms may play a role in resistance of sunflower to broomrape. Sources of resistance carrying quantitative or qualitative, or both, resistance mechanisms have been described (Sukno et al., 1999; Pérez-Vich et al., 2004). Qualitative resistance is characterized by dominant race-specific genes, and has played an important role in breeding for resistance in the last fifteen years. The *Or5* gene confers resistance in sunflower to race E, but also to the previous A through D races of less virulence. The nature of this dominant race-specific gene is still unknown. It has been hypothesized that it might be a cluster of resistance (R) genes encoding proteins characterized by the presence of leucine-rich repeat (LRR) motifs and a nucleotide binding site (NBS) N-terminal to the LRR domain (Lu et al., 2000), similar to those NBS-LRR clusters on LG 8 (Bouzidi et al., 2002) and LG 13 (Radwan et al., 2003) conferring resistance to different races of downy mildew. Alternatively, studies focused on the expression patterns of defense-related genes in race E compatible and incompatible reactions, suggested that a salicylic acid-responsive gene, *def.* (defensin) was characteristic of the race E resistant genotype, and that it might play a major role in *Orobanche* cell death (de Zélicourt et al., 2007; Letousey et al., 2007). In order to determine in more detail the nature of the *Or5* gene, different strategies could be used. One is a candidate gene approach, based on the genes hypothesized or related to race E resistance mechanisms. Another approach is a map-based cloning strategy, for which the identification of *Or5* closely linked and flanking markers would be necessary.

This study, combined with the previous results of mapping the TRC27133 TRAP marker (Hu, 2006), and those of identifying SSR markers close to *Or5* (Tang et al., 2003a), indicates that *Or5* is probably located in the TRC27133 to ZVG406/CRT392c marker interval. ZVG406 is the uppermost RFLP marker on LG 3 from the high density RFLP map of Berry et al. (1996), and CRT392c is the uppermost SSR marker on LG 3 described to date (Tang et al., 2003b; Yu et al., 2003). Both ZVG406 and CRT392c markers map 5.1 and 6.2 cM to *Or5* on its centromeric side, respectively. TRC27133 is on the LG 3 telomere, and has been mapped 4.9 cM distal to CRT392 (Hu, 2006). Therefore, *Or5* must be very close to TRC27133, and this marker probably flanks the gene. Unfortunately, we were not able to determine the exact position of TRC27133 in relation to *Or5*, since this marker when run in the entire F_2 P-21 x P-96 population produced bands of different intensities which were not scorable. The TRC27133 marker will be converted into a more useful sequence-tagged-site (STS) marker by cloning and sequencing the 133 bp fragment. The *Or5* marker interval TRC27133 — ZVG406/CRT392c covers a region of about 5-6 cM. This distance may be physically shorter, as suggested by Tang et al. (2003a). Therefore, this set of markers close to *Or5* may provide a foundation for *Or5* map-based cloning strategies.

Another approach to investigating the nature of the *Or5* gene could be a candidate gene analysis. In this study, we have tested genes differentially expressed in resistant genotypes when infected with race E of broomrape. Despite we have been able to map *ef1-α* (elongation factor 1-α), *chit.* (chitinase, PR3 protein), and *HaAC1* (aldoketo reductase) loci to LGs 7, 9, and 17, respectively, none of them co-located to *Or5*. These results were partially expected, since the *chit.* and the *HaAC1* genes play a role in defense responses and *ef1-α* is a housekeeping gene, and dominant race-specific genes such as *Or5* are hypothesized as essentially playing a role in an early stage of the plant-pathogen interaction (i.e. pathogen recognition). Many other candidate genes could be tested, including NBS-LRR type ESTs present in the Compositae Genome Database and the NCBI database.

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Tribenuron-methyl resistance in accessions of annual wild sunflower species from the Novi Sad germplasm collection

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ABSTRACT

The trial was performed to check whether the tribenuron-methyl (Express) resistance is a novel trait or that it was already present in the wild sunflower. The majority of tested accessions were collected in the USA between 1980 and 1991 and some accessions were obtained through exchange from Argentina. Two treatments with tribenuron-methyl at a 1X rate (17.3 g/ha) were applied one week apart. Moderately resistant plants with slight chlorosis or leaf damage were found in 12 of the total 73 tested accessions. Resistant plants with no visible herbicide damage symptoms were found in three accessions of *H. argophyllus* and one accession of *H. annuus*. At least one plant in 63 of 73 tested accessions continued to grow by branching after the vegetation cone was destroyed. The obtained results suggest that resistance was present in wild sunflower before tribenuron-methyl application started.

Key words: acetolactate synthase – herbicide resistance – wild sunflower.

INTRODUCTION

The genus *Helianthus* is native to North America and rich in genetic diversity with annual and perennial species of sunflower (Schiling and Heiser, 1981). The common sunflower (*Helianthus annuus*) can frequently be found along crop fields and roads. It is a major weed in corn (*Zea mays*), soybean (*Glycine max* (L.) Merr.), wheat (*Triticum aestivum* L.) and sugar beet (*Beta vulgaris* L.).

The use of herbicides for the control of common sunflower has been encouraged because it reduces the cost of labor-intensive manual control. Herbicide families like sulfonylureas, imidazolinones, triazolopyrimidines and pyrimidinyl thiobenzoates inhibit activity of acetolactate synthase (ALS, also known as acetohydroxyacid synthase AHAS). ALS inhibitors proved to be successful in control of common sunflower and were therefore used extensively. Such use resulted in the first occurrence of imazethapyr resistant plants of common sunflower in a soybean field in northeast Kansas where imazethapyr had been applied continuously for the previous seven years (Al-Khatib et al., 1998).

Sulfonylurea herbicides were used for broad-leaf weed control in Canada for cereal crops since 1982 (PMRA EDDENet, Product Information). Tribenuron-methyl resistance has been reported for native *Helianthus annuus* L. populations found mostly in or close to crop fields in Canada (1994) and USA (2002) with 52% and 57% of the total tested respectively (Miller and Seiler, 2005; Olson et al., 2004).

The objective of this study was to determine whether resistance to tribenuron-methyl (Express) is a novel trait or that it was already present in the wild sunflower species.

MATERIALS AND METHODS

After reviewing the seed reserve status for all the accessions of wild annual species in the sunflower collection at Novi Sad, it was found that 73 of them were available for the trial (Table 1.).

The seeds were collected during six collecting trips in the USA from 1980 to 1991. After that period, seven populations of *H. annuus* were collected in 2001, five in the USA and two in Mexico, and three populations of *H. petiolaris* were obtained through exchange from Argentina in 2005 and labeled as GRR accessions. Seven annual species were represented in this trial with 1 to 30 accessions per species.

Table 1. Accessions of wild *Helianthus* species used in the trial.

No.	Accession	Plant Introd. No.	Collect. date	Country	State/province	Habitat/Locality
1	ANN-1	-	2001	USA	Texas	Roadside ditch
2	ANN-10	-	2001	USA	Ohio-Idaho border	Highway No. 20, near cultivated field
3	ANN-13	-	2001	Mexico		Palmitos
4	ANN-14	-	2001	Mexico		Torreon
5	ANN-2	-	2001	USA	Texas	Seymour, highway 114
6	ANN-3	-	2001	USA	Texas	Roadside ditch
7	ANN-9	-	2001	USA	Wyoming	Riverton
8	ANN 1965	PI 531011	1987	USA	Idaho	Roadside ditch, near lava beds
9	ANN 1977	PI 531017	1987	USA	Washington	Disturbed moist roadside ditch
10	ANN 1983	PI 531022	1987	USA	Montana	Roadside ditch near sagebrush rangeland
11	ANN 1992	PI 531027	1987	USA	Idaho	Roadside ditch near sagebrush rangeland
12	ANN 2034	PI 547165	1989	USA	Illinois	Waste area, roadside ditch
13	ANN 2101	PI 586807	1991	USA	North Dakota	Roadside ditch by James River
14	ANN 2104	PI 586809	1991	USA	North Dakota	Poor sandy loam soil in roadside ditch
15	ANN 2114	PI 586815	1991	USA	North Dakota	Disturbed area of roadside ditch
16	ANN 2125	PI 586820	1991	USA	Montana	Disturbed soil in roadside ditch
17	ANN 2134	PI 586827	1991	USA	Wyoming	Disturbed area in roadside ditch
18	ANN 2138	PI 586830	1991	USA	Wyoming	Disturbed roadside ditch
19	ANN 2141	PI 586833	1991	USA	South Dakota	Disturbed backslope of roadside ditch
20	ANN 2144	PI 586835	1991	USA	South Dakota	Roadside ditch, along guard rail
21	ANN 2150	PI 586839	1991	USA	Wyoming	Roadside ditch
22	ANN 2165	PI 586845	1991	USA	Colorado	Disturbed roadside ditch
23	ANN 2168	PI 586846	1991	USA	Colorado	Disturbed roadside ditch
24	ANN 2169	PI 586847	1991	USA	Colorado	Disturbed roadside ditch
25	ANN 2170	PI 586848	1991	USA	Colorado	Disturbed area along roadside ditch
26	ANN 2183	PI 586860	1991	USA	Kansas	Roadside ditch
27	ANN 2187	PI 586864	1991	USA	Kansas	Roadside ditch, along corn field
28	ANN 2191	PI 586866	1991	USA	Nebraska	Along railroad track
29	ANN 2206	PI 586877	1991	USA	Nebraska	Roadside ditch
30	ANN 2223	PI 586884	1991	USA	South Dakota	Along edge of cultivated sunflower
31	ARG 1317	PI 468649	1980	USA	Texas	Sandy roadside area
32	ARG 1575	PI 468651	1980	USA	Florida	Two sandy, empty lots and yard
33	ARG 1677	-	-	-	-	-
34	ARG 1805	PI 494571	1984	USA	Texas	4.8 km south of Rodfield Road
35	ARG 1807	PI 494573	1984	USA	Texas	Along Highway P53
36	ARG 1812	PI 494576	1984	USA	Texas	Along Highway 181
37	DEB 1134	PI 468678	1979	USA	Texas	Along Highway 43
38	DEB 1810	PI 494583	1984	USA	Texas	Along Highway 59
39	DEB 1565	PI 468690	1980	USA	Florida	Beach, east edge of Carrabelle
40	GRR 269	-	2004	Argentina	San Luis	Highway 7, near Villa Mercedes
41	GRR 276	-	2004	Argentina	Buenos Aires	Quenuma
42	GRR 283	-	2004	Argentina	La Pampa	Highway 5, Anguil
43	NEG 1181	PI 468765	1979	USA	Texas	Along Highway 18
44	NEG 1183	PI 468865	1979	USA	New Mexico	Along Highway 18
45	NEG 457	PI 435761	1976	USA	Texas	Along Highway 18
46	NIV 1452	PI 468788	1980	USA	California	Along Highway 99
47	PET 2004	PI 531056	1987	USA	Montana	Ditch by the road,
48	PET 2009	PI 531057	1987	USA	Montana	Ditch by the road, on sandy soil,
49	PET 2011	PI 531058	1987	USA	North Dakota	Sandy soil, by the road
50	PET 2119	PI 586912	1991	USA	Montana	Roadside ditch close to small grain field
51	PET 2126	PI 586916	1991	USA	Montana	Roadside ditch, extending to rangeland
52	PET 2158	PI 586922	1991	USA	Colorado	Sandy roadside ditch
53	PET 2163	PI 586924	1991	USA	Colorado	Sandy roadside ditch
54	PET 2164	PI 586925	1991	USA	Colorado	Sandy roadside ditch
55	PET 2167	PI 586927	1991	USA	Colorado	Sandy soil in disturbed roadside ditch
56	PET 2178	PI 586928	1991	USA	Kansas	Sandy roadside ditch
57	PET 2208	PI 586931	1991	USA	Nebraska	Sandy roadside ditch
58	PET 71	-	1984	USA	North Dakota	S. W. Kindred
59	PET 722	PI 435831	1977	USA	Kansas	Junction of Highways 83 and 50
60	PET 74	-	1984	USA	North Dakota	Northwood
61	PET 1383	PI 468811	1980	USA	New Mexico	Along Highway 18
62	PET 1910	PI 503232	1985	USA	New Jersey	sandy waste area
63	PRA 1142	PI 468851	1979	USA	Texas	Along Highway 146
64	PRA 1145	PI 468852	1979	USA	Texas	Along Alternate 90
65	PRA 1168	PI 468847	1979	USA	Texas	Along Highway 44
66	PRA 1819	PI 494609	1984	USA	Texas	Along Highway 281
67	PRA 1821	PI 494610	1984	USA	Texas	Along Highway 281
68	PRA 1824	PI 494601	1984	USA	Texas	Along Highway 3005
69	PRA 1826	PI 494603	1984	USA	Texas	Along Highway 87
70	PRA 1340	PI 468848	1980	USA	Texas	Along Highway 83
71	PRA 1341	PI 468849	1980	USA	Texas	Along Highway 2644
72	PRA 1342	PI 468850	1980	USA	Texas	Along Highway 2644
73	PRA 1333	PI 468865	1980	USA	Texas	Along Highway 285

One hundred seeds of each accession were used for the study. The seeds were placed in petri dishes with filter paper saturated with distilled water for 24h at +25˚C in the dark. The seed coat was then removed and the seeds were placed on a new filter paper in the same manner. The dishes were

checked daily for germination and formation of the roots. Germinating seedlings were each planted into separate jiffy 7 pots expanded from compressed peat moss disks and placed in the greenhouse with 16/8-h day/night periods. At the two- to three-leaf stage the plants were transferred to the field.

When plants reached the six- to eight-leaf stage they were treated with tribenuron-methyl (Express) at 1x rate (17.3 g ai/ha). Susceptible and total plants were counted one week later and the surviving plants were again treated with the same dosage of tribenuron. After another week, plants with slight chlorosis or leaf damage were noted as moderately resistant and plants with no herbicide damage symptoms were noted as resistant, as described by Miller and Seiler (2005). Plants that continued growing through branches even though their vegetation cone was destroyed were also noted.

RESULTS AND DISCUSSION

Plants in the majority of tested accessions were susceptible, showing symptoms of herbicide damage like chlorosis, growth slowdown and necrosis of young leaves. Moderately resistant plants were found in accessions of three wild species *Helianthus annuus*, *H. argophyllus* and *H. petiolaris*. After the second treatment the number of accessions with healthy plants was reduced to three in *H. argophyllus* and one in *H. annuus* (Table 2.).

Table 2. Number of accessions per wild *Helianthus* species that were susceptible (SUS), moderately resistant (MR), or resistant (R) to tribenuron (Express) herbicide and which survived both treatments

Wild species	Total No. of accessions	No. SUS	No. MR	No. R	No. of survived accessions
Helianthus annuus	30	24	6	1	28
Helianthus petiolaris	19	18	1		14
Helianthus praecox	11	11			8
Helianthus argophyllus	6	1	5	3	6
Helianthus debilis	3	3			3
Helianthus neglectus	3	3			2
Helianthus niveus	1	1			1

Plants that were scored as resistant had no visible herbicide damage and continued to develop as if there was no herbicide treatment. The resistance that was recorded in *H. argophyllus* accessions may have been influenced and increased by the leaf hairiness as well.

If the survival of a plant is considered as resistance and plant death as susceptibility, then the number of resistant plants in this trial was much higher. At least one plant in 63 out of 73 tested accessions continued to grow by branching after the vegetation cone was destroyed and all tested species had at least one accession with plants that survived the full treatment (Table 3.). This is due to the fact that the majority of plants that showed symptoms of herbicide damage started to regenerate and formed lateral branches after the treatment. The decrease in sensitivity of ALS to herbicide active ingredient has been found to contribute to whole-plant resistance more than the differences in absorption, translocation and metabolism (Al-Khatib et al., 1998). The resulting whole-plant resistance may be of interest from the aspect of weed control in wild sunflower-infested crop fields.

This research confirmed that tribenuron-methyl resistance was present in the wild annual species of sunflower before the release of Express-tolerant cultivated sunflower. It occurred in low frequencies which will now probably increase because of the expected use of Express herbicide and applied selection pressure.

Table 3. Number and percentage of plants from wild *Helianthus* species which were susceptible (SUS), moderately resistant (MR), or resistant (R) to tribenuron (Express) herbicide and the number of plants that survived both treatments

No.	Accession population	Total No. of plants	No. SUS	No. MR	% MR	No. R	% R	No. of survived plants
1	A-1	22	22		0.0%		0.0%	17
2	A-10	15	15		0.0%		0.0%	1
3	A-13	17	17		0.0%		0.0%	11
4	A-14	11	11		0.0%		0.0%	5
5	A-2	17	14	3	17.6%		0.0%	11
6	A-3	11	11		0.0%		0.0%	8
7	A-9	9	9		0.0%		0.0%	3
8	ANN 1965	16	16		0.0%		0.0%	0
9	ANN 1977	9	9		0.0%		0.0%	3
10	ANN 1983	12	12		0.0%		0.0%	6
11	ANN 1992	8	7	1	12.5%		0.0%	7
12	ANN 2034	29	28	1	3.4%		0.0%	13
13	ANN 2101	21	21		0.0%		0.0%	5
14	ANN 2104	28	28		0.0%		0.0%	8
15	ANN 2114	10	10		0.0%		0.0%	4
16	ANN 2125	14	14		0.0%		0.0%	0
17	ANN 2134	11	8	3	27.3%		0.0%	8
18	ANN 2138	15	15		0.0%		0.0%	9
19	ANN 2141	22	22		0.0%		0.0%	9
20	ANN 2144	17	17		0.0%		0.0%	1
21	ANN 2150	20	20		0.0%		0.0%	14
22	ANN 2165	21	19	2	9.5%		0.0%	12
23	ANN 2168	24	24		0.0%		0.0%	17
24	ANN 2169	10	9	1	10.0%	1	10.0%	3
25	ANN 2170	24	24		0.0%		0.0%	14
26	ANN 2183	11	11		0.0%		0.0%	4
27	ANN 2187	12	12		0.0%		0.0%	11
28	ANN 2191	9	9		0.0%		0.0%	4
29	ANN 2206	11	11		0.0%		0.0%	7
30	ANN 2223	24	24		0.0%		0.0%	10
31	ARG 1317	17	0	17	100.0%		0.0%	17
32	ARG 1575	15	2	13	86.7%		0.0%	12
33	ARG 1677	35	0	35	100.0%	35	100.0%	35
34	ARG 1805	31	7	24	77.4%	7	22.6%	31
35	ARG 1807	27	9	18	66.7%	9	33.3%	22
36	ARG 1812	27	27		0.0%		0.0%	27
37	DEB 1134	20	20		0.0%		0.0%	1
38	DEB 1810	13	13		0.0%		0.0%	6
39	DEB 1565	23	23		0.0%		0.0%	1
40	GRR 269	17	17		0.0%		0.0%	0
41	GRR 276	9	9		0.0%		0.0%	3
42	GRR 283	24	24		0.0%		0.0%	4
43	NEG 1181	20	20		0.0%		0.0%	4
44	NEG 1183	11	11		0.0%		0.0%	2
45	NEG 457	9	9		0.0%		0.0%	0
46	NIV 1452	16	16		0.0%		0.0%	6
47	PET 2004	25	25		0.0%		0.0%	6
48	PET 2009	14	14		0.0%		0.0%	0
49	PET 2011	17	17		0.0%		0.0%	1
50	PET 2119	14	14		0.0%		0.0%	3
51	PET 2126	17	17		0.0%		0.0%	0
52	PET 2158	11	11		0.0%		0.0%	4
53	PET 2163	17	17		0.0%		0.0%	1
54	PET 2164	16	16		0.0%		0.0%	0
55	PET 2167	15	15		0.0%		0.0%	1
56	PET 2178	23	23		0.0%		0.0%	3
57	PET 2208	21	21		0.0%		0.0%	7
58	PET 71	13	13		0.0%		0.0%	1
59	PET 722	16	16		0.0%		0.0%	1
60	PET 74	25	24	1	4.0%		0.0%	7
61	PET 1383	21	21		0.0%		0.0%	2
62	PET 1910	23	23		0.0%		0.0%	0
63	PRA 1142	13	13		0.0%		0.0%	2
64	PRA 1145	9	9		0.0%		0.0%	1
65	PRA 1168	12	12		0.0%		0.0%	0
66	PRA 1819	21	21		0.0%		0.0%	12
67	PRA 1821	17	17		0.0%		0.0%	5
68	PRA 1824	10	10		0.0%		0.0%	0
69	PRA 1826	21	21		0.0%		0.0%	4
70	PRA 1340	11	11		0.0%		0.0%	3
71	PRA 1341	10	10		0.0%		0.0%	0
72	PRA 1342	10	10		0.0%		0.0%	1
73	PRA 1333	11	11		0.0%		0.0%	3

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Hybridization between cultivated sunflower *Helianthus annuus* L. and wild perennial species *Helianthus pumilus* Nuttall

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ABSTRACT

Helianthus pumilus Nuttall was included in hybridization programs with the cultivated sunflower *Helianthus annuus* L. The investigation encompassed the period 2000-2007. The results showed that the two species crossed, but the crossability rate was low. Seeds were obtained at both directions of crossing and hybrid plants only in the direct crosses. The F_1 plants had an intermediate type of heritability, but they resembled the wild species in their most important biomorphological characteristics. All plants showed an annual cycle of growth in contrast to the wild perennial species. It was established that *H. pumilus* carried *Rf* genes for CMS PET-1, genes controlling the resistance to diseases such as downy mildew and phomopsis and the parasite broomrape, and genes controlling quantitative seed oil content. As a result of self-pollination, sib-pollination of the F_1 plants and backcrossing with cultivated sunflower, F_2, BC_1 and the next hybrid progenies (F_3-F_7 and next to F_5BC_1) were obtained. Some of the obtained hybrid forms were included in a program for developing lines for heterosis breeding in sunflower.

Key words: *Helianthus annuus* – *Helianthus pumilus* – interspecific hybridization –b sunflower.

INTRODUCTION

H. pumilus Nuttall belongs to section *Ciliares*, series *Pumili* (Schilling and Heiser, 1981). Species of this section appear to be well isolated genetically from species of other sections (Seiler and Rieseberg, 1997). Habitats are dry, often rocky soils, from 1200 to 1800 m elevation in central Colorado northward through southeastern and central Wyoming (Rogers et al., 1982) and in 2005 covered 5150 km^2 (Seiler et al., 2007). *H. pumilus* (dwarf sunflower) is a perennial species with potential genes for oil improvement based on its xerophytic habitat. The higher concentrations of linoleic acid in *H. pumilus* could be a potential source of genes for increasing the concentration of this fatty acid in traditional sunflower oil. The *H. pumilus* populations had an average oil content of 254 g/kg, considerably lower than cultivated sunflower which has an average of 470 g/kg. The linoleic acid concentration approached 750 g/kg, much higher than the 540 g/kg /Seiler et al., 2007/. An antitumor drug, desacetyleupaserrin, has been identified from this species (Rogers et al., 1982*). H. pumilus* was hybridized with several species of genera *Helianthus*, but part of them were unsuccessful or the information from the results was insufficient (Heiser, 1965; Heiser et al., 1969; Krauter et al., 1991) used a modified embryo culture to cross *H. pumilus* with cultivated sunflower. Successful interspecific hybridization was carried out between the wild perennial diploid species *Helianthus pumilus* and cultivated *H. annuus* (Nikolova et al., 2004).

MATERIALS AND METHODS

The investigation encompassed the period 2000 - 2007. It included the cultivated sunflower *H. annuus* and the wild perennial species *H. pumilus*, accession GT-M-172. The cultivated sunflower was represented by two varieties, Peredovik and VNIIMK 6540, and by two lines, 2607 and 6116.

Hybridization was carried out through reciprocal crosses realized under field conditions. The sterile analogues of lines 2607 and 6116 (cytoplasmic male sterile lines in CMS PET-1) were used as a female parent of the cultivated sunflower in direct crosses. In the reciprocal crosses, the florets in the inflorescences of the wild species were emasculated manually and pollinated with pollen from a single line or with mixed pollen from varieties and lines. Hybrid plants were grown under field conditions, too. To obtain F_2 and BC_1, self-pollination, sib-pollination and back-crossing of F_1 to cultivated sunflower were made. Phenological observations of the F_1 hybrids were made during the vegetative period. Biometric parameters and description of the main morphologic characters and biologic peculiarities of the F_1 hybrids were performed. Similar investigations were carried out with the next hybrid generations as well. The seed set was calculated as a ratio between the seeds obtained (the number of inseminated disk flowers) and total number of disk flowers in the inflorescence. 1000 seed weight was calculated by

measuring two samples, each of 10, 25 or 50 seeds. Back-crossing with cultivated sunflower as a mother was used with the aim of confirming the presence of fertility restorer genes (*Rf* genes) in F_1 hybrids, transferred from *H. pumilus*. The reactions to diseases were studied, using standard methodologies (Panchenko, 1975; Acimovič, 1979; Vear and Tourvieille, 1987; Encheva and Kiryakov, 2002). Oil content of seeds was estimated. Cytological analyses were carried out on the mitosis, particularly on the chromosome number (Georgieva-Todorova, 1976). Pollen viability was determined by a standard methodology (Alexander, 1969; Atlagic, 1990).

RESULTS

The analysis of the results presented in Table 1 shows that the two diploid (2n = 34) sunflower species could be crossed. The crossability rate was low. Seed set per cultivated sunflower inflorescence after compulsory pollination with pollen from the wild species was low (0.52%) for the direct crosses and 3.57% for the reciprocal. The slightly higher level of this index in the cross *H. annuus x H. pumilus* was due to the difference in the inflorescence size of the cultivated and wild species, as well as to the number of the seeds. In comparison to the results obtained from the hybrids, the parental seed set after free pollination was 77.9% and 88.2% for lines 6116B and 2607B, respectively and varied from 3.3 to 8.8% for the wild species.

Table 1. Crossability of cultivated sunflower *H. annuus* and wild perennial *H. pumilus*.

Crosses	Pollinated inflorescences			Total number of seeds	Seed set		Hybrid plants	
	total number	with seed number	with seed %		mean number	%	number	%
H. annuus x *H. pumilus*	6	4	66.67	28	7	0.52	5	17.86
H. pumilus x *H. annuus*	20	11	55.00	23	2	3.57	0	0

Seeds were obtained at both directions of crossing, and hybrid plants only in the direct crosses. A total of 28 seeds and 5 F_1 plants, 4 from the cross combination *H. annuus* line 2607A x *H. pumilus* and one from the cross *H. annuus* line 6116A x *H. pumilus* were obtained from the hybridization of *H. annuus* with *H. pumilus*. Hybrid plants were not grown in the reciprocal crosses *H. pumilus* x *H. annuus*, although mixed pollen from varieties (Peredovik and VNIIMK 6540) and lines (2607B and 6116B) was also used.

The number of chromosomes for F_1 plants ranged from 2n = 31 to 2n = 34. This was probably due to a difference between the genotypes of the parents. Five satellite chromosomes per cell were observed (Fig. 1). The karotype's formula of *H. pumilus* was 1 sat + 5 m + 5 sm + 7 st, according to Kulshreshtha and Gupta (1981), and that of *H. annuus* (cultivated sunflower) - 3 sat + 10 m + 3 sm + 4 st according to Raicu et al. (1976); 3 sat + 4 m + 8 sm + 5 st, according to Al-Allaf and Godward (1977) and 2 sat + 5 m + 8 sm + 4 st, according to Georgieva-Todorova and Bohorova (1980).

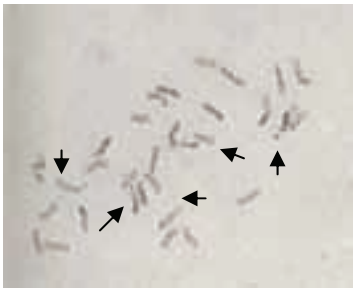
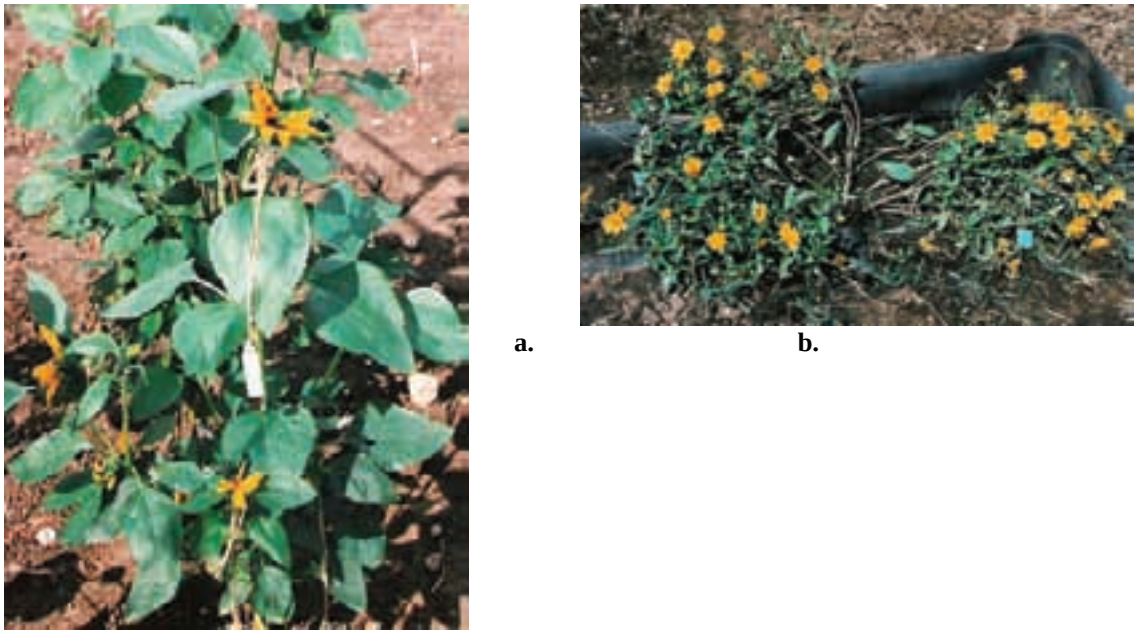


Fig. 1. Metaphase cell from F_1 plant with 5 satellite chromosomes.

The F_1 hybrid (Fig. 2a) had an annual growth habit like that of the cultivated sunflower (2 lines) in contrast to wild perennial species (Fig. 2b, Table 2).



a. **b.**

Fig. 2. Plants of: a) F_1 hybrid; b) *H. pumilus* GT M 172.

Table 2. Characterization of F_1 hybrids.

Characteristics	*H. annuus* L. 2607 A	F_1: L. 2607 A x M 172	*H. pumilus* GT M 172	F_1: L. 6116 A x M 172	*H. annuus* - L. 6116 A
Phenological characteristics					
Life cycle	annual	annual	perennial	annual	annual
Vegetation period, days	118	122 - 128	215	120	109
Morphological characteristics					
Plant height, cm	130 - 135	70 - 75	45	75	135 - 140
Number of branches	0	7 - 15	32 – 38	17	0
Leaf length, cm	24 - 27	16 - 18	4 – 15	20	21 - 27
Leaf width, cm	22 - 26	13 - 19	1 – 4	17	20 - 23
Length of leaf petiole, cm	11 - 13	2 - 3	0.7	3	14 - 17
Head diameter, cm	17 - 19	4.5 - 8	1.3	6.5	21 - 23
Technological characteristics					
1000 seed weight, g	61.4	x	2.9	x	54.2
Oil, %	43.0	x	25.1	x	45.10

The vegetation period of hybrids was similar to *H. annuus* and varied from 120 to 128 days in contrast to 215 days of *H. pumilus*.

All F_1 plants were branched along the entire stem, dark green with anthocyanin coloration and with trichomes. The stem of *H. pumilus* was low, fine, rugged, branched, green and with anthocyanin coloration at the top. The height the branches of F_1 hybrids exceeded the central stem (Table 2). This character was typical for *H. pumilus* in contrast to the cultivated sunflower, which was not branched. The presence of branches in the hybrid materials suggested dominant heritability and it was used as a

morphological marker for successful hybridization, similar to the anthocyanin coloration. They proved a transfer of genetic material from *H. pumilus* to the genotype of the F_1 plants.

The leaves of F_1 plants were green, with a glossy surface, similar to the wild species. They were distichous at the base and alternate along the other part of the stem. The lamina shape was cordate, slightly elongated, with a sharp peak. The leaf margins were serrate. The leaves presented anthocyanin coloration with time.

Heads of F_1 plants were small, like those of *H. pumilus,* with dark purple disk flowers and stigma, and orange pollen and ray flowers.

All F_1 plants were fertile. This showed that the genotype of *H. pumilus* had *Rf* genes for CMS PET-1. The mean percentage of pollen viability of F_1 hybrids was low (from 2.1 to 17.9 %), while in the wild species it varied from 75.5 to 87.2 %. After free pollination, from 1 to 11 seeds were obtained. As a result of the self-pollination of 5 central inflorescences, a total of 14 seeds were produced. After pollination of the sterile analog of line HA89 with pollen from F_1 plants, from 9 to 67 seeds were produced. The seed set after backcrossing was from 0.67 to 5.01 %.

Two fertile F_2 plants from the total of 14 seeds were obtained that differed in plant height. One of them was 85 cm high, and the other (from cross *H. annuus* line 6116A x *H. pumilus* M 172) was 112 cm high (Table 3). There were other differences in the form and size of branches, leaves, inflorescence and seeds. In F_2 plant from the cross *H. annuus* line 6116A x *H. pumilus* M 172, initially three branches occurred at the base of the stem, and another four branches developed on the main inflorescence at the beginning of flowering. These were short and situated above the middle part of stem. The lamina shape was cordate, slightly elongated, with a sharp peak and glossy surface. Seed color of F_1 was from gray-brown to anthocyanin-black.

The total number of obtained BC_1 was 198, 102 of them being fertile plants. The value of χ^2 in BC_1 generation was lower (χ^2 = 0.182) than that at level of significance of 5% (3.841). This determined an accidental nature of the differences between the observed and expected values. This result showed that *Rf* genes from *H. pumilus* were transferred in *H. annuus* and the control for recovery of male fertility at CMS Pet-1 was dominant and monogenic.

All BC_1 plants were branched with anthocyanin along the stem, branches and petiole. There were a few plants with leaves with a glossy surface. The branches were mainly in the middle and on the top part of the stem. Seeds possessed a dark brown, black and anthocyanin-black coloration.

Fig. 3. BC_1 plant.

Table 3. Characterization of plants of BC_1, F_2, and F_3 generations.

Generation	Plant height, cm	Head diameter, cm	1000 seed weight, g	Oil, %	Vegetation period, days
BC_1	95 - 130	15	36.2	44.2	126
F_2	85 and 112	12	30.3	39.9	123
F_3	145	12	31.2	45.5	125

F_3-F_7 and next to F_5BC_1 generations were produced. As a result of the selection, new forms suitable for R and B lines were developed. Genes were transferred from *H. pumilus*, which controlled the following characters: 100% resistance to downy mildew races 300 and 700, phomopsis, powdery mildew, rust and broomrape; *Rf* gene for CMS Pet-1; type of branching suitable for the R lines, high oil content in seed (from 51.25 to 59.05 %) and high combining ability.

DISCUSSION

The results obtained showed that the crossability level of the perennial diploid wild species *H. pumilus* with cultivated sunflower was low. The incompatibility was high regardless of the equal chromosome number in their genomes. This result was probably due to the more distant relationship or to some other reason, for example the higher cytoplasmic effect of the perennial species on the chromosome

conjugation, etc. Seeds were obtained from the reciprocal cross, although their percent was very low, but, however, no F_1 plants were produced. Viable plants that could reproduce were obtained only from the direct cross. The F_1 plants had an intermediate type of heritability, but they resembled the wild parent in the most important biomorphological characters. The positive result for seed set at back-crossing showed that the pollen from F_1 hybrids was viable.

Nuclear genetic material was transferred into cultivated sunflower through the direct cross. This is very important for heterosis breeding in sunflower because, besides the stability of the CMS source, the genetic potential of the nuclear material was also essential, as its material was enriched with new content in this case. According to Seiler and Gulya (2004), wild species have contributed many agronomically important traits to cultivated sunflower.

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Studies on some morphological characters of wild *Helianthus annuus* L. accessions with different origin

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ABSTRACT

This study presents the evaluation of populations of wild annual *Helianthus annuus* L., the closest relative of cultivated sunflower, the variability and similarities in their morphological characters as well as the possible effect of cultivation in different environments on the traits of this species. The investigation included 73 accessions of wild *H. annuus* with a different origin from the wild species sunflower collection of Dobroudja Agricultural Institute, General Toshevo, Bulgaria. The phenotypic correlations between characters signified the importance of the studied cases and variables. The genotypes were distributed into eight clusters. The simultaneous testing of the significance of the differences in mean values between genotypes revealed significant differences among the studied genotypes of wild *H. annuus* with a different origin.

Key words: cluster analysis – correlation coefficient – wild *Helianthus annuus* L.

INTRODUCTION

The *Compositae* (*Asteraceae*) is the largest and most diverse family of flowering plants. Collection and exploration of wild annual sunflower species have represented difficult and challenging activities in the process of conserving genetic diversity. Over the past several decades genes for resistance to some economically important diseases, parasites and pests have been successfully transferred into cultivated sunflower together with new valuable morphological characters.

Many investigations of scientific researchers in the field of interspecific hybridization in sunflower *Helianthus annuus* L. have confirmed that the wild species constitute an abundant source of genes determining important agricultural characters in sunflower (Pustovoit, 1975; Laferriere, 1986; Škorić, 1988, 1992; Seiler, 1988, 1992; Christov, 1996a, b; Christov et al., 1996).

Helianthus annuus, the wild ancestor from which the domesticated sunflower originated, is a diverse species in its center of origin and is also more variable in places where it was introduced. Wild *Helianthus* species possess not only a considerable variability for most of the traits, but also excellent environmental survival mechanisms (Thompson et al., 1981). They were widely used in sunflower breeding programs (Christov, 1999, 2004; Seiler and Brothers, 1999; Škorić et al., 1999).

This study evaluated populations of wild annual *H. annuus*, the closest relative of cultivated sunflower, the variability and similarities in their morphological characters as well as the possible effect of cultivation in different environments on the traits of this species.

MATERIALS AND METHODS

In this investigation were included 73 accessions of wild *Helianthus annuus* from the wild species sunflower collection of Dobroudja Agricultural Institute, General Toshevo, Bulgaria. They originated from USA, France, Russia, Germany, England, Romania, China, Czech Republic, Serbia, Canada and Holland (Table 1). Their morphological traits were evaluated according to IBPGR Descriptor (IBPGR, 1985). These were: leaf length, leaf width, ratio length to width of leaves, length of leaf petiole, number of ray florets, length of ray florets, width of ray florets, number of bract leaves, length of bract leaves, width of bract leaves, ratio length to width of bract leaves, head diameter, length of the longest branch, plant height, 1000 seeds weight and seed oil content.

The morphological characterization of the wild *H. annuus* accessions has been performed for the last three years and the data presented were .averaged over years. The observations were made on 10 plants, grown under field conditions. Oil content was determined by nuclear magnetic resonance (NMR).

The statistical estimations were performed by STATISTICA for WINDOWS 95 and included correlation coefficient, grouping into clusters and determining the Euclidean distances. In the method for grouping we used group analysis and the Euclidean units for estimating the differences between the groups. Groups of accessions were separated using single linkage method.

Table 1. Origin of studied *H. annuus* L. accessions.

Country	Accession number:
USA	GT-E-002, GT-E-003, GT-E-004, GT-E-088, GT-E-092, GT-E-093, GT-E-109, GT-E-110, GT-E-111, GT-E-112, GT-E-113, GT-E-114, GT-E-115, GT-E-116, GT-E-117, GT-E-118, GT-E-119, GT-E-120, GT-E-121, GT-E-122, GT-E-123, GT-E-124, GT-E-125, GT-E-126, GT-E-127, GT-E-128, GT-E-129, GT-E-153, GT-E-154, GT-E-155, GT-E-171, GT-E-172, GT-E-173, GT-E-174, GT-E-175, GT-E-176, GT-E-177, GT-E-178, GT-E-179, GT-E-180
France	GT-E-035, GT-E-042, GT-E-043, GT-E-044, GT-E-045, GT-E-055, GT-E-056, GT-E-057, GT-E-058, GT-E-059, GT-E-060, GT-E-061, GT-E-062, GT-E-063, GT-E-064
Russia	GT-E-040, GT-E-049, GT-E-103, GT-E-104, GT-E-105, GT-E-106
Germany	GT-E-066, GT-E-079, GT-E-184
England	GT-E-182, GT-E-183
Romania	GT-E-077, GT-E-081
China	GT-E-170
Czech Republic	GT-E-078
Serbia	GT-E-046
Canada	GT-E-053
Holland	GT-E-005

RESULTS AND DISCUSSION

Groups of variables could be usually determined in these investigations. They were correlated due to complex interactions that were uncontrolled and obscured. The degree of association of such variables can be determined through correlation analysis. Correlations between characters illustrated the importance of the studied cases as pleiotropic action of genes, linkages, improvements brought about by selection through related characters and natural selection (Table 2).

Table 2. Correlation coefficients of studied morphological traits.

	Leaf length	Leaf width	Length of leaf petiole	No. of ray florets	Length of ray florets	Width of ray florets	No. of bract leaves	Length of bract leaves	Width of bract leaves	Head dia-meter	Length of long-est branch	Plant height	1000 seed weight	Seed oil con-tent	Ratio length/ width leaves
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	1														
2	**0.86**	1													
3	**0.52**	**0.49**	1												
4	0.21	0.30	0.18	1											
5	**0.30**	**0.30**	**0.35**	0.06	1										
6	0.01	0.06	0.07	0.17	-0.16	1									
7	0.26	0.27	0.24	**0.53**	0.22	-0.03	1								
8	**0.46**	**0.59**	0.19	0.26	**0.34**	0.06	0.11	1							
9	**0.36**	**0.54**	0.19	0.28	0.16	0.03	0.20	**0.57**	1						
10	**0.37**	**0.49**	0.17	**0.35**	0.25	0.02	0.31	**0.59**	**0.49**	1					
11	0.29	0.24	0.02	-0.11	0.16	-0.05	0.01	0.05	0.14	0.07	1				
12	0.17	0.16	0.00	0.03	-0.28	0.34	-0.10	0.07	0.18	0.16	0.12	1			
13	0.02	0.01	-0.07	0.05	-0.02	0.10	-0.01	0.02	-0.06	-0.11	-0.19	-0.05	1		
14	0.09	0.08	0.10	**0.27**	-0.11	-0.13	0.09	-0.12	0.05	-0.05	-0.10	-0.06	**0.34**	1	
15	-0.27	**-0.64**	-0.37	-0.35	-0.11	-0.10	-0.26	-0.27	-0.35	-0.29	-0.08	-0.07	0.04	-0.12	1
16[1]	0.13	0.03	-0.03	-0.03	0.29	0.01	-0.08	0.44	-0.43	0.09	0.01	-0.16	0.05	-0.16	0.15

[1]Ratio length/width bract leaves

There were several high and positive correlations, which are presented in Table 2. The phenotypic correlation between leaf length and leaf width was positive (r=0.86*) and higher than those with length of leaf petiole (r=0.52*) and length of bract leaves (r=0.46*).

The phenotypic correlation between leaf width and length of bract leaves was significantly high and positive (r=0.59*) as well the correlations with width of bract leaves (r=0.54*), with length of leaf petiole (r=0.49*) and head diameter (r=0.49*). The phenotypic correlation between number of ray florets and number of bract leaves was positive (r=0.53*). Length of bract leaves correlated positively with their width (r=0.57*) as well as with head diameter (r=0.59*). The width of the bract leaves only correlated positively with head diameter (r=0.49*). The analysis of variance showed significant differences among most of the genotypes.

Seed oil content correlated positively with 1000-seed weight (r=0.34*) and to a smaller degree with the number of ray florets (r=0.27*), which may be due to the reported positive correlation with the number of disk florets and seed set and yield (Doddamani et al.,1997) .In general, in this study the association of oil content with most of the characters was either non-significant or the few significant correlations were of a lower magnitude, suggesting that indirect selection for oil content would be rather a difficult proposition.

The simultaneous testing of significance of difference in mean values between genotypes revealed significant differences between the studied genotypes. The 73 genotypes were grouped into eight clusters using Tree Diagram for 73 cases, unweighted pair-group average with Euclidean distances but the dendrogram obtained was too large, which was why the distribution of genotypes was presented in Table 3.

Table 3. Distribution of 73 genotypes of *H. annuus* L. into different clusters.

Cluster	Genotype	Number of genotypes
I	GT-E-003; GT-E-044; GT-E-002; GT-E-035; GT-E-105; GT-E-116; GT-E-056; GT-E-093; GT-E-112; GT-E-123; GT-E-106; GT-E-043; GT-E-154; GT-E-182; GT-E-046; GT-E-175; GT-E-184; GT-E-113; GT-E-178; GT-E-005; GT-E-045; GT-E-111; GT-E-004; GT-E-173; GT-E-118; GT-E-153	26
II	GT-E-040; GT-E-092; GT-E-110; GT-E-121; GT-E-124; GT-E-120; GT-E-042; GT-E-119; GT-E-078; GT-E-170; GT-E-053;	11
III	GT-E-079; GT-E-171; GT-E-114; GT-E-055; GT-E-117; GT-E-058; GT-E-109;	7
IV	GT-E-049; GT-E-061; GT-E-063; GT-E-066;	4
V	GT-E-125; GT-E-173; GT-E-172; GT-E-177; GT-E-077; GT-E-127; GT-E-060; GT-E-104; GT-E-062;	9
VI	GT-E-057; GT-E-059; GT-E-179; GT-E-174; GT-E-155; GT-E-183;	6
VII	GT-E-103; GT-E-129; GT-E-064; GT-E-081; GT-E-088; GT-E-126; GT-E-128; GT-E-122;	8
VIII	GT-E-115; GT-E-180;	2

The maximum number of genotypes was included in cluster I. Most of them were of USA origin and part of them from France, Russia, Germany, Serbia and England, which indicated that there was a slight association between clustering pattern and the influence of agro-climate conditions and the cultivation of the genotypes. Insignificant linkage distances according to the dendrogram obtained were found between GT-E-112 and GT-E-123 (origin USA), GT-E-043 and GT-E-044 (origin France) and GT-E-045 and GT-E-105 (origin France and Russia). The significant distances for all accessions included in this cluster as well in cluster 2, cluster 3 and cluster 4 were not high as a whole. The lack of significant inter-cluster linkage distance could be due to the closely related genotypes and similar climate conditions and introduction. There were significant linkage distances for the accessions in clusters 5, 6, 7 and 8.They differentiated on many of the studied characters. The accessions in cluster 8 distinguished themselves in most of the studied morphological traits. GT-E-115 and GT-E-180 of USA origin, could not be integrated to any of the other clusters because of the great linkage distance between them. These results showed that the purposive genetic drifts and an extended and prolonged introduction into different environments could cause a greater diversity among genotypes than their origin.

The wild *Helianthus annuus* L. collection of DAI-General Toshevo possesses great diversity for the studied morphological characters

CONCLUSIONS

The similarities of some morphological traits of the studied populations of different origins with the populations from the USA as well as a clear separation of some groups from the rest of the accessions supported the hypothesis that there was some different and repeated introduction of the wild *H. annuus* from its native places of origin.

Using the correlation analysis, which presented the nature and degree of association among various characters, further investigations could be made on the independent character with the aim of improving the dependent one. These results, together with the research carried out by Christov et al. (2004) and Encheva et al. (2006) on the resistance of these accessions to *Scleotinia sclerotiorum, Phomopsis helianthi* and other diseases and pests gave a brief view of the possibilities for inclusion of the studied accessions into future breeding programs.

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Weedy sunflowers in France: Prevalence and first inferences on their origin

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ABSTRACT

Sunflower is not a native of Europe. Weedy forms morphologically close to American wild *Helianthus annuus* have, however, been observed in France since 2004. In order to describe the situation and test hypotheses regarding their origin and evolution, we surveyed the infestation of sunflower fields by weedy sunflower in the Lauragais region (South of France) from 2005 to 2007, and described more precisely five weedy populations using morphological, agronomic and genetic descriptors. Weedy sunflowers affected around 15% of sunflower fields and caused yield losses that reached 50% in strongly infested patches. The five weedy populations surveyed were composed of a wide diversity of morphotypes showing an association of wild and domesticated traits in proportions that differed between populations. The genetic diversity was stronger in weedy populations than in volunteer populations and than in a pool of representative conventional and ornamental varieties. A wide diversity of original alleles was detected and their frequency varied between populations. All weedy plants carried the PET1-cytoplasm conferring male-sterility. These results suggest that weedy sunflowers may have arisen through the hybridization of cultivated and wild sunflower, either during the creation of improved inbred lines or during the seed production process; precursors of the weeds would have then been introduced through the seed lots. Until now, the only available methods to control these weeds are mechanical weeding at the very beginning of the infestation of a field. Studies are underway to get a better understanding of the formation and the spread of weedy populations in the agro-ecosystem.

Key words: genetic diversity – microsatellites – weeds – wild hybridization.

INTRODUCTION

Wild *Helianthus annuus* is a native of North America and does not occur naturally in Europe. However, weedy forms, showing morphological traits of wild sunflower (e.g. branching, anthocyanins in stem and disk, seed shattering) have been observed for decades in and around agricultural fields in Spain, Italy and Central Europe (Faure et al., 2002; Holec et al., 2005). They can compete with crops and are thus potentially problematic for cultivation. In North America, where weedy sunflowers also occur, they can decrease substantially yields of corn and soybean (Kane and Rieseberg, 2008). Moreover, the potential intercrossing between cultivated and weedy sunflower raises the question of the transfer of advantageous crop traits to the weeds (Mercer et al., 2007), which can contribute to the evolution of more aggressive weeds (case of Johnsongrass; Morrell et al., 2005). In 2004, patches of weedy sunflower within sunflower fields were officially observed for the first time in France, in the East of Toulouse. Interviews with farmers and a first survey revealed that they occurred in a consequent number of fields and in some cases for more than 10 years (Muller et al., 2006). However, they seemed to occur almost only within sunflower fields, and no other crops were strongly concerned. The main questions raised by these observations were: (i) what is the origin of the French weedy populations: are they issued from the reversion of the crop to weedy forms, through the evolution of volunteers, or from the introduction of original wild forms or crop-wild hybrids? (ii) What are the prevalence and dynamics of weedy sunflower in the agro-ecosystem in France? (iii) What impact do these weedy forms have on sunflower cultivation and how can they be controlled? To start answering these questions and make a first description of weedy sunflowers in France, we conducted studies across the agro-ecosystem and within cultivated fields, using morphological traits, and molecular markers.

MATERIALS AND METHODS

During summers 2005, 2006 and 2007, we followed the same roads every year over an area of approximately 1200 km² in the East of Toulouse, a region called Lauragais. Along these roads we

observed every sunflower field and scored the infestation level on the following scale: 0 (no weedy sunflower), 1 (less than 10 plants), 2 (a few patches) and 3 (very infested).

In summer 2006, we surveyed in detail 5 sunflower fields. These fields were selected at the beginning of July, as they showed a strong infestation with weeds (level 3). In 4 of these fields, an experiment was conducted to estimate the impact of weeds on yield. Weeds were manually destroyed on 6 rows of cultivated sunflower (weeded - 30m²) and the density of weeds in an adjacent area of the same size was estimated (infested). At the end of season, crop heads were manually harvested on the weeded and the infested plots, on 24m² only to avoid border effect. The seed production, seed weight and oil content of the crop were determined.

On 18 and 25th July, during the flowering time period of the weeds, 30 branched weeds were randomly chosen on each of the 5 fields. The following traits were scored: male-sterility and anthocyanin pigmentation of petiole, disk and stem. One head per plant was bagged before the opening of the first flower. At the end of August, bagged heads were collected, and on each of the surveyed plants, at least one open-pollinated head was collected for DNA analysis. The number of seeds produced by the bagged head was determined in the laboratory. A plant was scored as self-incompatible if less than 5 seeds had been produced, and as self-compatible in the other case.

In spring 2006, 6 open-pollinated seeds (achenes) per maternal plant were placed on filter paper and soaked in tap water. After 3 days at 25°C, the number of germinated seeds was counted. If no seed had germinated in a maternal family, we removed the seed coat and allowed two more days to germinate. We scored the need to remove the seed coat to obtain germination of at least one seed per maternal plant.

As a whole, 6 traits had been scored. For each trait and each population, the frequency of the wild type (e.g. anthocyanins in 3 locations, self-incompatibility and seed-coat removing) was computed. For male-sterility, we computed the frequency of male-sterile plants. The significance of differences in frequency across the set of populations was tested with a chi-square test on contingency tables. The correlation between traits within each population was investigated with exact test on contingency table. All tests were performed with SAS software (SAS Institute, Cary, NC, USA).

For molecular analyses, in addition to the 5 weedy populations described above (one open-pollinated seed per maternal family), we also included (i) two natural volunteer populations, one located in the Lauragais (FR006) and the other in the Gard (FR001). These populations occurred in fallows and exhibited only domesticated traits. Sampling was made of open-pollinated seeds taken from distinct maternal plants (9 for FR006, 44 for FR001). (ii) 18 conventional F_1-hybrid varieties historically or currently grown in the Lauragais region (iii) 6 ornamental varieties. Between 3 and 5 seeds per variety were analyzed. DNA was isolated from 100mg of young leaves with the standard protocol of Dneasy kit (Qiagen). Thirteen microsatellite markers from Tang et al. (2002) were used. The amplification reaction consisted of 50ng DNA, 4pmol of unlabelled reverse primer, 2pmol of forward primer, fluorescently labelled with NED, HEX or FAM, 1x reaction buffer, 2 mM MgCl2, 200µM dNTP, 0.15U Taq DNA polymerase, in a total volume of 25µL. The amplification method was as follows: 95°C for 2 minutes, 36 cycles of 94°C for 30s, Tx for 30s (Tx is initially 63°C and decreases of 1°C per cycle for the 6 first cycles, until it reaches 57°C), and 72°C for 45s; followed by a final extension for 20 minutes at 72°C. Amplification products were analyzed on an ABI 3130xl Genetic analyzer. The GENEMAPPER (Applied Biosystems) software was used to score the genotypes.

Standard statistics of genetic diversity were computed on groups of populations and per population: the number of detected alleles (*A*), unbiased genetic diversity H_e (Nei, 1987) and allelic richness (number of alleles standardized for the same sample size for each population, Petit et al. 1998), using the program FSTAT (Goudet, 2001). For each natural population (weeds and volunteers), the frequency of original alleles (e.g. absent from the conventional varieties) was computed.

Additionally, we used a polymerase chain reaction-based marker system that identifies sunflower plants carrying the mitochondrial DNA conferring male-sterility (PET1). PET1 is found in all male-sterile plants used for F1-hybrid seed production and thus in all F1-hybrid plants; it has been introduced from *H. petiolaris* and is theoretically absent from wild *H. annuus* (Rieseberg et al., 1994). We applied the protocol described in Rieseberg et al. (1994).

RESULTS

Field surveys over 3 years

In the Lauragais region, the frequency of sunflower fields infested with weedy sunflower varies between 22% in 2005 to 11% in 2006 (Fig. 1.). As the standard rotation in Lauragais is sunflower-wheat, comparison between 2005 and 2007 deals roughly with the same sunflower fields. However in 2005, the

frequency of fields with a low level of infestation seems to be an overestimation. Indeed, in this first year of the survey, sporadic volunteers may have been confused with weedy sunflower. Considering this, the prevalence of infested fields seems to have been stable. Between 1 and 4% of the fields were strongly infested. In these cases, the density of weeds could reach 15 plants/m² over some patches (Table 1).

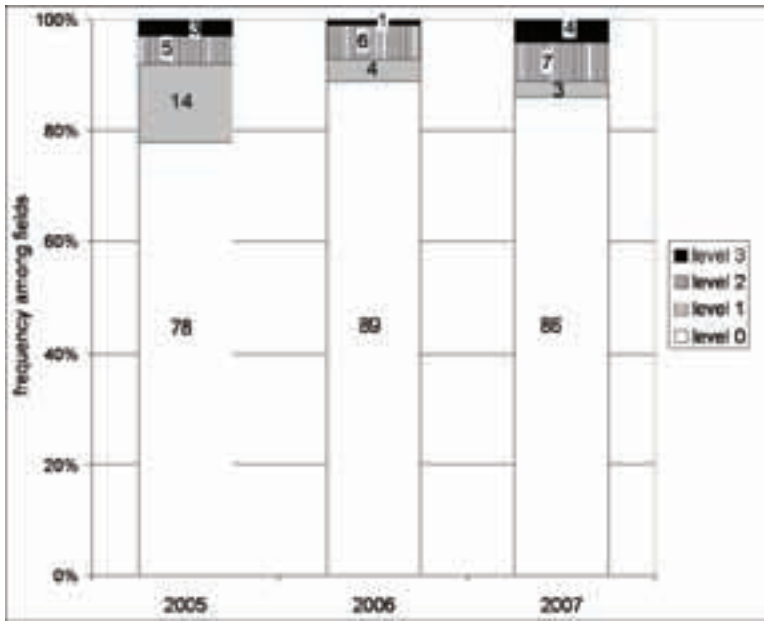

Fig. 1. Frequency of fields showing the different levels of infestation. The total number of fields surveyed for 2005, 2006 and 2007 were, respectively, 231, 227 and 196.

Impact on yield

High densities of weedy sunflowers greatly decreased crop yield (Table 1): Losses can reach more than 50%. These losses are due to a reduction in seed number and seed weight. But the seed oil content was unaffected. Visually, weedy sunflowers largely overtopped the crop. In strongly infested fields, harvest became impossible and some farmers have given up sunflower cultivation.

Table 1. Measures of the impact of weeds on sunflower cultivation.

Population	Weed density infested plot (plants/m²)	Yield infested plot (t/ha)	Yield weeded plot (t/ha)	Sw[1] Infested plot (g)	Sw[1] weeded plot (g)
Baziège	12	1.79	2.74	50.9	59.6
Gardouch	12	1.41	2.73	43.8	49.8
Fourquevaux	13	1.04	2.59	30.5	44.4
Odars	15	1.72	2.99	44.7	58.1

[1]Sw: weight of 1000 achenes

Morphological traits

The weedy forms are morphologically clearly different from the volunteers issued from the segregation of hybrid-F_1 varieties. Our observations confirmed that they exhibit traits typical of wild forms of *H. annuus*: pigmentation of disk, stems and petioles, self-incompatibility, and seed dormancy (Fig. 2). A trait more typical of a cultivated variety, although sometimes observed in wild populations, male-sterility, was also observed at a varying rate in the weedy populations. For 5 out of 6 traits, the frequency varied significantly between populations (chi-square). These variations were correlated between traits, namely Baziège was the population with the lowest frequency of wild-like traits for all traits, whereas Villefranche was the population with most wild phenotypes. This tendency was also observed for less quantifiable traits such as seed shattering, or amount of branching.

Significant correlations between traits were only observed between the scoring of anthocyans in different locations (stem, petioles and disk). No other correlation was detected, confirming the visual observation that all kinds of combination of traits were present in the fields. Weedy populations are

characterized by a high morphological diversity with plants combining in different proportions domesticated and wild traits, from typical F2 plants to typical wild-like phenotypes.

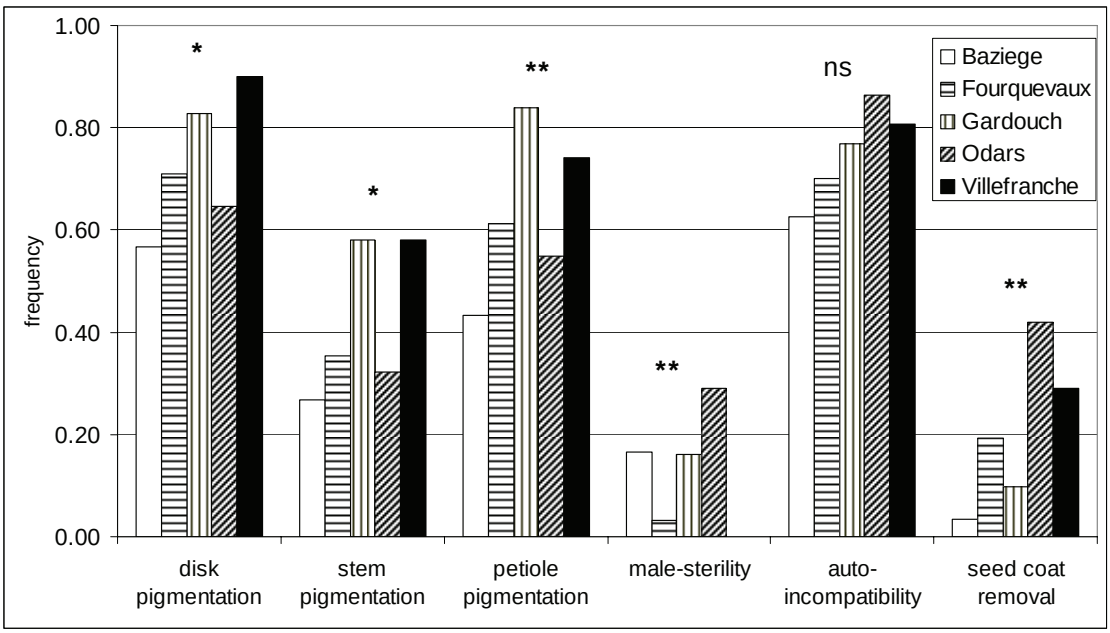

Fig. 2. Frequency of phenotypic traits in the different populations. Significance of frequency differences between populations: *: P<0.05 ; **: P<0.01 ; ns: not significant

The flowering time periods of cultivated and weedy sunflowers overlap, the first weeds starting flowering during the flowering of the crop. But the flowering time period of the weeds extends almost until harvest time.

Genetic analyses

The total genetic diversity was much greater over the weedy populations than in all the other groups analyzed: 137 different alleles were detected over 13 loci in the weedy populations, compared to 45 in the volunteer populations, 50 over the conventional varieties and 82 over the ornamental varieties. Many alleles were thus detected in the weeds, that were absent from the varieties. We call these alleles “original alleles” in the following. This pattern of genetic diversity is similar when comparing intrapopulation diversity between weedy and volunteer populations (Table 2).

Original alleles are relatively rare: their frequency within weedy populations varies from 7 to 23% (Table 2). The main alleles detected in the weeds are thus shared with the conventional varieties, and apart from the original alleles, the genetic composition of the weedy populations is rather similar to the pool of conventional varieties analyzed. Interestingly, in Villefranche, the population showing the most originality at the molecular level was the one that also exhibits more wild-like phenotypes, whereas in Baziège, showing the lowest frequency of wild-like phenotypes, it had a genotypic composition closer to that of the cultivated pool (Fig. 2, Table 2).

All weedy plants, conventional variety and volunteers analyzed carry the PET1-cytoplasm. Except one (SunrichF1), all ornamental varieties carried the fertile cytoplasm.

Table 2. Genetic diversity statistics within 5 weedy populations and 2 volunteer populations.

Accession	Baziège	Fourquevaux	Gardouch	Odars	Villefranche	FR001	FR006
Sample size	30	31	31	31	31	44	9
H_e	0.504	0.568	0.602	0.633	0.680	0.466	0.410
Allelic richness	3.34	3.97	3.89	4.21	4.81	2.36	2.43
Frequency of original alleles	0.072	0.127	0.163	0.165	0.232	0.002	0.009

DISCUSSION

This first description of weedy sunflower in France shows that it is a non negligible phenomenon. It affects around 15% of fields in the Lauragais region, and has also been observed in other areas in the South-West and West of France (unpublished results). Weedy plants can reach a locally high density and compete strongly with the crop leading to over 50% of loss of yield. Oleic acid content for high oleic varieties had also been shown to be affected when the field was strongly infested. This decrease was not detected at the single crop head level but on a sample of the whole harvest. It was thus probably due to the mixing of crop and weed seeds during harvest rather than to pollination by weed pollen (unpublished results). Seeds are dormant and can stay in the seed bank for many years (Alexander and Schrag, 2003). Some farmers have given up sunflower cultivation in strongly infested fields. Weedy sunflowers have also been observed in other spring crops such as sorghum, but never in winter crops such as wheat or oilseed rape.

Preliminary observations and interviews of farmers have revealed that the infestation level within a field generally increases from one generation to the other; this is due to the high seed production of weeds and to the fact that seeds are dispersed at maturity and fall readily on the soil compared to the seeds produced by the crop. By contrast, the dispersion of weeds to adjacent fields seems more restricted: quite often, a sharp contrast is observed between a strongly infested field and a neighbouring perfectly clean one. This suggests that seed and pollen flow from field to field is either rare or not sufficiently efficient compared to the spread of a weedy population within a given field. A more thorough survey of the infestation of a network of fields over time is underway: the results after a few years may give precise answers to the potential spread of weedy populations across the agro-ecosystem.

Morphologic and genetic data suggest that the origin of these populations is the hybridization between crops and wild plants. To be specific, as all weedy plants analyzed carry the PET1-cytoplasm, they all have in their maternal lineage a cultivated plant. This hybridization may for instance have occurred in seed-producing fields, when wild *H. annuus* occurs at proximity and can pollinate the female line. As the number of original alleles per locus is high, the diversity of wild forms involved in this origin is probably strong (more than a few plants are probably involved). But crop-wild hybridization also occurs in sunflower breeding, when wild genetic resources are used to introduce valuable traits into cultivated lines. The use of lines insufficiently inbred in the F_1-variety creation process could have led to varieties containing wild genetic variability. Rare remaining wild traits could have constituted a starting point for later weed evolution in a field. More detailed analyses are required to attempt to discriminate between these hypotheses and to infer if weedy sunflower evolved only once or multiple times within a region and over the different French areas.

As the frequency of cultivated-like alleles is strong, and as the combinations of phenotypic characters are important, a lot of crop-weed intercrossing may have occurred in the history of these populations. Further genetic analyses are planned to precisely estimate the rate of crop-weed hybridization within a field and the rate of pollen and seed dispersal between adjacent or more distant fields. Understanding in more details the process of formation and spread of weedy populations both at the field and at the agro-ecosystem levels is important to be able to predict the risk of establishment of new populations.

Methods to control these weeds are currently very limited. As weedy sunflowers affect cultivated sunflower fields, the methods used to control the weed may have an impact on the crop itself. Varieties resistant to herbicides such as imidazolinone and sulfonylurea are currently used in other countries. These herbicides could be used to eradicate weedy sunflowers in fields cultivated with resistant varieties. However, the risk of crop-weed gene flow and of transfer of the resistance to the weed has to be considered (Massinga et al., 2003). The only method that can now be advised to farmers is thus mechanical weeding: as soon as the first weeds are observed on a field, they have to be destroyed, before they produce seeds and give rise to a bigger population which is more difficult to control.

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Characterization of hybrids from crosses between cultivated *Helianthus annuus* L. and subspecies *rydbergii* (Britton) Long of perennial diploid *Helianthus nuttallii*

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ABSTRACT

The subspecies *rydbergii* (Britton) Long of the perennial diploid species *Helianthus nuttallii* was included in hybridization with the cultivated sunflower *Helianthus annuus* L. The investigation encompassed the period 1999-2007. *H. nuttallii* ssp. *rydbergii* could be crossed with the cultivated sunflower, but hybridization was difficult and the crossability rate was low. Seeds were obtained at both directions of crossing and hybrid plants - from the direct crosses. All F_1 plants showed an annual growth cycle. The heritability in first generation was intermediate but the plants strongly resembled the wild species in their most important biomorphological traits. The polymorphism between *H. annuus*, *H. nuttallii* spp. *rydbergii* and their F_1 hybrids was studied by RAPD. The F_1 plants were also cytologically investigated. From *H. nuttallii* ssp. *rydbergii* were transferred in F_1 genes that controlled such characters as, period of vegetation, plant height, type of branching, size and form of inflorescence and seeds, degree of anthocyanin coloration, seed oil content (60.84%), resistance to *Plasmopara helianthi*, races 300 and 700, *Phomopsis helianthi*, *Phoma macdonaldii* and *Orobanche cumana*. It was established that the subspecies was a source of *Rf* gene for CMS Pet-1 and the control was dominant and monogenic. As a result of self-pollination, sib-pollination of the F_1 plants and back-crossing with cultivated sunflower, F_2, BC_1 were obtained. F_3 - F_8 and next to F_6BC_1 generations were produced. Some of the obtained hybrid forms were included in a program for developing lines for heterosis breeding in sunflower. The hybrids with the perennial *H. nuttallii* ssp. *rydbergii* constitute a real contribution to interspecific hybridization because successful hybridization with this wild species had not yet been reported.

Key words: *Helianthus nuttallii* spp. *rydbergii* – interspecific hybridization – RAPD – sunflower.

INTRODUCTION

H. nuttallii Torrey and Gray. is a perennial diploid (2n = 34) species, belonging to section *Divaricati*, series *Corona Solis* (Schilling and Heiser, 1981). It has three subspecies: *H. nuttallii* ssp. *nuttallii* Torrey and Gray, *H. nuttallii* ssp. *parishii* (Gray) Heiser and *H. nuttallii* ssp. *rydbergii* (Britton) Long (Rogers et al., 1982). The hybridization of cultivated sunflower with diploid perennial species in comparison to diploid annual species *Helianthus* is difficult (Christov, 1991, 1996; Seiler and Rieseberg, 1997). No information regarding the obtaining of successful interspecific hybridization between *H. nuttallii* ssp. *rydbergii* and the cultivated sunflower had hitherto been found.

MATERIALS AND METHODS

The investigation encompassed the period 1999-2007. The subspecies *rydbergii* (GT-M-173) of the perennial species *H. nuttallii* was included in hybridization with cultivated sunflower *H. annuus* L. Hybridization was carried out through reciprocal crosses realized under field conditions. The sterile analogues of lines 2607, 6075, HA89 and HA402 (cytoplasmic male sterile lines in CMS PET-1) were used as female parent of the cultivated sunflower in direct crosses. In the reciprocal crosses, the florets in the inflorescences of the wild species were castrated manually and were pollinated with pollen from line 2607B. To obtain F_2 and BC_1, self-pollination, sib-pollination and back-crossing of F_1 to cultivated sunflower were made. Phenological observations of the F_1 hybrids and the next hybrid generations were conducted during the vegetation period. Biometric parameters and description of the main morphologic characters and biologic peculiarities of all F_1 hybrids were performed. The seed set (the number of inseminated disk florets) was calculated as a ratio between the seeds obtained and total number of disk florets in the inflorescence. 1000 seed weight was calculated by measuring two samples, each of 10, 25 or 50 seeds. Back-crossing with cultivated sunflower as a mother was used with the aim to confirm the presence of fertility restorer genes (*Rf* genes) in F_1 hybrids transferred from species *H. nuttallii* ssp. *rydbergii*. The reactions to diseases were studied using standard methodologies (Acimovič, 1979; Vear and Tourvieille, 1987; Encheva and Kiryakov, 2002). The seed oil content was determined by nuclear

magnetic resonance (NMR). Cytological analyses were carried out on the meiosis of pollen mother cells (PMC) according to Georgieva-Todorova (1976). Pollen viability was determined by a standard methodology (Atlagic, 1990). RAPD analyses were performed in order to determine the hybrid nature of the new F_1 forms. Total DNA was isolated from the youngest sunflower leaves by the method of Dellaporta et al. (1983) with some modifications. Kits for PCR analyses (Ready To Go PCR Beads, Amersham Pharmacia Biotech Inc.) and for amplification of random DNA sequence, RAPD decamer primers from Operon Technologies, USA: OPA-01, OPA-02, OPB-01 and OPB-07 were used. PCR program was: 5 min. in temperature 95^0C; 45 cycles of 1 min in 95^0C, 1 min in 36^0C and 2 min in 72^0C; 5 min in 72^0C. DNA marker 50 *bp* Amersham Biosciences, USA was used.

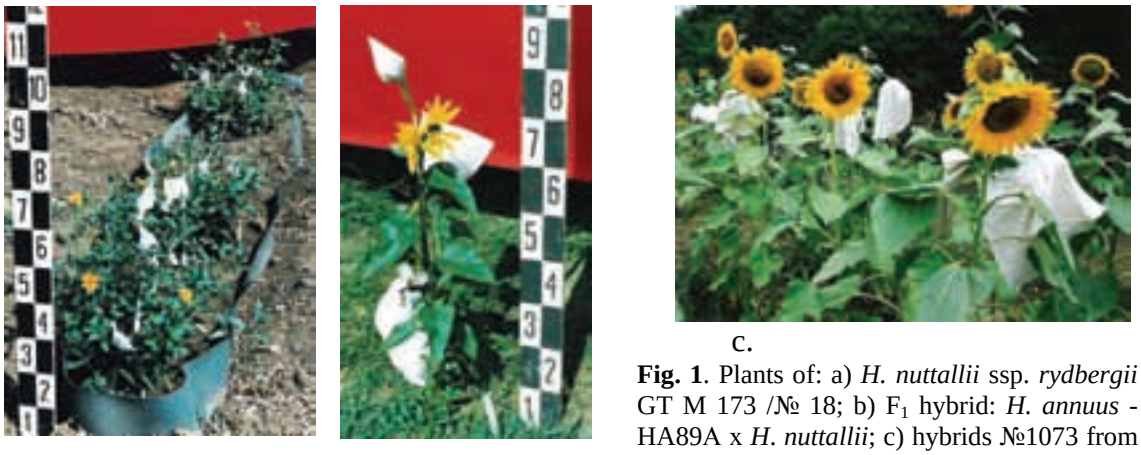
RESULTS

The analysis of the results presented in Table 1 showed that the *H. nuttallii* ssp. *rydbergii* could be successfully crossed with *H. annuus*. The percentage of successful crosses (inseminated heads) of the direct crosses was from 72.7 to 80.0 % and of the reciprocal crosses - 58.3 %. In the combination *H. annuus* x *H. nuttallii* ssp. *rydbergii* 15 seeds and 4 F_1 hybrids and in the combination *H. nuttallii* ssp. *rydbergii* x *H. annuus* 28 seeds and any hybrid plant were obtained.

Table 1. Crossability of cultivated sunflower *H. annuus* and wild perennial *H. nuttallii* ssp. *rydbergii*.

Crosses	Pollinated inflorescences			Insemination inflorescence, %	Total number seeds	Hybrid plants	
	total number	with seed					
		number	%			number	%
H. annuus x *H. nuttallii*	24	18	75.00	0.47	113	36	32.99
H. nuttallii x *H. annuus*	12	7	58.33	3.31	28	0	0

The F_1 hybrid (Fig. 1a) had an annual growth habit and a vegetation period similar to that of cultivated sunflower, in contrast to the wild perennials (Fig. 1b, Table 2). All F_1 plants had anthocyanin coloration, especially at cotyledon phase and along the stem. The intensity was similar to that of wild species. In cultivated sunflower anthocyanin coloration was absent. Its presence in hybrids proved the transfer of genetic material from *H. nuttallii* ssp. *rydbergii* to the genotype of the F_1 hybrids and was a suitable morphological marker.



a. b. c.

Fig. 1. Plants of: a) *H. nuttallii* ssp. *rydbergii* GT M 173 /№ 18; b) F_1 hybrid: *H. annuus* - HA89A x *H. nuttallii*; c) hybrids №1073 from cross 2607B x F_1 (HA 89A x *H. nuttallii*).

RAPD analyses of F_1 plants and their parents were performed to prove their hybrid nature. The analysis was carried out on those fragments that were well visible (Fig. 2). Out of 4 primers used for amplification of the parents and the hybrid, all showed specific fragments for the wild or cultivated species and the hybrid and fragments represented in the three genotypes. The comparison of the amplification profiles of F_1 plants and the father genotype was based on the presence or absence of the fragments. Primer OPA-01 allowed to amplify two specific fragments for the wild species and the hybrid (size: 400 *bp* and around1300 *bp*), the primer OPA-02 - specific fragment with size over 1300 *bp*, the

Table 2. Characteristics of parents and F_1 hybrids.

Characters	*H. annuus* line HA89 A	F_1: *H. annuus* - HA89A x *H. nuttallii* ssp. *rydbergii*	*H. nuttallii* ssp. *rydbergii* M 173
Physiological development			
Life cycle	annual	annual	perennial
Period of vegetation, day	114	132	195
Flowering central head, day	9 - 10	6 - 8	6 - 7
Flowering full plant, day		36	59
Morphological characters			
Hypocotyl			
Anthocyanin coloration	absent	medium	medium
Stem			
Plant height, *cm*	105 - 110	90 - 150	70 - 140
Branched	absent	in top half-past	in top half-past
Position of lateral heads		below	below
Number of branches	0	6 - 12	9 - 37
Coloration	green	dark green with anthocyan	dark green with anthocyan
Intensity of hairiness	weak	medium	medium
Leaf			
Number of leaves	24 - 28	32 - 39	74 - 81
Length of leaves, *cm*	25 - 27	13 - 18	11 - 12
Width of leaves, *cm*	24 - 25	6 - 9	4 - 5
Shape	cordate	oblong-cordate	lanceolate wide to oval
Coloration	green	green	dark green
Serration of entire	medium	medium	fine
Length of leaf petiole, *cm*	12 - 15	2 - 4	1
Anthocyanin coloration	absent	medium	medium
Inflorescence			
Attitude of the central head	half-turned down	half-turned down	half-turned down
Shape of grain side	convex	convex	convex
Head diameter, *cm*	22 - 25	7 - 12	1.6 - 1.9
Number of bracts	52	16 - 19	29
Coloration	green	dark green	dark green
Number of ray flowers	36	11 - 19	21
Coloration	yellow	orange-yellow	orange-yellow
Shape	ovate	ovate	ovate
Number of disk flowers	1211 - 1465	91 - 152	86 - 97
Disk flowers color	yellow	purple	purple
Anthocyan of stigma	absent	medium	medium
Coloration of pollen	yellow	orange	orange
Seed			
Friability	absent	at full ripeness	at full ripeness
Length/width/thickness, *cm*	1.1 / 0.7 / 0.4	0.9 / 0.4 / 0.3	0.5 / 0.2 / 0.1
Size	medium	small	small
Shape	ovate wide	ovate wide	ovate
Trichomes	very weak	weak	medium
Main color	gray-black	gray-brown / black	gray-brown
Technological characters			
Seeds after self-pollination	0	3 - 6	0 - 1
Insemination:			
- self-pollination, %	0	2.48 - 4.80	0 - 1.09
- free pollination, %	71.5	0.83 - 15.70	38.5
1000 seed weight, *g*	57.6	x	5.9
Oil, %	49.8	x	31.2

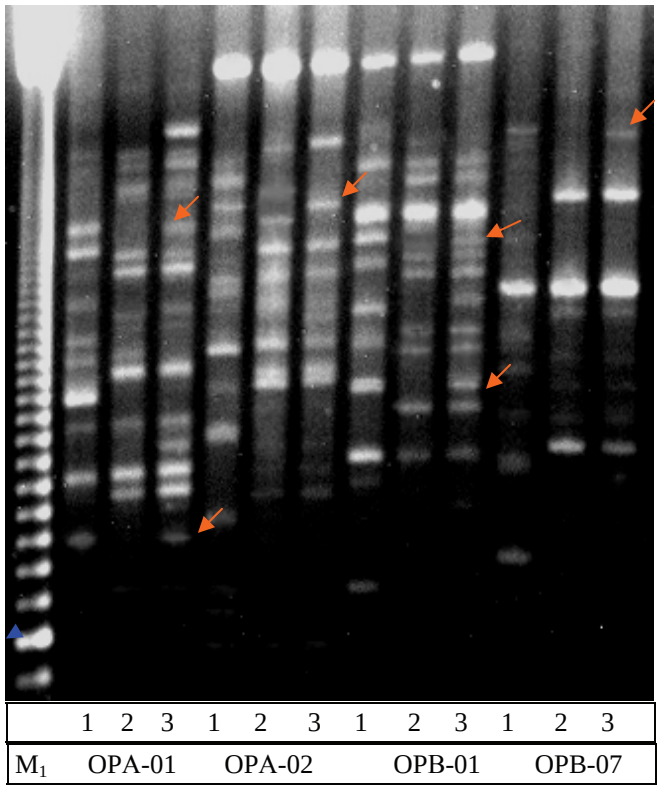


Fig. 2. Electrophoresis spectra of amplification profiles of the three genotypes with OPA-01, OPA-02, OPB-01 and OPB-07 primers.

1. *H. nuttallii* ssp. *rydbergii* (M 173)

2. *H. annuus* - line 6075

3. F_1 *H. annuus* - line 6075 x *H. nuttallii* ssp. *rydbergii*

primer OPB-01 – two specific fragments with size 750 *bp* and 1300 *bp* and the primer OPB-07 - one specific fragment with size over 1300 *bp*. The results confirmed that there was a polymorphism in the amplification PCR profiles of *H. annuus*, *H. nuttallii* and *H. annuus* x *H. nuttallii ssp. rydbergii*, i.e. the RAPD analysis confirmed the hybrid nature of the F_1 material obtained from crosses between the line 6075 and the wild species.

All F_1 hybrids had erect and branched stems, colored from dark green to dark anthocyanin and were covered with prickly hairs (Table 2). The branches were situated in the center and the top of the stem, with the exception of 3 out of 4 plants from cross *H. annuus* (HA89A) x *H. nuttallii* ssp. *rydbergii*, with branches and in lower half-stem (Fig. 1a). The size of the inflorescence in F_1 was intermediate and the number of disk florets - similar to those of *H. nuttallii* ssp. *rydbergii*. The disk florets and the stigmas were colored dark purple, and pollen and ray florets were orange. These characters were typical of the wild species. The number of bracts in F_1 was smaller than that in the two parents. The bracts were colored dark green like the male parent form. All F_1 plants with four different sterile analogues were male fertile. The mean percentage of pollen viability of F_1 hybrids was low (31.62%: from 26.38 to 34.96%) and three times lower than the value of *H. nuttallii* ssp. *rydbergii* (92.73%: from 87.62 to 97.32%).

The meiosis in *H. annuus* - line HA89B was normal, and in *H. nuttallii* ssp. *rydbergii* with few aberrances. In the greater part of the cells in diakinesis the chromosomes conjugated completely with 17 bivalents: open from 2 to 7 and closed from 10 to 15 were recorded. Cells with 1 to 3 “X” bivalents were also recorded. Single cells were 15-16 bivalents and 2 univalents or 1 quadrivalent. The cells in metaphase I with aberrances - lagging chromosome were 0.96%. In anaphase I 1.67% there were cells with chromosome bridges and lagging chromosome. The tetrads were observed during telophase II. Reduction division of PMC in the F_1 plants occurred with deviations. Cells with 17 bivalents, cells with 14-16 bivalents and 2-6 univalents and cells with 11-13 bivalents and 2-3 quadrivalents were recorded in diakinesis. The meiotic analyses showed that the mean frequency of closed bivalents per cell in F_1 hybrids was 1.88 and was much lower than the value for *H. annuus* (6.20) and for the wild species (12.30). The highest mean frequency of chiasmata per cell was registered for *H. nuttallii* ssp. *rydbergii* (28.80). It was lower for cultivated sunflower (23.20) and lowest (19.21) for the hybrid. The mean frequency of chiasmata per bivalent for hybrids was lower than that of the parents. The values were 1.13 for F_1, 1.36 for *H. annuus* and 1.69 for wild species. In metaphase I there were cells with 2 to 5 fast chromosomes, which were located on one or two sides of the lamella. In the F_1 hybrid there were cells with non-included chromosomes during anaphase I. The number of lagging chromosomes was up to 8. Cells with 2

chromosome bridges and fragments were recorded. Cells with non-regular distribution during telophase I were observed (19:15), and during telophase II - tetrads, tetrads with up to 6 lagging chromosomes, and tetrads with one micronucleus. It is interesting to note that the different phases of meiosis occurred simultaneously in the same preparation, in the same field of vision. This fact showed the non-synchronous occurrence of the meiosis.

As a result of self-pollination and sib-pollination of the central inflorescences of 3 F_1 plants from the cross *H. annuus* line HA 89A x *H. nuttallii* ssp. *Rydbergii*, a total of 21 seeds were obtained, and from the crosses of lines HA 402A, 6075A and 2607A with *H. nuttallii* ssp. *Rydbergii*, a total of 73 seeds after self-pollination. Seed color of F_1 was from gray-brown to black. The seed shape was similar to that of cultivated sunflower, and its size to that of wild species. This is a negative character typical of the wild sunflower species. The number of obtained F_2 plants was 81.13 F_2, all originating from cross HA 89A x *H. nuttallii*, 17 from cross 6075A x *H. nuttallii*, 32 from HA 402A x *H. nuttallii* and 19 from 2607A x *H. nuttallii*. The observed diversity among the F_2 plants was a result of the segregation of the following characters: vegetation period, plant height, type of branching, size of branches, size and shape of leaves, inflorescence and seeds, degree of anthocyanin coloration, oil content in seed (Table 3). Twenty-six out of all 81 F_2 plants were branched along the entire stem, 36 plants had branches in the middle and at the upper half of the stem, and 19 plants had branches near the top. Of all the F_2 plants, 21 were male sterile - 3 F_2 from cross HA 89A x *H. nuttallii*, 9 from HA 402A x *H. nuttallii*, 4 from 6075A x *H. nuttallii* and 5 from 2607A x *H. nuttallii*. The segregation of the characters fertility/sterility was in a correlation close to 3:1 fertile:sterile (Table 4), and in the above groups of crosses the ratio was 10:3; 23:9; 13:4 and 14:5, respectively. The inflorescence of all male fertile F_2 plants (a total of 60) was self-pollinated. Seed color was from gray-brown to anthocyanin-black.

Table 3. Characteristics of F_2, BC_1, F_3, F_1, BC_1 and F_4 hybrids.

Characters	*H. annuus* - HA 89A x *H. nuttallii* ssp. *rydbergii*				
	F_2	BC_1	F_3	F_1BC_1	F_4
Physiological development					
Period of vegetation, day	139	131	118 - 124	113 – 116	115 - 117
Morphological characteristics					
Plant height, *cm*	150 - 180	160 - 220	140 - 180	140 – 160	130 - 150
Number of branches	4 - 9	4 - 8			
Head diameter, *cm*	16	18	15 - 16	16 – 20	15 - 16
Technological characteristics					
1000 seeds weight, *g*	42.2	43.4	43.1	43.6	42.8
Oil %	39.8 - 44.5	48.2 - 52.9	46.5 - 53.0	47.1 - 50.4	49.9 - 53.6

Table 4. χ^2 for F_1, F_2 and BC_1 generations.

Generation	Number of plants		Expected ratio	χ^2*
	fertile	sterile		
F_1	36	0	- (Rfrf)	0
F_2	60	21	3:1 (Rf- : rfrf)	0.037
BC_1	81	76	1:1 (Rfrf : rfrf)	0.159

* χ^2 at level of significance 0.05, 0.01 and 0.001 was 3.841, 6.635 and 10,827.

The total number of BC_1 plants was 234 and all were branched (Table 3). Seed color of BC_1 plants was from dark brown to black. All 77 BC_1 plants from the combination 2607B x F_1 (HA 89A x *H. nuttallii*) were with normal male fertility. About 50 % of BC_1 plants were with normal cytoplasm and the recessive gene *rf* and about 50% with normal cytoplasm and the dominant gene *Rf*. B lines with important characters transferred from *H. nuttallii* ssp. *rydbergii* can be produced from the hybrids with normal cytoplasm and a recessive gene. The hybrid forms with dominant genes were suitable for developing R lines. These BC_1 plants were morphologically similar to the plants from the combination 2607A x F_1 (HA 89A x *H. nuttallii*); from the latter cross 45 fertile and 40 male sterile plants were produced. For cross combinations 2607A x F_1 (6075A x *H. nuttallii*) and 2607A x F_1 (HA 402A x *H. nuttallii*), the number of fertile and male sterile plants was 15:13 and 21:23, respectively. The ratio fertile/sterile BC_1 plants was almost 1:1 (81:76, Table 4).

All F_1 plants *H. annuus* x *H. nuttallii* with four different sterile analogues were male fertile. The presence of fertile F_1 plants indicated that in the genotype of subspecies *rydbergii* there were genes that

controlled restoration of male fertility for CMS Pet-1. Restored genes were transferred from *H. nuttallii* to the hybrid material. In wild species the *Rf* genes were probably homozygous because all F_1 plants were fertile in the formula: S *rfrf* (male sterile) x N *RfRf* (fertile) = S *Rfrf* (fertile), where in the nucleus of the plants from the sterile line the genes controlling the restoration of male fertility were only recessive - *rfrf*, and in *H. nuttallii* ssp. *rydbergii* - only dominant, *RfRf*. The F_1 progeny was fertile and heterozygous. The segregation of the characters fertility/sterility in the F_2 plants was at a ratio close to 3:1 fertile:sterile, and in the BC_1 plants - close to 1:1 (Table 4). Back-crossing with cultivated sunflower as a mother was used with the aim of confirming the presence of *Rf* genes in F_1 hybrids, transferred from species *H. nuttallii* ssp. *rydbergii*. B lines can be developed from the new forms obtained. The value of χ^2 in F_2 and BC_1 generations was lower than the level of significance 5 % (3.841). That determined the accidental nature of the differences between the observed and expected value. This result showed that *Rf* genes from *H. nuttallii* ssp. *rydbergii* were transferred in *H. annuus* and the control for recovery of male fertility at CMS PET-1 was dominant and monogenic.

F_3 - F_8 and next to F_6BC_1 generations were produced. As a result of the selection, new forms suitable for R and B lines were developed. Genes were transferred from *H. nuttallii* ssp. *rydbergii* that controlled such characters as: 100 % resistance to *Plasmopara helianthi*, races 300 and 700 (№ 990, №2505, №2558 and №2559), *Phomopsis helianthi* (№990, №1073, №2497, №2505 and №2558), *Phoma macdonaldii* (№2505) and *Orobanche cumana* (№955, №1066 and №1073), *Rf* gene for CMS Pet-1, suitable type of branching for R lines, high oil content (as in №991 - 53.43 % and №2505 - 60.84 %) and high combining ability.

DISCUSSION

H. nuttallii ssp. *rydbergii* could be crossed with the cultivated sunflower, but hybridization was difficult. The hybrids with this perennial diploid species were a real contribution to interspecific hybridization because successful hybridization with this wild species had not been reported up to now.

ACKNOWLEDGEMENTS

We would like to thank Dr. Seiler who gave us the possibility to work with the wild species *H. nuttallii* ssp. *rydbergii*, which led to the successful production of the new hybrids described in this paper.

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Preventing botanical contamination risk of sunflower hybrid seed in the Valle Bonaerense del Río Colorado, Argentina

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ABSTRACT

River Colorado Valley in southern Buenos Aires province (VBRC) is the main sunflower seed production area in Argentina, with over 8000 hectares, standing for 85% of the controlled fields over the country. The wild annual species *Helianthus annuus* and *H. petiolaris* naturalized in Argentina can pollinate crop plants giving fertile progeny. As in the US and in Europe, the presence of these species limits seed production because they represent a pollen contamination source. However, at present none of them have invaded the VBRC area. An ecological characterization was performed in order to assess the invasiveness of the VBRC. To test agro-ecological dissimilarity, habitats of both wild species in Argentina and the center of origin were compared using macro-abiotic (climatic and soil taxa), micro-abiotic (soil composition), and biotic (plant community) variables. Ecological conditions were found not to restrict the establishment of wild *Helianthus* invaders in the VBRC. An active alertness was initiated, comprising an exhaustive cleaning of machinery coming from other regions, roguing for off-type plants within seed production fields, to avoid annual *Helianthus* species culture for ornamental purposes, and removal of all feral forms of annual *Helianthus* within the protected region.

Key words: ferality – *Helianthus annuus* – *H. petiolaris* – invasiveness – pollination – wild sunflower.

INTRODUCTION

The potential of the River Colorado Valley in southern Buenos Aires province (VBRC) for sunflower production has been known since the 1980s (Hernandez and Orioli, 1982; Rivas et al., 1987) however it was not profitable until some crop constraints - such as parakeet (*Cyanoliseus patagonus*) damage - were overcome through an increment of acreage and early sowing. The valley comprises ca. 90,000 hectares with gravitational watering devoted to onion and forage crops. Sunflower seed production comprises 10% of valley acreage that constitutes the main sunflower seed production area in Argentina, this being more than 85% of the controlled fields over the country (INASE, 2007).

Two wild annual species, *Helianthus annuus* and *H. petiolaris* have established themselves in Argentina on a transitional area between the Pampean steppe and Espinal phyto-geographical provinces (Burkart et al., 1999). *H. petiolaris* invaded sandy soils, usually degraded by wind erosion, whereas wild *H. annuus* has spread on more fine-textured soils, with hydric constraints such as flooding and salinity (Cantamutto et al., 2008). Their migration followed land moving routes, probably facilitated by transportation and machinery although the location of *H. annuus* populations in the irrigated areas of Mendoza and San Juan (Poverene et al., 2002) seems to demonstrate that seeds have moved through the irrigation channels. Stable populations of both species were located 200 km north from the VBRC (Fig. 1) but isolated plants were frequently observed in the surroundings of Bahía Blanca harbor complex, at 100 km of distance from the valley (unpublished data).

The annual *Helianthus* species established in Argentina can pollinate crop plants giving fertile progeny (Poverene et al., 2004; Ureta et al., 2008). *H. annuus* (Deines et al., 2004; Rosales-Robles et al., 2005) and *H. petiolaris* (Grichar et al., 2004) are also crop weeds. In the US, the presence of these species in roadsides, slopes, and fireguards limits seed production because they are a pollen contamination source (Anfinrud, 1997). Wild contaminant and off-type seed imported from invaded areas has been suggested as being the origin of Europeean wild sunflower populations (Bervillé et al., 2005; Vischi et al., 2006; Muller et al., 2007) and a study of their expansion trend has been recommended (Stankovic-Kalezic et al., 2007).

The use of beehives to improve seed production is frequent in the VBRC, though this practice represents a threat to seed purity because bees often visit wild flowers in the vicinity, outside crop fields (Andrada et al., 2004). Given that most inbred lines used for commercial hybrids production flower at the same time as wild invaders, undesired pollination risk in the VBRC would be very high.

There are a few known cases where it has been possible to anticipate and avoid a biological invasion. A proper environmental management is proposed herein with prevention rules in order to characterize the VBRC and prevent the establishment of the two annual *Helianthus* species already present in other Argentine regions. This would place a severe constraint on sunflower seed production, threatened by wild sunflower gene flow. It would not only affect seed inspection by increasing seed lots and field rejection, but also would be a risk for other regions in the country not yet invaded, due to contaminated sunflower hybrid seed usage, which could originate new feral populations.

To test agro-ecological dissimilarity, habitats of both wild species in Argentina and the center of origin were compared using three analysis scales. If available habitats in the VBRC measured by each scale were different to those colonized habitats in other regions, the null hypothesis of similar environment would be rejected, and there would be no risk of invasion. If not, it would have to be accepted that the two wild *Helianthus* species could invade the VBRC and the employment of prevention rules would be worthwhile.

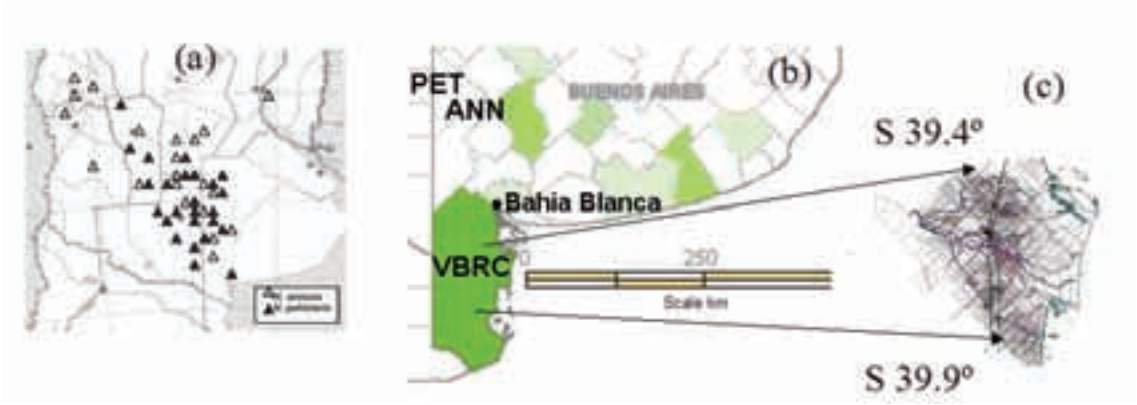

Fig. 1. Distribution (a) and nearest stable populations of *Helianthus petiolaris* (PET) and wild *H. annuus* (ANN) (b) with respect to the VBRC region (c). Shaded counties in Buenos Aires province are devoted to sunflower seed production in Argentina (Maps from Poverene et al., 2002, INASE and CORFO).

MATERIALS AND METHODS

Argentinean habitats were characterized through a representative sample of nine *H. annuus* and 13 *H. petiolaris* stable populations, geo-referenced by Poverene et al. (2002). The macro-abiotic variables altitude, annual rainfall, hottest month mean temperature, coldest month mean temperature, and soil taxa were obtained from De Fina (1992) and INTA (1990). Micro-abiotic conditions were estimated by soil composition within each habitat, according to methods described in Cantamutto et al. (2008). Plant community was characterized through a semi-quantitative technique in February 2007. Data were collected in each collection site from 10 points on a uniformly spaced grid coordinate system. At each grid point (a 2 m^2 circle), abundance was visually qualified from 0 (absent) to 5 (very abundant).

Climate variables in the center of origin corresponded to 49 *H. annuus* and 19 *H. petiolaris* populations registered in the GRIN-USDA (www.ars-grin.gov2/cgi-bin/npgs/html) representative of the different states where germplasm has been collected in the Northern Hemisphere. Climate parameters were taken from the www.worldclimate.com site, for series of more than 20 years obtained from meteorological stations located 4.3 ± 3.9 km and 8.4 ± 6.5 km far away from *H. annuus* and *H. petiolaris* populations, respectively.

Macro-abiotic climate variables for VBRC were estimated in four localities (Mayor Buratovich, Pedro Luro, Hilario Ascasubi, and Villalonga) using De Fina (1992). Parameters of soil taxa and soil composition of representative cartographic units were taken from INTA (1990). Plant community on roadsides was estimated as previously described, in 10 points over a transect between Mayor Buratovich and Villalonga (ca. 60 km) in March 2007.

On the macro and micro-abiotic scales, variables were compared through the non-parametric Kruskal-Wallis test and graphical analysis. Soil similarity was estimated through the frequency of *H. annuus* and *H. petiolaris* population occurrence in soil units (taxa) also present in the VBRC. On the biotic scale, plant community of Argentine habitats where *H. petiolaris* and *H. annuus* have established

themselves, was compared to that of the VBRC through contingency tables. All the analyses were performed with the InfoStat (2002) statistics package.

RESULTS AND DISCUSSION

Macro-environment geographical and climate conditions would not be a restrictive factor for the establishment of wild *Helianthus* invaders in the VBRC. The valley is located at a latitude that almost corresponds to the mean value of the latitudinal distribution range in the US. The VBRC latitude did not differ from those recorded for both species in the center of origin, but it was different from *H. annuus* in Argentina (Fig. 2) probably because the invasion began in the northern part of the country, though it progressed towards the South (Cantamutto et al., 2008). The climate variables did not differentiate the habitats of VBRC and the US either. Although the habitat altitude is different for *H. annuus* environments - given that in the US this species grows even below sea level - the VBRC does not seem to have any altitude restriction.

Soil cartographic units did not show differences on a macro-abiotic scale. There are 15 soil groups in the VBRC, among which Mollisols and Entisols predominate. These orders were found to be closely associated with both annual *Helianthus* populations in Argentina and in the US (Cantamutto et al., 2008). More than one fifth of *H. annuus* and *H. petiolaris* populations in Argentina were associated with soil taxa present in the VBRC. Given that soil taxonomy can be used as an ecosystem processes index (Mann et al., 1999; Bouma, 2003) the taxa associated with Argentinian populations would show that soil genesis processes taking place in the VBRC are similar to those in other invaded regions elsewhere in the country.

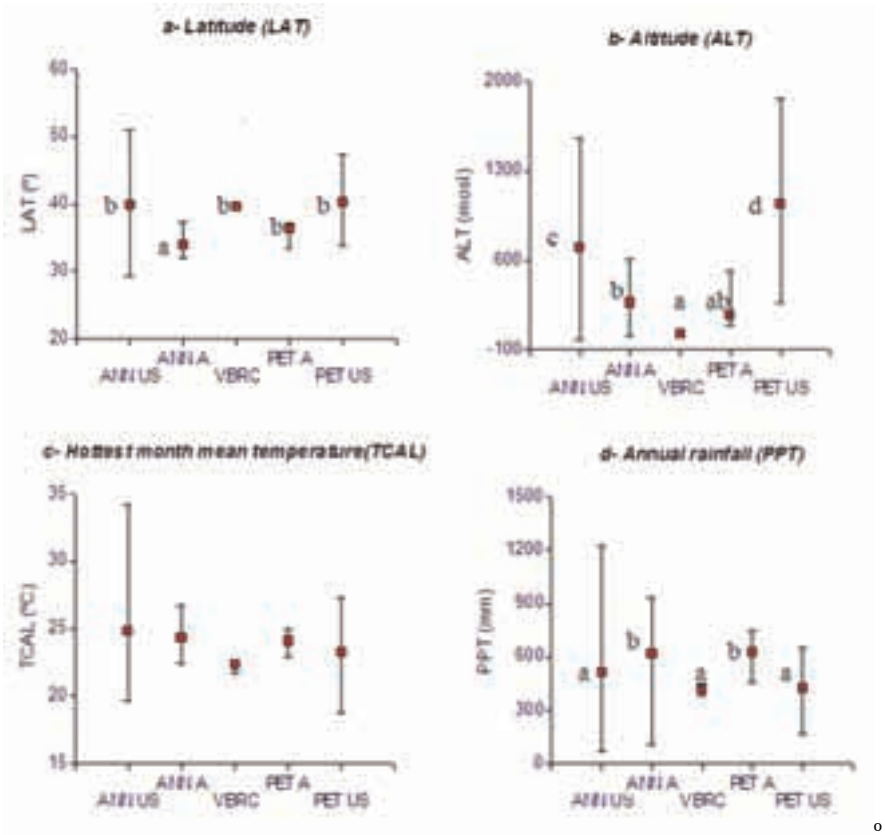

Fig. 2. Geographical and climatic distribution (mean, range) of *Helianthus annuus* (ANN) and *H. petiolaris* (PET) populations in North America (US) and Argentina (A) compared to that observed in River Colorado Valley in Buenos Aires province (VBRC). Means significantly different are followed by different letters.

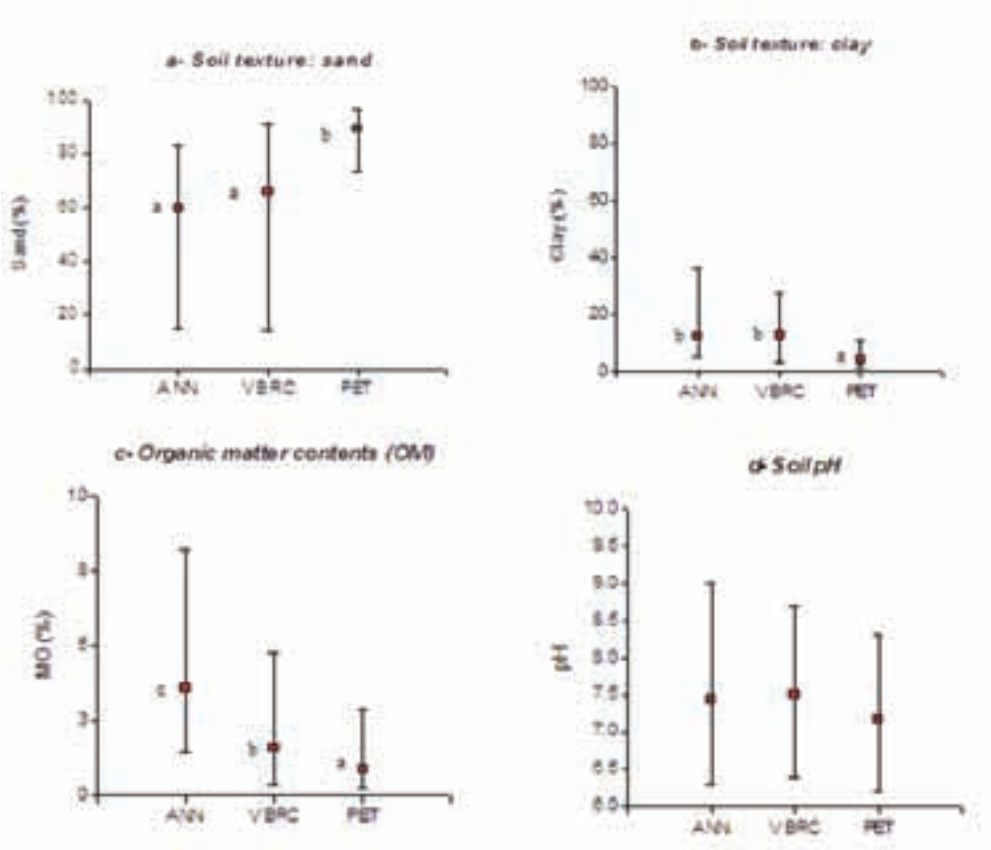

Fig. 3. Soil physical and chemical composition in *Helianthus annuus* (ANN) and *H. petiolaris* (PET) habitats in Argentina (mean, range) compared to those observed in River Colorado Valley (VBRC) using Kruskal-Wallis test. Means significantly different are followed by different letters.

At a micro-abiotic level, soil physical and chemical composition in the VBRC did not show differences with other annual *Helianthus* micro-habitats in Argentina, except for organic matter content (Fig. 3). However, a canonical discriminant analysis for these parameters showed that 10 out of 22 representative soils in the VBRC corresponded to those where the two invaders had previously established themselves in Argentina (Cantamutto et al., 2007).

On a biotic scale, no differences were found in plant community composition accompanying annual *Helianthus* species in Argentina and the sampled sites in the VBRC. More than two thirds of plants species considered as invaders found in the VBRC were associated with both annual *Helianthus* in their habitats in Argentina (Table 1). The high association with cosmopolite species would point to the invasion capacity of the VBRC by the two annual *Helianthus* species.

According to our results, the hypothesis of environmental similarity between the VBRC and the *H. annuus* and *H. petiolaris* habitats in Argentina and the US could not be discarded. As a consequence, the valley would be vulnerable to the invasion of both species.

The obtained data would be useful for helping set guidelines for commercial seed production. In order to derive recommendations for handling ecological aspects of invasion risks, the analysis results were disseminated to 24 of the main seed companies responsible for sunflower hybrid seed production in the VBRC, and to centres involved in science and technology within the region. An active alertness was initiated, comprising an exhaustive cleaning of machinery coming from other regions, roguing for off-type plants within seed production fields, to avoid annual *Helianthus* species culture for ornamental purposes, and removal of all feral forms of annual *Helianthus* within the protected region.

Table 1. Plant community main species found in disturbed lands at roadsides and channels in the River Colorado Valley (VBRC) also present in wild *Helianthus annuus* and *H. petiolaris* Argentine habitats, grouped by botanic family.

Plant species	Habit[1]	Origin[2]	Distribution %[3]
Asteraceae			
Centaurea solstitialis (L.)	A	E	50
Cichorium intybus (L.)	P	E	50
Solidago chilensis (DC.) Baker	P	N	88
Hyalis argentea D. Don ex Hook. & Arn.	P	N	30
Verbesina encelioides (Cav.) Benth. & Hook. f. ex A. Gray	A	N	83
Xanthium spinosum (L)	A	N	88
Brassicaseae			
Diplotaxis tenuifolia (L.) DC.	P	E, A	54
Chenopodiaceae			
Kochia scoparia (L.) Schard.	A	E, A	50
Salsola kali L.	A	E, A	20
Chenopodium album (L)	A	E	80
Convolvulaceae			
Convolvulus arvensis (L)	P	E	58
Fabaceae			
Melilotus albus Desr.	B	E	75
Poaceae			
Agropyron elongatum (Host) P. Beauv.	P	E	nd
Cynodon dactylon (L.) Pers.	P	A	88
Distichlis spicata (L.) Greene	A	N	80
Sorghum halepensis (L.) Pers.	P	E, A	54
Cenchrus pauciflorus (Benth)	A	N	63
Eleusine indica (L.) Gaertn	A	A	63
Eragrostis curvula (Schrad.) Nees	P	F	33
Solanaceae			
Solanum elaeagnifolium (Ort.) Dun.	P	N	85
Zygophyllaceae			
Tribulus terrestris (L)	A	E	63

[1]A = annual; B = biannual and P = perennial.
[2]E = Europe; A = Asia; F = Africa; N = Native or America
[3]Proportion of invaded provinces in Argentina, nd = not determined

ACKOWLEDGEMENTS

We are indebted to Ings. Agrs. E. Gaido, J. Irigoyen, J. Rivas y R. Matarazzo for their comments and suggestions. Dr. N. Peinemann helped in soil analysis. MAC is a fellow of Fundación Carolina (Spain).

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Seed morphology and oil composition of wild *Helianthus annuus* from Argentina

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ABSTRACT

Wild *Helianthus annuus* naturalized in Argentina constitutes a potential genetic resource for use in sunflower crop breeding. Seed morphology, oil content and fatty acid composition of nine stable Argentine wild populations were characterized and compared to 17 wild accessions from the US. The achenes were harvested from an experimental field at Bahia Blanca (S 38º41', W 62º14') during February, in three successive summers. Seed dimensions of Argentine accessions were within the range of US accessions, but showed less variability. The lower mottling and higher frequency of stripes in Argentinean populations would be an indication of crop introgression. The oil content, fatty acid composition and iodine value did not differentiate from the wild species origins. None of the Argentine populations showed a fatty acid composition similar or better than the improved mutant lines reported by other authors. All measured seeds traits showed significant differences, pointing to the existence of high variability in this new wild germplasm from Argentina.

Key words: achene – fatty acid – ferality – genetic resources – oil quality – sunflower.

INTRODUCTION

The quality of sunflower oil which contributes to about 80% of the total value of the crop has received considerable breeding efforts in the last 30 years (Fick and Miller, 1997). The main use of sunflower oil is as a salad and cooking oil, being also used as a major ingredient in some vegetal butter and shortening products, but it and for industrial non edible purposes in paints, varnishes, plastics, soap, and detergent (Seiler 2007). Sunflower oil has a high potential as a source for biodiesel production to satisfy the demand for renewable energy (Vannozzi, 2006).

Oil physical and chemical properties determine its end-use, with the fatty acid composition and iodine value being indicative of the oil characteristics. Traditionally, sunflower has been considered as having a polyunsaturated oil because of its high content of linoleic acid, but breeding selection, sometimes helped by chemical mutagenesis, has produced several lines with altered fatty acid composition (Fernandez-Martinez et al., 2006). Low saturated fatty acid content oils are chosen for edible purposes, high oleic mono-unsaturated acid oils are used for high temperature processes (as frying or bio-lubricants), whereas high saturated acid oils are preferentially used for margarine production, because they reduce the need for hydrogenation (Jan and Seiler, 2007).

The wild *Helianthus annuus* naturalized in Argentina grows as extended populations in a wide area across the boundary between humid and sub-humid regions (Poverene et al., 2002). Wild and weedy relatives of crops are genetically much more diverse than cultivated lineages and constitute a genetic resource that has not been fully exploited (Maxted et al., 2006). Wild *Helianthus* species provide a resource for improving oil quality in cultivated sunflower (Thompson et al., 1981) and a potential source of altered fatty acid composition (Seiler, 2004, 2007). The potential of wild sunflower naturalized in Argentina as genetic resource for oil quality improvement is unknown.

The objective of this work was to characterize wild *Helianthus annuus* from Argentina as a potential source for sunflower crop oil composition improvement.

MATERIALS AND METHODS

The wild germplasm was represented by nine stable populations from the diverse agro-ecological conditions where it grows in Argentina (Cantamutto et al., 2008). The accessions were from Rio Cuarto (RCU) S 33º09', W 64º20', Juarez Celman (JCE) S 33º40', W 63º28', Colonia Barón (BAR) S 36º10', W 63º53', Rancul (RAN) S 35º04', W 64º46', Adolfo Alsina (AAL) S 37º16', W 62º59`, Carhué (CHU) S 37º16', W 62º55', Diamante (DIA) S 32º03', W 60º38', Media Agua (MAG) S 31º57', W 68º27', and Las

Malvinas (LMA) S 34º47', W 68º15'. The accessions were collected by M. Poverene and M. Cantamutto in 2002-2003 during exploration trips, regenerated in the experimental field in Bahía Blanca (S 38º41', W 62º14') during the summer of 2004 and stored in the Sunflower Germplasm Active Bank at INTA Manfredi Experimental Station (Córdoba, Argentina) as code numbers 832 to 840.

Wild germplasm from North America (US) represented by 17 populations provided by the USDA-ARS GRIN germplasm system was studied for comparison. States of origin and passport numbers were: Arizona PI 468571, California PI 468580, Colorado PI 468621, Illinois PI 435540, Indiana PI 468633, Iowa PI 597901, Kansas PI 586851, Montana PI 586821, Nebraska PI 586867, Nevada PI 468596, New Mexico PI 468537, North Dakota PI 586807, Oklahoma PI 468483, South Dakota PI 586835, Texas PI 468504, Utah PI 468607, and Wyoming PI 586824 (for more information see www.ars-grin.gov/cgi-in/npgs/acc/display.pl?1080516).

Seedlings were grown in a greenhouse for one month and then transplanted by accessions in the experimental field at 1.9 plants/m^2 density during three successive summers (2003-2006). Drip-irrigation was applied to satisfy plant water demands. To regenerate the populations, heads of 20-30 individuals of each accession were bagged prior to open and sib-pollinated by hand during flowering. Bulk seed of mature heads were collected before achene shattering during the last week of February for sibbed and open pollination heads to minimized flowering date effects (Seiler, 1983).

A sample of 30 completely developed achenes from both pollination systems was used for seed description. Seed length, width, and thickness were measured using 10X magnification. The individual seed fresh weight was estimated by the total mass of the achenes. Qualitative traits, shape, pubescence, stripe presence, pericarp colour and mottling were individually determined and computed as frequencies. Argentine qualitative traits were determined using the original seed. Oil composition, fatty acid content and iodine value were evaluated at the EEA INTA Manfredi laboratory by AOCS (2007) approved methods (Ai 3-75, Ce 1-62 and Tg 1a-64) on a 10 g sample of seeds harvested from the experimental field under two pollination systems. Methyl esters of fatty acids were analyzed by Gas Chromatograph Hewlett Packard 6890 with a fire lionization detector and a capillary column HP-INNOWax (Crosslinked Polyethylene Glycol), of 0.32 mm x 30 m x 0.5 mm thick film. Each population was grown for at least two years.

To compare all the accessions, the ANOVA considered country, populations nested in countries, and year as variation sources. For seed qualitative traits of Argentine wild accessions, population and year were considered as sources of variability for the ANOVA. The oil content and fatty acid composition of Argentine accessions were analyzed for open-pollinated and sib-pollinated seed and the pollination system was considered as a source of variability for the ANOVA. LS means were calculated for each parameter and pair-compared using a linear combination of the model using the GLM procedure of SAS (2002). The linear regression between metric parameters was calculated and compared using an ANOVA (Quinn and Keough, 2005). Box-plot graphics were obtained with the InfoStat package (InfoStat, 2002).

RESULTS AND DISCUSSION

Argentine seed dimensions possessed about a half of the variability observed in the sample of US wild sunflowers, with no differences in the relationships between width, length, thick and weight, and were within the extreme values observed in the US populations (Fig. 1). Achene weight, length and width of accessions from both hemispheres corresponded to the expected values for wild and weedy populations (Heiser, 1978; Seiler, 1997).

The frequency of sparse pubescence and grey pericarp was not able to discriminate the between the groups, but stripes and mottling frequency differentiated both wild species origins (Fig. 2). The ranges of all qualitative traits overlapped for the Argentine and US wild origins (Fig. 2). A possible crop introgression in Argentine populations was suggested by their lower mottling (Fig. 2.b) and higher stripes frequency (Fig. 2.d) compared to the US accessions.

Though not included for botanical classification by Heiser (1978), mottling could be considered a wild trait. Stripes are typical of confectionary sunflower (Jan and Seiler, 2007) and characterized the first Argentine varieties (Bertero and Vazquez, 2003). If introgression happened during the colonization process, a strong selection pressure for small seed size would be expected (Alexander et al., 2001) but not for pericarp traits, that seem to be neutral. This could explain the absence of complete separation using seed dimensions, being larger in Argentine wild accessions but within the range of acceptable sizes for wild sunflower (Heiser, 1978). Hybridization with cultivated sunflower, also suggested by a phenotypic study of a number of plant traits (unpublished data), likely took place during the invasive process as a

result of the intense gene flow documented in Argentina landscape (Ureta et al., 2008). The introgression process was probably followed by a strong selection for small seed.

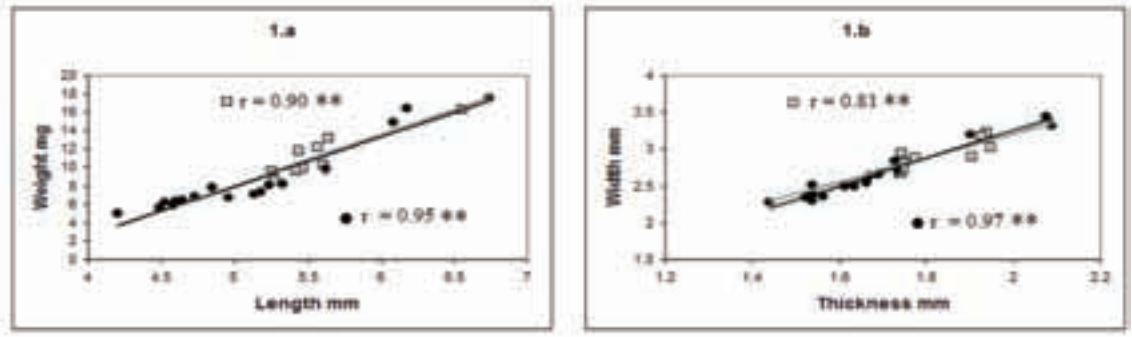

Fig. 1. Morphological relationships in wild *Helianthus annuus* seeds grown for three years in an experimental field. Argentine (grey squares) and North American (black circles) populations showed no differences in linear correlation between parameters.

Within the Argentine accessions, an ANOVA showed that populations differed for all the analyzed morphological traits (Table 1). Year effect was evident only in seed weight and length, probably due to differences in climatic conditions during grain filling. The significant effect of year on pericarp colour could be due to differences in achene size making it difficult to clearly visualize this trait in small seeds. The Argentine accession, CHU had the smallest seed dimensions, significantly different from LMA and MAG, which had the largest achenes (Table 1). The CHU accession also had a higher ovoid shape and grey pericarp frequencies. RCU, RAN, and JCE showed mottling in all seeds, significantly different from LMA, MAG and AAL with low mottled seed frequency. Considering all the traits together, RCU, BAR and CHU seemed to be a pure wild strains as opposed to LMA, AAL and MAG which showed introgressed crop-related traits (big seeds, presence of stripes, low mottling). These findings agree with the hypothesis that Rio Cuarto was as an entry point of wild *Helianthus annuus* before 1950s (Bauer, 1991) from where the invasive process progressed (unpublished data).

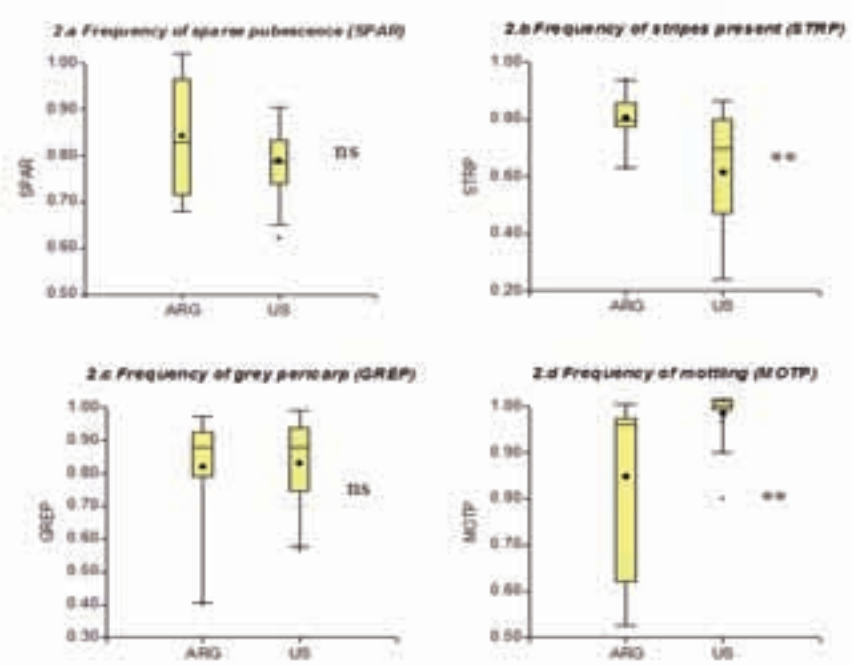

Fig. 2. Seed morphological descriptors of 26 wild sunflower populations from Argentina and the US grown for three years in an experimental field. Box-plots show the LS means distribution, ANOVA differences between both sources are indicated in each case. Year effect was not significant for all traits

Table 1. Morphological seed traits of nine wild *Helianthus annuus* populations from Argentina.

Wild population[1]	Seed dimensions[2]				Seed traits frequency[3]				
	Weight mg	Length mm	Width mm	Thickness mm	Ovoid shape	Sparse pubescence	Stripes	Grey pericarp	Mottling
AAL	11.7 bc[4]	5.6 bc	2.9 bd	1.9 a	0.92 a	0.67 c	0.93 a	0.94 ab	0.64 b
BAR	9.3 d	5.5 bc	2.9 bd	1.7 b	0.98 a	0.74 bc	0.77 b	0.99 a	0.98 a
CHU	8.8 d	5.2 c	2.6 e	1.7 b	0.96 a	0.66 c	0.89 a	0.97 a	0.98 a
DIA	10.2 c	5.5 bc	2.8 ce	1.8 b	0.92 a	1.00 a	0.85 a	0.81 bc	0.97 a
JCE	9.4 d	5.4 bc	2.8 ce	1.8 b	0.90 a	0.69 bc	0.62 c	0.95 ab	1.00 a
LMA	17.4 a	6.7 a	3.3 a	2.0 a	0.90 a	0.96 a	0.79 ab	0.41 c	0.53 b
MAG	13.2 d	5.7 b	3.0 ac	2.0 a	0.87 a	1.00 a	0.85 a	0.75 c	0.64 b
RAN	11.4 bc	5.4 c	3.1 ab	1.9 a	0.63 b	0.92 a	0.71 b	0.83 ac	1.00 a
RCU	9.0 d	5.3 c	2.7 de	1.8 b	0.95 a	0.84 b	0.80 a	0.90 ac	1.00 a
					ANOVA				
Population	**	**	**	*	*	**	**	**	**
Year	**	**	ns	ns	ns	ns	ns	*	ns

[1]See text for population codes. [2]Achenes harvested during three years in the experimental field. [3]Original seed accessions and achenes harvest in the experimental field. [4]LSmeans with different letters showed differences at $p < 0.05$

The oil content, fatty acid composition and iodine value did not show differences between the wild species origins (Fig. 3) but showed a year effect in fatty acid composition and iodine value. A higher palmitic acid concentration (Fig. 3.b) and a lower oleic acid concentration (Fig. 3.d) was found in Argentine accessions, with the other chemical parameters within the ranges observed for the US wild populations.

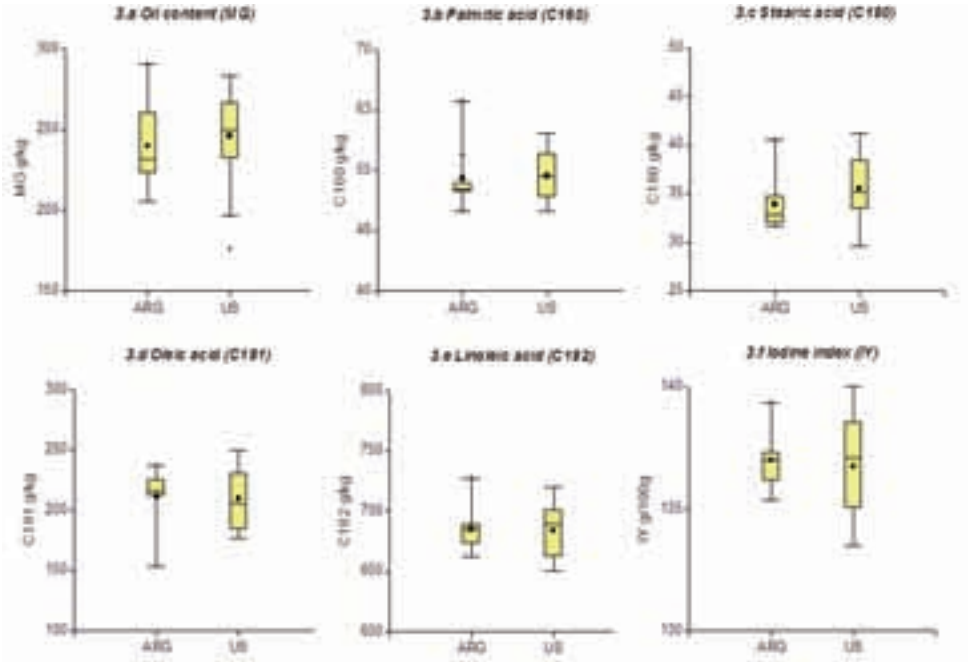

Fig. 3. Oil composition of wild *Helianthus annuus* open pollinated populations from Argentina (ARG) and North America (US) grown in Bahia Blanca, Argentina over three years. No differences were observed between both groups. Box-plots show the LS means distribution of 26 wild populations.

Argentine populations showed differences between the accessions for all the chemical parameters (Table 2). An oil content variation between 21.4 to 28.2% was typical of wild seeds and was only affected by population variability. The year had a significant effect on palmitic acid content and highly significant effects on oleic, linoleic, linolenic concentration, oleic/linoleic ratio and iodine value. Even though the grain filling of all analyzed achenes correspond to the same month, a variation between 35.4 to 40.5ºC of maximum temperature registered during this period could explain the year effect since the oil content and fatty acid composition are influenced by temperature (Harris et al., 1978). Slight variations in nitrogen

availability (Steer and Seiler, 1990), water regime (Flagella et al., 2002) and night minimum temperature (Izquierdo et al., 2006) can have an effect on oleic and linoleic sunflower concentrations and maybe responsible for the observed year effect.

Table 2. Oil content and chemical composition of nine wild *Helianthus annuus* from Argentina. Achenes correspond to grain-filling in February with sibbed and open pollination systems. Data are LS means of three years.

Wild `population[1]	Oil content	Fatty acid composition						Iodine value
		palmitic 16:0	stearic 18:0	oleic 18:1	linoleic 18:2	linolenic 18.3	oleic: linoleic	
	g/kg DM	g/kg						g/100g
AAL	282 a[2]	52 cd	32 cd	218 a	684 c	0.71 d	0.32 a	137 cd
BAR	238 bd	52 cd	33 cd	205 ab	696 bc	0.77 cd	0.29 ab	138 bd
CHU	261 ac	54 bc	31 d	199 ab	701 bc	0.86 ac	0.28 ab	139 ac
DIA	217 d	65 a	42 a	135 c	743 a	1.01 a	0.18 c	141 a
JCE	236 bd	52 cd	32 cd	201 ab	700 bc	0.83 bd	0.29 ab	139 ac
LMA	226 cd	51 cd	31 cd	211 a	690 bc	0.76 cd	0.31 a	138 bd
MAG	228 cd	57 b	34 c	181 b	713 b	0.89 ac	0.25 b	139 ac
RAN	214 d	54 bc	37 b	211 a	681 c	0.87 ac	0.31 a	136 d
RCU	270 ab	50 d	31 d	194 ab	711 b	0.92 ab	0.27 ab	140 ab
Pollination								
Sibbed	242	54	33	180	718	0.84	0.25	140
Open	241	54	34	210	686	0.86	0.31	137
ANOVA								
Population	*	**	**	**	**	*	**	*
Year	ns	*	ns	**	**	**	**	**
Pollination	ns	ns	ns	**	**	ns	**	**
Population x pollination	ns	ns	ns	ns	ns	ns	ns	ns

[1] See text for population code. [2] LSmeans with different letters showed differences at $p < 0.05$

There was a high significant effect of the pollination system on oleic and linoleic concentration, their relationship and the iodine value (Table 2) as expected considering both parent influence. Given the general inverse relationship, sibbed seeds produced lower oleic acid and higher linoleic acid concentration than open pollinated seeds. The 15% gain for oleic acid from open pollination was insufficient to reach the maximum value observed in AAL. The cause of the increased in average oleic content from open pollination could be addressed in future studies.

In general, the fatty acid composition did not show values of interest with respect to those reported for improved mutant lines with altered fatty acid composition (Fernandez-Martinez et al., 2006). None of the Argentine accessions showed less than 39 and 26 g/kg of palmitic and stearic acid content, nor more than 300 g/kg of palmitic acid to be considered low or high in saturated acid content. None of the Argentine accessions showed oleic acid over 860 g/kg or linoleic concentration over 780 g/kg, similar to values of improved mutant lines.

The AAL accession had the highest oleic concentration, but was only different from MAG, RAN and DIA. Among Argentine germplasm, DIA showed the most variability in fatty acid composition, with higher palmitic, stearic, linoleic, linolenic, and iodine values and the lower oleic acid content. This population from Diamante represented a life cycle that is significantly longer than the other North America and Argentine accessions (unpublished data) and could constitute a unique germplasm of potential value.

ACKOWLEDGEMENTS

Drs. Juan Antonio Martín Sanchez and Lluis Torres (IRTA-Spain) made valuable comments. MAC is a fellow of Fundación Carolina (Spain). This research was supported by grants INTA-PNCER 1339, ANPCYT-PICT 08-9881 and UNS-PGI 24A106.

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Helianthus species in breeding research on sunflower

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ABSTRACT

This investigation presents the results from the study on new sunflower forms obtained through hybridization between cultivated sunflower (*Helianthus annuus*) and 38 wild *Helianthus* species. Valuable genetic diversity in sunflower was developed by transfer of genetic material. The genetic system CMS - restorers of fertility was enriched with 15 new CMS sources and a lot of new Rf-gene sources. New sunflower forms, lines and hybrids with high seed oil content and high productivity, with resistance to economically important diseases and new plant architecture were produced, together with new sunflower forms and lines with high protein content in seeds suitable for birds and human consumption.

Key words: cytoplasmic male sterility – disease resistance – intergeneric hybridization – interspecific hybridization – oil – *Rf* gene.

INTRODUCTION

The increase in sunflower production and seed quality has been largely connected to the inclusion of wild *Helianthus* species into the improvement work on sunflower. Using this approach, the diversity in sunflower was enriched and the genetic system CMS - restorers of fertility was established. A real possibility for a complete practical application of heterosis breeding was created. The transfer of useful characters from *Helianthus* species to the cultivated sunflower started at the beginning of the 20th century and continues nowadays purposefully and at accelerated rates. The results from the investigations made by Satzaiperov (1916), Pustovoit (1960), Putt and Sackston (1963), Pustovoit .(1975), Georgieva-Todorova (1976), Leclercq (1969), Leclercq et al. (1970), Fick et al. (1974), Jan and Chandler (1985), Vrânceanu et al. (1986), Christov (1990, 1999), Christov et al. (1993), and many other authors showed that the wild *Helianthus* species are rich sources for genes determining resistance to different diseases, parasites, pests, drought and other important traits. Some of these genes were already transferred to the cultivated sunflower and, on this basis, the new sunflower forms, resistant to some diseases (downy mildew, rust, phomopsis, etc., pests, broomrape and drought were developed, which also possess high production potential, high oil content in seed, presence of *Rf* genes, etc. (e.g. Morizet et al., 1984; Laferriere, 1986; Serieys and Vincourt, 1987; Seiler, 1992; Christov, 1991; Christov, 1996; Christov, 1996a; Christov et al., 1996; Perez-Vich et al., 2002; Christov et al., 2004; Hristova-Cherbadzi, 2004 and 2007, Hristova-Cherbadzi et al., 2006). This investigation aims to demonstrate that by transfer of genes controlling important economic characters from wild *Helianthus* species to cultivated sunflower, new sunflower forms, lines and hybrids were developed, which possess: resistance to diseases and parasites, good combining ability, high production potential and high oil content in the seeds, and which are suitable for growing in Bulgaria and other sunflower production regions worldwide.

MATERIALS AND METHODS

Plant material: New sunflower hybrid forms and completed B and R lines obtained from hybridization between sunflower cultivars and lines and 37 wild species from genus *Helianthus*.

Methods and directions of the investigation: The methods and directions of the investigation were: 1) Investigation on F_1 obtained from hybridization between sunflower and wild *Helianthus* species and the hybrid material from the next generations; 2) Individual selection and self-pollination or backcrossing; 3) Developing B and R lines and sterile analogues (A lines) of B lines; 4) Developing hybrid varieties by production and testing of hybrid combinations (A x R); 5) Developing other interesting sunflower forms.

Evaluation of hybrid progenies and comparison to the parental forms - accessions from wild Helianthus *species and cultivars and lines of cultivated sunflower:* The methods used in this investigation were practised, adapted to and confirmed for the conditions of DAI (Christov, 1990a; Christov, 1996a,b; Christov et al., 2004).

Biomorphological characterization: It was made on the basis of phenological and biometrical observations during the vegetation period, as well as on the basis of laboratory measurements of whole plants and seeds.

Phytopathological characterization: It included evaluation of the resistance to mildew (*Plasmopara helianthi* Novot.), phoma (*Phoma helianthi Munt*.-Cvet. et al.), phomopsis (*Phomopsis helianthi Munt*.-Cvet. et al.), downy mildew (*Erisiphe cihoracearum* D. C.*)*, sclerotinia (*Sclerotinia sclerotiorum* Lib.), alternaria (*Alternaria helianthi* (Hanasf.) Tub. and Nish.), rust (*Puccinia helianthi* Schw.), verticilium (*Verticilium helianthi* Keeb.) and to the parasite broomrape *(Orobanche cumana* Wallroth).

Biochemical characterization: Study on the percentage and quality of oil and protein content in the seeds of interspecific hybrids and new sunflower lines and hybrids. The methods used for evaluation were confirmed and adapted by the biochemistry laboratory in DAI.

Cytological characterization: The investigations were mainly carried out with the aim of studying the meiosis of pollen female cells of F_1 interspecific hybrids special and pollen viability of the hybrid materials.

Cytoplasmic male sterility and restorer genes: CMS sources could be found among the hybrids obtained from crossing wild species x cultivated sunflower. For obtaining sterile inflorescence, the pollination was carried out with pollen from B lines or cultivars. After maintaining the sterility and the determination of its cytoplasmic type, the comparative study of other CMS sources began. These sources had already been established in DAI - General Toshevo as well as elsewhere in the world. Evaluation of the cytoplasmic effect on some agricultural characteristics of lines and hybrids included in several CMS sources was made. *Rf* genes could be found primarily in crosses cultivated sterile sunflower x wild species. The presence of *Rf* genes in the genome of a wild species was established in F_1. Forms with *Rf* genes were found in the materials obtained from crosses cultivated sunflower (B line or cultivar) x wild species and wild species x cultivated sunflower. The determination was done when the pollen of these materials was used for pollination of sterile plants from cultivated sunflower on the basis of CMS source. In all these cases *Rf* genes were identified in several CMS sources and the genetic basis of restoration was studied.

Developing of sunflower B and A lines. Developing of sunflower B lines was carried out by purposeful selection in the hybrid materials, which began after the third hybrid combination and lasted usually up to the 9-12 generation. The evaluation and the selection were done on the basis of their biomorphological, biochemical, phytopathological and entomological characteristics, lack of *Rf* genes and their good combining ability. For determining the combining ability of B lines, two variants were used: a) well-known (confirmed) sterilized R lines in CMS Rig 1 (Vulpe, Romania, Vulpe, 1972) and CMS Falax 1 (Serieys, France) were crossed with new B lines; b) Sterile analogue of new B line in BC_3 or BC_4 was crossed with familiar R lines. The second variant was predominantly used. Developing of sterile analogues (A lines) of B lines began with determining the fact that in the studied hybrid material Rf genes were absent. After BC_3 or BC_4, the study on the common combining ability of the developed A lines began, and after that on the specific combining ability for determination of the best hybrid combinations.

Developing of hybrid combinations and candidate cultivars for the State Variety Commission. Hybrid combinations were obtained when the sterile analogues (A lines) of B lines were used. They were obtained as a result of interspecific hybridization and experimental mutagenesis and were included in some CMS sources and R lines. Other Bulgarian and foreign A lines were also used. The new hybrids (A x R) were checked in preliminarily tests. The best of them were tested and submitted to the Executive Agency for Variety Testing, Field Inspection and Seed Control and to other organizations all over the world. Obtaining of seeds from the hybrid combinations was carried out by growing of plants in nurseries covered with netting (net-house) or isolation of plants with paper isolators. The pollination was done manually. Bees could be used for plant pollination.

RESULTS

On the basis of the obtained interspecific hybrids, useful characters were transferred into cultivated sunflower from 183 accessions of 38 *Helianthus* species (Table 1). In many of the hybrids genes controlling different useful characters such as resistance to diseases, parasites and other stress factors

were identified. Forms with new plant architecture, with different vegetative periods and different seed colors were produced. Many of the forms were with high combining ability and high seed oil content, higher than that of the cultivated sunflower included in the hybridization. A lot of CMS sources were found in the cultivated sunflower, as well as fertility restorer genes (*Rf* genes).

Table 1. Sources of new characters transferred into cultivated sunflower.

Character	Species
Resistance/tolerance to: *Plasmopara helianthi*	*H.annuus* (w.f.), *H.argophyllus, H. bolanderi, H.debilis, H.exilis, H. neglectus., H. paradoxus, H.petiolaris, H.praecox, H.divaricatus, H.doronicoides, H.giganteus, H.glaucophyllus, H.grosseserratus, H.mollis, H.maximiliani, H.microcephallus, H.multiflorus, H.nuttallii, H.occidentalis, H.orgialis, H.pumilus, H.salicifolius, H.smithii, H.decapetalus, H.hirsutus, H.laevigatus, H.scaberimus, H. tomentosus, H.eggertii, H.californicus, H.ciliaris, H.pauciflorus, H.resinosus, H.strumosus, H.tuberosus, H.x laetiflorus.*
Phomopsis helianthi	*H.annuus* (w.f.), *H.argophyllus, H.debilis, H.eggertii, H.pauciflorus, H.glaucophyllus, H.laevigatus.*
Erysiphe cichoracearum	*H.decapetalus, H.laevigatus, H.glaucophyllus, H.ciliaris.*
Orobanche cumana	*H.tuberosus, H.eggertii, H.smithii, H.argophyllus, H.pauciflorus, H.strumosus*
Phoma helianthi	*H.argophyllus, H.laevigatus, H.eggertii, H.debilis*
Sclerotinia sclerotiorum	*H.praecox, H.argophyllus, H.annuus* (w.f.), *H.petiolaris, H.eggertii, H.pauciflorus, H.smithii.*
Earliness	*H.praecox, H.scaberimus, H.glaucophyllus, H.giganteus, H.rigidus, H.nuttallii, H.ciliaris* and *H.annuus* (w.f.)
Seed size	*H.annuus* (w.f.), *H.argophyllus, H.tuberosus, H.strumosus*
High oil content	*H.annuus* (w.f.), *H.debilis, H.petiolaris, H.praecox, H.pauciflorus, H. x laetiflorus*
Genes controlling CMS	*H.annuus* (w.f.), *H.argophyllus, H.debilis, H.petiolaris, H.praecox, H.pauciflorus* and *H.strumosus*
Rf genes	*H.annuus* (w.f.), *H.argophyllus, H. bolanderi, H.debilis, H.exilis, H.neglectus, H. paradoxus, H.petiolaris, H.praecox, H.divaricatus, H.doronicoides, H.glaucophyllus, H.giganteus, H.grosseserratus, H.maximiliani, H.microcephallus, H.mollis, H.multiflorus, H.nuttallii, H.occidentalis, H.orgialis, H.pumilus, H.salicifolius, H.smithii, H.decapetalus, H.hirsutus, H.laevigatus, H.scaberimus, H.tomentosus, H.eggertii, H.ciliaris, H.resinosus, H.pauciflorus, H. strumosus, H.tuberosus, H. californicus* and *H. x laetiflorus*.

New sunflower forms

- Resistance/tolerance to diseases and parasites:

*Resistance to *Plasmopora helianthi*: Full resistance to *Plasmopora helianthi* (races 300, 330, 700) was shown by 784 lines (765 R and 19 B lines). More than 850 new sunflower forms obtained also by interspecific hybridization (from F_6 to F_{13}) showed full resistance to the pathogen and possessed restorer genes (*Rf* genes) for the first sunflower CMS source - CMS Pet 1 and for some new CMS sources, obtained at DAI.

*Resistance to *Sclerotinia sclerotiorum*: In this case the term tolerance could be used. Using the species *H. eggertii, H. pauciflorus, H. smithii, H. praecox, H. petiolaris, H. argophyllus* and *H. annuus* (w.f.), plants with tolerance to *S.sclerotiorum* forms were obtained on the head as well as on the stem and roots. Lines 7043 R, 7083 R and 7092 R were of considerable importance, besides the obtained ones.

*Resistance to *Phomopsis helianthi*: New sunflower forms resistant to the pathogen *Ph. helianthi* were obtained using *Helianthus annuus* (w.f.), *H. argophillus, H. debilis, H. glaucophyllus, H. laevigatus, H. eggertii, H. pauciflorus (rigidus*). Lines 6066B, 6748B, 7041 R, 7043 R obtained from *H. argophillus, H. decapetalus, H. eggertii, H. pauciiflorus* transfer resistance/ tolerance to the F_1 hybrids included.

*Resistance to *Phoma helianthi*: New sunflower forms resistant to *Phoma helianthi* were developed using *H. eggertii, H. laevigatus, H. argophillus* and *H. debilis.*

*Resistance to *Erysiphe cichoracearum*. There are two types of resistance to this pathogen. The first one is controlled by a dominant gene. This type of resistance was found in *H. decapetalus*. The second one is controlled by a group of genes. Some accessions of *H. glaucophyllus, H. ciliaris, H. laevigatus, H. debilis, H. tuberosus* (M 004), *H. resinosus* possess such resistance. New sunflower forms resistant to this pathogen were obtained using *H. decapetalus, H. laevigatus, H. glaucophyllus* and *H. ciliaris*.

*Resistance to *Orobanche cumana*. Resistance to the parasite *O. cumana* found in the cultivated sunflower is controlled by one dominant gene. Several perennial *Helianthus* species also showed 100%

resistance to *O. cumana*. New sunflower forms resistant to the parasite were obtained using *H. tuberosus*, *H. eggertii*, *H. smithii*, *H. decapetalus*, etc. Full resistance to the parasite was demonstrated by some new lines such as 7009 R, 7019 R, 7203 R, etc.
Many lines showed resistance to two or three pathogens (downy mildew, phomopsis, phoma and sclerotinia) and the parasite broomrape.

- Cytoplasmic Male Sterility (CMS):

Untill 2005, 72 CMS sources in sunflower have been reported and described by different authors. Most of these CMS sources were derived from interspecific crosses (Serieys and Christov, 2005). On the basis of the hybridization carried out till 2005, 14 new CMS sources were found plus one new type of CMS - CMS ARG3-M1. The greater part of the new CMS sources were distinguished by the first CMS source, obtained from *H. petiolaris* - CMS Pet 1 (Leclercq, 1969), as well as between them (Table 2).

Table 2. Sources of CMS produced by interspecific hybridization.

Origin	Obtained in generation	Year of observation	Year of report	DAI code	F.A.O. code
H. annuus E-067	F_1	1985	1992	AN-67	ANN-10
H. annuus E-058	F_6	1988	1994	AN-58	ANN-11
H. annuus E-002	F_5	1991	1991	AN-2-1	ANN-12
H. annuus E-002	F_6	1992	1992	AN-2-2	ANN-13
H. argophylus E-006	F_1	1984	1990	ARG-1	ARG-1
H. argophylus E-006	BC_1	1987	1990	ARG-3	ARG-3
H. argophylus E-007	F_1	1985	1992	ARG-2	ARG-2
H. debilis E-010	F_2	1990	1994	DV-10	DEB-1
H. petiolaris E-034	BC_1F_6	1991	1991	Pet-34	PET-4
H. praecox E-027	F_2	1990	1990	PHIR-27	PRH-1
H. praecox E-029	F_4	1989	1989	PRUN-29	PRR-1
H. rigidus M-028	BC_1F_2	1991	1991	Rig-28	RIG-2
H. strumosus M-056	BC_1F_5	1991	1996	Strum-56	STR-1
H. argophyllus E-007	BC_1F_7	1995	1998	ARG-4	ARG-4
H. argophyllus E-006	new BC_1	1997	2000	ARG-3-M-1	ARG3M1

*Sources of new fertility restorer (*Rf*) genes:*

The investigation was directed towards the discovery of *Rf* genes for CMS from *H. petiolaris* (PET-1) and for the new CMS sources obtained at DAI - General Toshevo. It was found out that 181 accession of 37 *Helianthus* species carried *Rf* genes. We also identified Rf genes in *H. argophyllus*, *H. debilis* and *H. rigidus* (*pauciflorus*) for CMS RIG-1. Our investigations showed that fertility restoration in CMS RIG-1 was controlled by two dominant genes. Similar results were obtained for fertility restoration in CMS ARG-3-M-1. The sources of genes for restoration of fertility of plants in CMS ARG-3-M-1 were different from those for CMS RIG-1. One of the sources was found in *Carduus acanthoides* widespread in Bulgaria.

New sunflower forms with normal cytoplasm (B lines): The total number of developed (fixed) B lines till 2007 was 268. The stem height varied from 60 to 180 cm, and the vegetation period - from 86 to 125 days. Thousand seed weight varied from 35 to 118 g, and seed oil content - from 40 to 54 % (Table 3). Some B lines showed resistance to phomopsis and others - to downy mildew and broomrape. Such lines were 6066B, 6101B, 6488B, 6748B, etc. New sunflower forms with *Rf* genes (R lines): Till now 765 R lines have been fixed and named. All of them are resistant to downy mildew. Some of them are resistant to phomopsis and broomrape. There are lines which show resistance to phoma and others - even tolerance to sclerotinia. All lines show high combining ability. A part of these lines are given in Table 4.

New sunflower hybrid varieties in registration: During the developing and investigation of new sunflower hybrid varieties, two groups of combinations were performed. The first combination included crosses between old, confirmed Bulgarian A (B) lines with R lines obtained from interspecific hybrids, and the second group included crosses between new A (B) lines obtained by using mutagenesis and R lines produced through wide hybridization. There was a small number of hybrid combinations developed from B lines through wide hybridization and R lines, obtained through the same method. Each year 280 to 320 hybrid combinations are produced for testing. New sunflower hybrids were developed which exceeded the standard hybrid Albena by seed yield and seed oil content per unit area. Five of these hybrids - Musala, Mura, Maritsa, Mesta and Magura, were already registered in the State Variety Commission at the end of 2006. At the beginning of 2008 the first type of hybrid for direct human consumption - hybrid Madan - will be released.

Table 3. Characteristics of B lines produced from interspecific hybridization.

No	Origin	Plant height (cm)	Head diameter (cm)	Seed oil content (%)	Vegetation period (days)
6101	*H.decapetalus* M-043	125	18	47.35	106
6159	*H.pauciflorus* M-028	155	15	48.79	105
6170	*H.strumosus* M-056	110	12	47.82	110
6202	*H.hirsutus* M-029	105	12	45.25	105
6215	*H.salicifolius* M-045	180	18	51.15	107
6275	*H.argophyllus* E-007	140	23	49.96	105
6134	*H.debilis* E-011	90	23	49.58	100
6149	*H.eggertii* M-001	130	24	49.88	105

Table 4. Characterization of R lines produced from interspecific hybridization.

No	Origin	Plant height (cm)	Head diameter (cm)	Vegetation period (days)	Seed oil content (%)	Generation
7009R	*H.tuberosus* M-037	80	13	92	45.99	17*
7015R	*H.debilis* E-011	120	15	102	52.73	15*
7026R	*H.smithii* M-008	140	14	106	45.34	16*
7027R	*H. x laetiflorus* M-005	135	17	102	48.86	15*
7041R	*H.eggertii* M-001	120	15	101	47.21	16*
7043R	*H.pauciflorus* M-028	135	16	106	52.46	15*
7203R	*H.decapetalus* M-043	110	16	102	49.56	15*
7082R	*H.glaucophyl*. M 012	110	16	107	49.91	16*
7090R	*H.paradoxus* E-019	120	15	104	52.12	15*
7091R	*H.ciliaris* M-092	70	21	105	45.31	16
7092R	*H.divaricatus* M-044	120	17	105	52.38	16*
7089R	*H.salicifolius* M-078	120	15	103	46.30	15*
7203R	*H.decapetalus* M-043	110	16	102	49.56	15*

*branched forms

CONCLUSIONS

As a result of a successfully ample hybridization program, new sunflower forms, lines and hybrids were developed. Many of these materials possess high productivity, high seed oil content and resistance to some economically important sunflower diseases. Besides, a great number of CMS sources and *Rf* genes were obtained. Forms and lines with oil type and protein type of seeds (for human consumption and poultry feeding) were produced.

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Wild sunflower species from the southeastern United States as potential sources for improving oil content and quality in cultivated sunflower

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ABSTRACT

Sunflower (*Helianthus annuus* L.) oil has the potential to be improved for nutritional and industrial purposes through selection and breeding. The narrow genetic base of cultivated sunflower has been broadened by the infusion of genes from wild species, resulting in a continuous improvement in agronomic traits. Interest in using wild species in breeding programs has increased, but information about oil concentration and fatty acid composition is lacking for a number of rare and threatened species. The objective of this study was to evaluate achenes of seven wild sunflower species, *H. eggertii*, *H. schweinitzii*, *H. porteri*, *H. verticillatus*, *H. smithii*, *H. angustifolius*, and *H. atrorubens*, from the southeastern USA for oil concentration and fatty acid composition of four major fatty acids, palmitic, stearic, oleic and linoleic acids. *Helianthus verticillatus* had the highest oil concentration of any species with 324 g/kg and was within the range expected for a wild perennial sunflower species. The high linoleic acid concentration in *H. porteri* of 815 g/kg is the highest concentration reported for a wild sunflower species. Linoleic acid concentrations for all seven species were higher than expected for populations grown in southern latitudes. The lower saturated fatty acid profile in several of the species indicates these species have the potential to reduce saturated fatty acids in commercial sunflower oil. Further research will be needed to determine the inheritance of the fatty acids and oil concentration. Other agronomic traits will need to be maintained during the introgression of these traits into sunflower.

Key words: *Helianthus* – linoleic acid- oil – oleic acid – palmitic acid – stearic acid.

INTRODUCTION

Vascular plants produce many compounds and secondary metabolites, one of which is oil resulting from plant lipid synthesis. Although oil concentrations of up to 37 g/kg have been reported in whole plants of wild sunflower (*Helianthus annuus* L.), the achenes are the primary storage tissue for oil (Seiler et al., 1990). The oil that accumulates in the achenes of wild and cultivated sunflower is composed of triacylglycerols that exist in the liquid form at room temperature and have a low melting point. The fatty acid composition of the achene oil determines its end use suitability. Sunflower oil is currently considered high quality edible oil; however, the potential for improved nutritional and industrial characteristics through selection and breeding has not been exhausted.

Since the development of high-oleic sunflower hybrids, sunflower oil has become a more important feedstock for the oleochemical industry, of which the cosmetics industry is a major user. Development of the mid-oleic NuSun® oil in the USA has increased the demand for this type of oil in the food processing industry due to its high oxidative stability (Kleingartner, 2002).

Oil concentration and fatty acid composition, especially oleic and linoleic fatty acids, of oil from wild and cultivated sunflower varies greatly mainly as a response to temperature during seed development (Harris et al., 1978; Seiler, 1986). A high temperature during seed maturation results in oil with high oleic acid concentration, and a low linoleic acid concentration. Generally, the cooler northern latitudes produce considerably higher concentrations of linoleic acid in the oil than the warmer southern latitudes (De Haro and Fernández-Martínez, 1991).

The genus *Helianthus* consists of 51 species and 19 subspecies with 14 annual and 37 perennial species (Schilling, 2006). The narrow genetic base of cultivated sunflower has been broadened by the infusion of genes from wild species. This has resulted in continuous improvement of agronomic and economic traits in cultivated sunflower (Thompson et al., 1981; Seiler, 1992; Seiler and Rieseberg, 1997; Jan and Seiler, 2007). Recent emphasis on the oil concentration and fatty acid composition of sunflower achenes has increased interest in using wild species in breeding programs to enhance oleic or linoleic acid, or to reduce saturated fatty acids.

While a few populations of some wild sunflowers have been collected and evaluated for oil concentration and fatty acid composition, many remain to be evaluated to fully characterize the available genetic diversity. There is an urgent need to collect and evaluate species that are endemic to limited geographic areas and may be at risk of being eliminated by the activities of man.

The objective of this study was to evaluate achenes of seven wild sunflower species, *H. eggertii*, *H. schweinitzii*, *H. porteri*, *H. verticillatus*, *H. smithii*, *H. angustifolius*, and *H. atrorubens* collected from the southeastern region of the USA for oil concentration and fatty acid composition of four major fatty acids, palmitic, stearic, oleic and linoleic acids.

MATERIALS AND METHODS

Plant materials. Populations of wild sunflowers were collected between 17 and 28 October, 2003. The expedition covered a distance of 4600 km in five states: Alabama, Georgia, North Carolina, South Carolina, and Tennessee. Details of this exploration can be found in Gulya et al. (2007). Heads of wild sunflowers were collected from 50 to 250 plants within each population and bulked into a single sample. Herbarium specimens were deposited at the USDA-ARS wild *Helianthus* herbarium at Fargo, North Dakota. Achene samples were sent to the USDA-ARS North Central Regional Plant Introduction Station, Ames, Iowa, USA where they are maintained and distributed.

All populations were collected throughout the broad distributional range of the species. Prior collection sites obtained from herbarium specimens and generalized distribution maps were used to locate populations. Population size (number and extent), habitat, soil type, achene set per head, the presence of diseases and insects, GPS coordinates including elevation, and the presence of other wild sunflower species located near the collection sites were recorded for each population.

Oil and fatty acid analyses. Achenes were stored at 5°C and low humidity (<20%) until analyzed. Each sample represented an isolated, open-pollinated segregating population. Two 6-ml portions from each achene sample were cleaned to remove empty achenes, and analyzed for oil concentration (expressed as a percent on a dry weight basis) by nuclear magnetic resonance (Granlund and Zimmerman, 1975). Fatty acid composition was determined from a 10- to 20-achene sample. A small portion of the pulverized sample (10 to 20 mg) was transferred to a disposable filter column (Fisher Scientific[1]) and eluted with 3.5 ml of diethyl ether. The oil in the diethyl ether solution was converted to methyl esters using an organic-catalyzed transesterification technique (Metcalfe and Wang, 1981). The sample was injected into a Hewlett-Packard 5890 gas chromatograph containing a DB-23 capillary column (25 m x 0.25 mm, J & W Scientific). A fatty acid standard, 21A (Nu-Chek-Prep, Inc.), containing methyl esters of the following acids was used as a reference: palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), arachidic (20:0), 11-eicosenoic (20:1), behenic (22:0), and lignoceric (24:0). Fatty acid and oil concentrations were means of two samples per population.

Data analysis. The data were analyzed using an analysis of variance (ANOVA). Means were separated using Duncan's New Multiple Range Test.

RESULTS

Achenes were collected for five rare species, *H. eggertii* from Alabama, South Carolina, and Tennessee, *H. schweinitzii* from North and South Carolina, *H. porteri* from North Carolina and Georgia, *H. verticillatus* from Alabama and Tennessee, and *H. smithii* from North Carolina (Table 1). Two additional species with wider distributional ranges, *H. angustifolius* from Georgia and Tennessee, and *H. atrorubens* from Alabama were also collected.

Helianthus porteri is an annual species that was recently transferred from the genus *Viguiera* (Pruski 1998). Eight populations of this species were collected from its known distributional range (Table 1). The average oil concentration was 288.7 g/kg (Table 2).

Among the seven species, the lowest average oleic acid concentration of 65.2 g/kg was observed in *H. porteri* (Table 2). This was accompanied by a high linoleic acid concentration. The 814.9 g/kg average linoleic concentration in *H. porteri* was the highest observed in any wild species, with one population having a concentration of 830 g/kg. The concentration of palmitic and stearic acids in *H. porteri* averaged 55.8 g/kg and 32.1 g/kg, respectively.

Table 1. Wild *Helianthus* populations analyzed for oil concentration and fatty acid composition of seven species collected from the southeastern USA

Species	Number of populations	State (Location)	Habitat
ANNUAL			
H. porteri	8	Georgia and North Carolina	Granite outcrops, Piedmont Region
PERENNIAL			
H. angustifolius	2	Georgia and Tennessee	Open to shaded woods, usually moist places
H. atrorubens	1	Alabama	Open mixed woods, dry roadsides, dry shaded hillsides
H. eggertii	13	Alabama, South Carolina, and Tennessee	Grassy openings, barrens, open oak-hickory woods
H. schweinitzii	14	North and South Carolina	Open woodlands, clearings, Piedmont region
H. smithii	1	North Carolina	Dry, open woods
H. verticillatus	2	Alabama and Tennessee	Moist, prairie-like openings, and edge of woodlands, clay soil

Helianthus eggertii is a hexaploid perennial species that was recently removed from the threatened species list by the U.S Fish and Wildlife Service (USFWS 2005). Thirteen populations were collected from all areas of the species range, except from Kentucky. Oil concentration of the populations averaged 285 g/kg, while oleic and linoleic acids averaged 154.6 and 721.4 g/kg, respectively (Table 2). The saturated fatty acids, palmitic and stearic acids averaged 55.6 and 28.4 g/kg, respectively.

Helianthus schweinitzii is a federally protected rare hexaploid species in the Piedmont region of North and South Carolina. Fourteen populations of this species were collected from throughout its range. The oil concentration of the 14 populations averaged 285 g/kg, the same as *H. eggertii* (Table 2). Saturated palmitic and stearic acid concentrations averaged 53.6 and 39.0 g/kg, respectively, while oleic and linoleic acids averaged 108.7 and 765.2 g/kg, respectively.

Helianthus smithii, a rare diploid perennial species, was collected from a single population in North Carolina, near the eastern edge of its distributional range. The oil concentration was 296 g/kg, while oleic and linoleic acid concentrations were 104.2 and 778.4 g/kg, respectively (Table 2). Palmitic and stearic acid concentrations were 50.7 and 32.7 g/kg, respectively.

Perennial *H. verticillatus* is a species which was described over 100 years ago (Small, 1898), but was not rediscovered or recollected until recently in Tennessee (Mathews et al., 2002). Two populations of this species were collected, one from Tennessee and one from Alabama. Oil concentration averaged 323.5 g/kg, the highest concentration of any species analyzed in the study (Table 2). The saturated fatty acids, palmitic and stearic, averaged 57.6 and 25.8 g/kg, respectively, while oleic acid and linoleic acids averaged 137.6 and 744.3 g/kg, respectively.

Oil concentration of one population of diploid perennial *H. atrorubens* and two populations of diploid perennial *H. angustifolius* averaged 277 and 297.5 g/kg (Table 2). Oleic acid and linoleic acids averaged 89.6 and 778.5 g/kg for *H. atrorubens* and 117.2 and 723.1 g/kg for *H. angustifolius*, respectively. Palmitic and stearic acids averaged 63.4 and 34.1 g/kg for *H. atrorubens* and 74.0 and 37.9 g/kg for *H. angustifolius*, respectively.

DISCUSSION

Wild *Helianthus* populations generally have oil concentrations of 250 to 300 g/kg, much lower than 450 to 500 g/kg in cultivated sunflower (Seiler, 1985; 1994). In one study, wild *H. annuus* averaged 258 g/kg and *H. petiolaris* 288 g/kg (Seiler, 1983). The oil concentration for the perennial sunflowers from Canada averaged 297 g/kg (Seiler, 1999) compared to 244 g/kg for perennial species from the central Great Plains of the USA (Seiler, 1994).

Table 2. Oil concentration (g/kg) and fatty acid composition (g/kg) of seven *Helianthus* species collected from the southeastern USA

Species	Oil concentration	Fatty acid composition[1]			
		Palmitic	Stearic	Oleic	Linoleic
ANNUAL					
H. porteri	288.7±19.1 NS	55.8±3.9ab[1] *	32.1±4.4ac **	65.2±11.0d **	814.9±13.3a **
PERENNIAL					
H. angustifolius	297.5±21.9 NS	74.0±0.8b *	37.9±0.1ab **	117.2±24.3bc **	723.1±42.0b **
H. atrorubens	277.0±0.0 NS	63.4±0.0ab *	34.1±0.0a-c **	89.6±0.0cd **	778.5±0.0ab **
H. eggertii	285.0±29.6 NS	55.6±16.2ab *	28.4±3.6bc **	154.6±17.6a **	721.4±48.7b **
H. schweinitzii	285.0±28.7 NS	53.6±4.1b *	39.0±6.3a **	108.7±13.0bc **	765.2±13.7ab **
H. smithii	296.0±0.0 NS	50.7±0.0b *	32.7±0.0a-c **	104.2±0.0c **	778.4±0.0ab **
H. verticillatus	323.5±27.6 NS	57.6±2.1ab *	25.8±2.0c **	137.6±26.1ab **	744.3±23.0b **

[1]Means followed by the same letter in a column are not significantly different at the P<0.05* and P<0.01** level according to the Duncan's New Multiple Range Test.

One previous report of oil concentration for a single population of *H. porteri* from Georgia was 174 g/kg (Seiler, 1985). This low concentration compared to the 288.7 g/kg concentration in the current study may be due to the achene quality or the one population examined was not representative of the species. The oil content of *H. eggertii* in this study was 285 g/kg, higher than the 222 g/kg concentration reported by Thompson et al. (1981). Achenes from the original populations were evaluated in both studies. The higher oil concentrations observed in the current study may be more representative of the potential genetic diversity of the species than the concentration reported by Thompson et al. (1981) because of the larger number of populations analyzed in the current study. A previous report of oil concentration in *H. schweinitzii* was only 82 g/kg, an extremely low value resulting from a very low achene weight, which may indicate incomplete achene filling (Seiler, 1985). The oil concentration of 285 g/kg in the current study is closer to that normally observed for a perennial sunflower species from this region. The reported oil concentration for *H. smithii* of 296 g/kg from a single population is within the high range of 247 to 296 g/kg previously reported for this species (Seiler, 1985).

Helianthus verticillatus had the highest average oil concentration of any species in the current study. This is the first report of oil concentration and fatty acid composition of this recently rediscovered species. This species is restricted to three sites, two of which were collected for this study. The mean oil concentration in *H. angustifolius* in the current study was 297.5 g/kg, slightly higher than the 252 g/kg

reported for the single population evaluated by Thompson et al. (1981). The few populations evaluated probably do not represent the total genetic variability since only a small portion of its distributional range was sampled. The oil concentration of *H. atrorubens* averaged 277 g/kg, which is within the range of 211 to 320 g/kg based on 15 populations reported by Seiler (1985).

Thompson et al. (1981) concluded that the environments (geographic location) in which collections of different species were made did not influence the oil percentage. The effect of temperature on oil content in sunflower has been shown to be variable. Seiler (1983) showed that environmental factors related to temperature are not related to oil concentration in wild *H. annuus*. Robertson et al. (1979) found that average temperature during full-bloom to harvest stages of field-grown sunflower in North America had no significant effect on oil content of seed obtained from 22 locations in 1976 or 35 locations in 1977. Harris (1978) concluded that oil content decreased as temperature increased; whereas Unger and Thompson (1982) observed a decrease in oil content as temperature decreased.

Oil concentration of interspecific hybrids can be rapidly increased to an acceptable level by backcrossing with cultivated sunflower lines (Seiler and Rieseberg, 1997). Based on this fact, there should be little concern about the lower oil concentration of the wild species when they are used as sources of genes for unique traits.

The 814.9 g/kg linoleic acid concentration for *H. porteri* is the highest of any sunflower species. Previous reports for linoleic acid for this species were 834 g/kg in one population from Georgia (Seiler, 1985) and one population regenerated in France with 818 g/kg (De Haro and Fernández-Martínez, 1991).

The fact that populations of all seven species had linoleic concentrations > 720 g/kg indicates that this trait should have a genetic basis because it is relatively stable in the different populations and species over a wide range of environments. High linoleic sunflower oil with > 700 g/kg is preferred for the production of soft margarine (De Haro and Fernández-Martínez, 1991).

The linoleic fatty acid concentration observed in the *H. porteri* populations and other rare species was unusually high for southern latitudes. High temperatures during flowering, achene filling, and maturation favor a low linoleic acid concentration and a high oleic acid concentration (Seiler, 1986). Generally, the cooler northern latitudes have higher concentrations of linoleic acid in the oil than the warmer southern latitudes (De Haro and Fernández-Martínez, 1991). A lower concentration of 540 g/kg of linoleic acid is more typical of the concentration expected in warmer southern latitudes.

There is a strong negative relationship between linoleic and oleic acid concentrations; i.e., if linoleic increases, oleic decreases (Seiler, 1983; 1986). This relationship is common to both wild and cultivated sunflower. Thus, the high linoleic concentration of 818 g/kg in *H. porteri* is accompanied by a corresponding low concentration of oleic acid near 65 g/kg. Low oleic acid concentrations for *H. porteri* from Georgia, USA were reported by Seiler (1985) with 55 g/kg and by De Haro and Fernández-Martínez (1991) with 60 g/kg for one population grown in France. Reports of oleic concentrations from other populations of *H. eggertii* were 210 g/kg compared to 285 g/kg in the current study (Thompson et al., 1981). For *H. smithii*, it was 190 g/kg for a single population grown in Spain, and 180 g/kg when grown in France (De Haro and Fernández-Martínez, 1991).

The environmental relations between saturated palmitic and stearic fatty acids are less clear than those for linoleic and oleic fatty acids. In a study based on a few wild sunflower species, those collected from northern latitudes had lower saturated fatty acids than those from further south (Seiler, 1994; 1999). The mean palmitic acid concentration ranged from 50.7 g/kg for *H. smithii* to 74 g/kg in *H. angustifolius*, while stearic acid ranged from 25.8 g/kg in *H. verticillatus* to 39 g/kg in *H. schweinitzii*. This is similar to wild *H. annuus* with 51 g/kg palmitic and 31 g/kg stearic acid (Seiler, 1983). The combined palmitic and stearic acid concentration ranged from 83.4 g/kg in *H. smithii* and *H. verticillatus* to a high of 111.9 g/kg in *H. angustifolius*. The saturated palmitic and stearic fatty acids totaling 83 to 88 g/kg are about 30 % less than typical cultivated sunflower oil with approximately 120 g/kg. Lower saturated fatty acids in sunflower oil may be possible by using these species.

CONCLUSIONS

The introgression of wild species into cultivated sunflower with different fatty acid profiles and a stable linoleic concentration could facilitate the expansion of commercial sunflower production into the southern latitudes. There appears to be sufficient variability to introduce and select for high linoleic acid concentration and reduced saturated fatty acid concentrations in cultivated sunflower oil. Further research will be needed to determine the inheritance of fatty acid composition and oil concentration. Other agronomic traits will need to be maintained during the introgression of these traits into cultivated sunflower. The addition of these wild species populations to the wild sunflower germplasm collection

significantly increases the available genetic diversity for improving cultivated sunflower and also insures their future preservation.

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Cytogenetic study of an F_1 sunflower interspecific hybrid (*Helianthus annuus* x *Helianthus praecox*)

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ABSTRACT

Annual species *H. praecox* (15 populations from the Novi Sad collection) has been crossed with cultivated sunflower (HA26). F_1 interspecies hybrids were obtained with three populations (1801, 1819 and 1824) and their morphology and cytology were studied. Plants of F_1 hybrids were most often more similar phenotypically to the wild parent or intermediate. The analysis of meiosis revealed that the crossed species differed for the chromosome level. Besides bivalents, multivalents (quadri- and hexavalents) were most often found in diakinesis. Trivalents and univalents were rarely observed. Frequently observed chromosome configurations in diakinesis were 15II 1IV and 13II 2IV. Fast and lagging chromosomes in metaphase I and anaphase, as well as chromosome bridges and fragments were found in a large number of meiocytes. Pollen viability in F_1 interspecies hybrids was low (8.3, 9.8 and 11.2%). The results suggested that the species *H. praecox* and *H. annuus* differ by 1-2 translocations and 1-2 inversions.

Key words: *H. praecox* – interspecies hybrid – meiosis – pollen viability.

INTRODUCTION

Helianthus praecox Engelm. and A. Gray (premature sunflower), is a species described by Heiser et al. (1969) and Rogers et al. (1982). It contains three subspecies, which are geographically isolated but all within the state of Texas (USA). The three subspecies *praecox* Engelm. and A. Gray, *runyonii* Heiser and *hirtus* Heiser, intercross easily and produce fertile hybrids. The flowering occurs from early summer to late fall. They are branched, with a large number of inflorescences on long peduncles and with rough to stiff hairs on slightly serrated leaves. They are very attractive as an ornamental species. Their natural and artificial hybrids with other annual species are well known. Haploid chromosome number is n=17.

According to the literature, *H. praecox* has shown resistance to two races of *Plasmopara halstedii*, to *Puccinia helianthi* and *Erysiphe cichoracearum* as well as tolerance to *Phomopsis helianthi*. High oleic acid content has also been reported for this species next to *H. argophyllus* and *H. annuus* (Seiler, 1992).

The hybridization potential of *H. praecox* has been tested by many authors, mainly to determine the similarities with other species of the *Helianthus* genus and to study their phylogenetic relations. Heiser et al. (1969) found that the species *H. praecox* is similar to species *H. debilis*, *H. petiolaris* and *H. neglectus*, which was confirmed by the results of Chandler et al. (1986).

The results of interspecific hybridization of *H. praecox* with cultivated sunflower has been published by Georgieva-Todorova (1990), Christov (1991), Jan (1997), Nikolova et al. (1998) and Iouras et al. (2002). A large hybridization program with the goal of transferring the desired traits from the species *H. praecox* has been done by Atlagić (1986) and Terzić (2006). A significant number of researchers obtained F_1, BC_1F_1 - BC_4F_1 hybrids between *H. praecox* and the cultivated sunflower. They gave a morphological description of the interspecies hybrids, but without a cytogenetic description of the hybrids.

The objective of this paper was to establish the possibility of *H. praecox* usage in cultivated sunflower breeding, through analyzing the cross compatibility and morpho-cytogenetic traits of F_1 interspecies hybrids.

MATERIALS AND METHODS

Fifteen populations of the species *H. praecox* (1145, 1151, 1168, 1181, 1142, 1333, 1340, 1341, 1342, 1801, 1819, 1812, 1824, 1826 and 1828) from the collection of wild sunflower species in Novi Sad were crossed in a classical fashion with cultivated sunflower lines (direct and reciprocal crosses). The F_1 seeds of the interspecific hybrids obtained were sown in the field. The plants of parental species and of F_1 interspecies hybrids were analyzed morphologically and samples for the analysis of meiosis and pollen viability were taken.

Meiosis was analyzed using acetocarmine method (Georgieva-Todorova, 1990) and the pollen viability with a coloring method (Alexander, 1969).

The results of meiosis are shown through chromosome configuration in diakinesis and the regularity of meiosis through other stages. Pollen viability is shown as a percentage of viable pollen grains in comparison to the total number.

RESULTS AND DISCUSSION

Of the 15 populations used in this study (71 inflorescences), 25 seeds were obtained on 11 inflorescences. Hybrid F_1 plants (2 to 12) were obtained in 3 hybrid combinations between the CMS line HA26 and the populations 1801, 1819 and 1824 of *H. praecox*.

The plants of F_1 interspecies hybrids were phenotypically similar to *H. praecox*. F_1 plants were higher than both parents; the disk diameter was similar to the wild parent; petiole length and leaf length were either similar to the cultivated sunflower or greater than both parents; leaf width was intermediate.

The analysis of meiosis of the *H. praecox* populations and the cultivated sunflower line HA26 showed that the meiosis proceeded normally with 17 bivalents in diakinesis and without irregularities in other stages. On the contrary, a high percentage of cells with irregularities was evident in F_1 plants. The analysis of diakinesis showed that the percentage of meiocytes with normal chromosome pairing (17II) was 12.5, 13.84 and 27.27%, while a significantly larger percentage of cells contained multivalent chromosomes (72.72, 73.84 and 87.50%). The most frequent multivalents were quadri- and hexavalents and the most frequent chromosome configuration in F_1 interspecies hybrids was 13II 2IV (27.69% of meiocytes in hybrids with *H. praecox* 1801), followed by 15II 1IV (31.81% of meiocytes in hybrids with *H.praecox* 1819 and 37.5% of meiocytes in hybrids with *H. praecox* 1824). Results of the meiotic analysis for each studied plant of the F_1 hybrids are shown summarized for each crossed population and given as a percentage (Table 1).

Table. 1. Analysis of meiosis and pollen viability of F_1 interspecific hybrids (*H. annuus* x *H. praecox*)

		F_1PRA - 1801	F_1PRA - 1819	F_1PRA - 1824
Chromosome configurations in diakinesis		17^{II}(9); **$13^{II}2^{IV}$(18)**; $15^{II}1^{IV}$(16); $14^{II}1^{VI}$(6); $12^{II}1^{IV}1^{VI}$(5);$12^{II}2^{IV}2^{I}$(4); $13^{II}1^{IV}1^{III}1^{I}$(3); $11^{II}3^{IV}$(1); $11^{II}1^{IV}2^{III}1^{I}$(1); $12^{II}3^{IV}$(1); $8^{II}3^{VI}$(1)	17^{II}(6); **$15^{II}1^{IV}$(7)**; $13^{II}2^{IV}$(4); $14^{II}1^{VI}$(4); $12^{II}1^{IV}1^{VI}$(1)	17^{II}(3); **$15^{II}1^{IV}$(9)**; $14^{II}1^{VI}$(6); $13^{II}2^{IV}$(4); $12^{II}1^{IV}1^{VI}$(2)
most frequent configuration (%)		27.69	31.81	37.50
meiocytes with bivalents (%)		13.84	27.27	12.50
meiocytes with multivalents (%)		73.84	72.72	87.50
Percentage of meiocytes				
Metaphase I	Normal	76.72	77.78	73.33
	Fast chromosomes	23.27	22.22	26.67
Anaphase I	Normal	64.81	66.67	69.44
	Lagging chromosomes	20.72	20.51	22.22
	Chromosome bridges and fragments	14.46	12.82	8.34
Telophase II	Normal	48.71	52.63	57.14
	Lagging chromosomes	14.72	21.05	9.52
	Apolar with micronuclei	34.97	26.31	33.33
Pollen viability (%)		8.30	11.47	9.80

The results showed that the number of quadri- and hexavalents was variable (1-3), while tri- and univalents were rare in diakinesis. The following stages of meiosis also had a high percentage of irregularities (Table 1). The metaphase I was usually with 1 or 2 fast chromosomes (22.22, 23.27 and 26.67% of meiocytes). The anaphase I showed high percentage of meiocytes with lagging chromosomes (20.51, 20.72 and 22.22%), as well as meiocytes with chromosome bridges and fragments (8.34, 12.82 and 14.46%). In telophase II, meiocytes with lagging chromosomes were found (9.52, 14.72 and 21.05%), as well as apolar ones with micronuclei (26.31, 33.33 and 34.97%).

Plants of the F_1 interspecies hybrids were male fertile. Considering that a male sterile cultivated line HA26 was used in the crosses (PET-1 sterility source) it can be concluded that the *H. praecox* populations (1801, 1819 and 1824) contain fertility restoration genes. Pollen viability in the analyzed interspecific hybrids was extremely low (8.30, 9.80 and 11.17%) in comparison to the parental species (> 90%).

The meiotic irregularities throughout the stages cannot be discussed in relation to the origin of the hybrids because the results were not conclusive enough (PRA 1801 and PRA 1819 from the same subspecies *runyonii*, while PRA 1824 is ssp. *praecox*).

While reviewing the phylogenetic relations in the *Helianthi* section, most of the authors found that the annual species are close to each other so that their hybridization is possible. The results of the presented work show that a small number of hybrid combinations and plants of F_1 interspecies hybrids was obtained after a large number of crosses had been made. Even though *H. praecox* is an annual diploid species like the cultivated sunflower, a certain degree of cross incompatibility is present. The results of hybridization between *H. praecox* and the cultivated sunflower reported so far also pointed to this conclusion. Škorić et al. (1988a) used 30 populations and obtained interspecific hybrids with only 2 of them, developing 7 hybrid combinations in F_1, 10 in BC_1F_1 and 9 in BC_1F_1 generations. A large hybridization program done by Bulgarian researchers resulted in 6 hybrid combinations between the species *H. praecox* and *H. annuus* (Nikolova et al., 1998) and with a note that the hybridization success is greater if *H. annuus* is used as a mother. Jouras et al. (2002) published results of interspecific crosses between wild and cultivated sunflower (ten year trial period) that had the objective of transferring the resistance to *Sclerotinia* and *Phomopsis*, where the species *H. praecox* was successfully crossed, and, consequently, F_1BC_1 and BC_2 generations were obtained. Interspecific hybrids between *H. praecox* and *H. annuus* shown in the papers of the quoted authors were not analyzed cytogenetically. The similarity of the crossed species was determined on the basis of cross compatibility and morphological similarity.

The results of cytogenetic analysis of F_1 hybrids obtained in this work from the crosses between *H. annuus* and *H. praecox* pointed to the existence of large differences between these two annual species. Besides bivalents, quadri- (1-2) and hexavalents (1) were the most frequent in diakinesis, with only occasional appearance of trivalents (1-2) and univalents (1-2). These findings confirm the results of Chandler et al. (1986) who, while studying the relations between annual species of the genus *Helianthus*, found that the most frequent multivalents in interspecific hybrids between *H. annuus* and *H .praecox* were VI + IV, VI, 2 IV and a IV. A high percentage of meiocytes with non included chromosomes in metaphase I, anaphase I and telophase II, as well as a high percentage of meiocytes with chromosome bridges and fragments, also suggest the existence of non homologous chromosomes between the species *H. praecox* and *H. annuus*. The results of Chandler (1979) indicated a high frequency of chromosome bridges and fragments in the same interspecies hybrid (0/5, 1/12, 2/6, 3/4, 4/4), which is an indication of chromosomal structural differences of the inversion type. After summarizing the results of cytogenetic analysis, this author divided the annual species into two groups by the frequency of chromosome bridges and fragments: I (*H. annuus, H. debilis, H. neglectus, H. niveus* and *H. praecox*), II (*H. annuus* and *H. argophyllus*). On the basis of quadrivalent presence for the species *H. annuus*, he stated that this differs in 2-6 translocations from the other annual species. The species *H. praecox* is, comparing its morphology and chromosome behavior, closely related to *H. debilis* and *H .petiolaris*. Chromosome pairing suggested that the species is related closely to *H.neglectus* (Heiser at al., 1969). While analyzing the meiosis in *H. praecox* x *H. annuus* hybrids, Georgieva Todorova (1990) found a configuration of 15II, 1IV in only 9 out of 269 PMC (pollen mother cells). Previous findings (Chandler, 1979; Chandler et al., 1986; Georgieva-Todorova, 1990), together with the results shown in this paper, indicate that the species *H. annuus* and *H. praecox* differ in 1-2 translocations and 1-2 paracentric inversions.

The fertility restoration by *H. praecox* for PET-1 source of cytoplasmatic sterility found in this work has previously been indicated by Škorić et al. (1988b), who reported that certain populations of the species *H. praecox* contain heterozygous genes for restoration of not only PET-1 but also of some other CMS sources (CMG-1, CMG-2, CMG-3, Indiana - 1). Low pollen viability in F_1 interspecific hybrids *H. annuus* x *H. praecox* is certainly influenced by a high percentage of meiotic irregularities and it has

previously been documented in the research performed by Chandler (1979) (2.1-4.2%) and Georgieva Todorova (1990) (11.1-61.80%).

Detailed cytogenetic analysis of F_1 interspecies hybrid *H. annuus* x *H. praecox* has shown that, even though it is an annual diploid species, *H. praecox* differs significantly chromosomally from *H. annuus*. The results of previous research together with the results given in this paper about low cross compatibility, dominant inheritance of some undesired traits, differences in chromosome structure that induce meiotic irregularities and lowered pollen viability are serious difficulties in the way of using *H. praecox* as a source of desired genes for the breeding of cultivated sunflower.

ACKNOWLEDGEMENTS

This work was supported by funds of the Ministry of Science. The authors are grateful to Jasminka Pilipović for technical assistance with the laboratory work.

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Sunflower nested core collections for association studies and phenomics

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ABSTRACT

In order to develop association studies and to improve the phenotypic description for abiotic and biotic stress related traits, nested core collections of 48, 96, 144 and 384 sunflower lines were built from a set of 752 diverse, public or private accessions. These 752 lines were genotyped with 51 SSR markers covering the genetic map (3 markers/linkage group). We then used MSTRAT software to construct 4 nested core collections as follows: we built a first core with 48 public lines and a kernel of 12 selected entries, accounting for 47% of the total diversity. This short core collection was then used as a kernel to define a second core with 96 public entries, accounting for 59% of the total diversity. Finally, the private entries were added to build a core collection of 144 and 384 entries, accounting for 78% and 100% of the total diversity, respectively. The INRA lines belonging to the "96" core collection are available for research to any public institution under a Material Transfer Agreement.

Key words: core collection – *Helianthus annuus* – MSTRAT software – sunflower.

INTRODUCTION

The development of association studies is becoming very popular to discover relationships between genetic polymorphism and functional variability, either on candidate genes or genome-wide, in human, animal and plant genetics (e.g. Sanguineti et al., 2007; Flint-Garcia et al., 2005; Mckhann et al., 2004). Although less robust than establishing such a relationship through Linkage Mapping in a recombinant population, this approach makes it possible to scan a wider diversity.

As a lot of genomic resources have been or will be developed for crop species, including sunflower, the pertinence and the accuracy of phenotypic description is becoming a potentially strong limiting factor when association studies are developed. Thus it appeared to be useful to establish core collections or panels of different sizes, to establish on a small or medium size panel a correlation between a low throughput phenotyping method and a higher throughput, more adapted to the phenotyping of large core collections.

This paper describes the method we used to build nested core collections of different sizes from 752 sunflower inbred lines, and to provide the list of two nested collections (48 and 96 lines) containing public lines or INRA lines made available to the scientific community.

MATERIALS AND METHODS

Genetic material

The initial genetic material used to build the core collections comprises:

- 350 inbred lines collected or created by INRA at Clermont-Ferrand (F. Vear, P. Leclercq) and at Montpellier (H. Serieys, G. Piquemal), and selected by F. Vear according to their *a priori* phenotypic diversity and to their interest for breeding, from a total list of 2,300 lines maintained by INRA.
- 500 private, inbred lines listed by three seed companies (RAGT Génétique, SOLTIS, SYNGENTA Seeds), some being elite lines, used as parental lines in hybrid development, the others being created as introgression of wild *Helianthus* ecotypes into an elite line.

Molecular data
All the 850 entries were genotyped with 51 single locus and highly robust SSR markers by the three seed companies involved in the project. A detailed list of these SSR is available at http://lipm-helianthus.toulouse.inra.fr/Web/core/marker_core.xml.

Computations
We firstly chose a set of 12 inbred lines for their *a priori* diversity and their interest as resources for different research purposes (Table 1). We checked whether this choice would affect the diversity of the core collections in locating these lines against the first axis of a Correspondence Analysis made on a table of qualitative data (presence/absence) having the number of genotypes as first dimension, and {locus*alleles} as second dimension.

Then we used MSTRAT (Gouesnard et al., 2000) on qualitative, molecular data, to define the following nested core collections:

- 48 lines with the constraint of including the kernel constituted by the 12 lines listed in Table 1 and of including only public or INRA lines,
- 96 lines with the constraint of including the core collection of 48 lines, and of including only public or INRA lines,
- 144 lines with the constraint of including the core collection of 96 lines, and no other constraint,
- 384 lines with the constraint of including the core collection of 144 lines, and no other constraint.

For each step, 30 iterations and 30 replicates were used, and, as recommended by the authors of MSTRAT, we chose the most represented accessions within the 30 replicates.

Table 1. Lines included as the kernel in the 48 core collection built with MSTRAT. 'Line' indicates the line code used for the genotyping, 'Type' indicates if lines restore the male sterility on PET1 cytoplasm (R) or maintain the PET1 cytoplasmic sterility (B), Origin indicates the line pedigree

Line	Type	Code	Origin	from
SF056	B	RU	Selection from Romanian origins	INRA
SF085	B	CD	HA89	USDA
SF107	B	92A6	*H. argophyllus* introgression	INRA
SF109	B	2603	Moroccan population	INRA
SF193	B	XRQ	HA89 * Progress (Russian open pollinated variety)	INRA
SF268	R	RHA266	RHA266	USDA
SF302	R	PAC2	PET1 restorer from H.petiolaris, HA61	INRA
SF306	R	PAZ2	PET1 restorer from H.petiolaris, AD66, HA61, Zambia population	INRA
SF310	R	PST5	Recurrent selection for *Sclerotinia* head-rot resistance	INRA
SF317	R	83HR4	Russian origin * RHA274	INRA
SF326	R	PSC8	Recurrent selection for *Sclerotinia* head-rot resistance	INRA
SF332	R	RHA274	RHA274	USDA

Finally, we checked with STRUCTURE (Pritchard et al., 2000) as to whether the groups identified by this MSTRAT were correctly represented.

RESULTS

From molecular data
As the lines were described as homozygous, we expected to find only a few heterozygous loci from the SSR data. Still, about 100 accessions, presented more than 10% of heterozygous loci according to SSR data. We discarded these lines for further analysis to obtain the highest confidence in genotypes – as described by SRR – of the resulting core collections.

Effect of the kernel content on the richness of core collections
The 12 lines chosen as members of the kernel in the first step of MSTRAT represented high diversity. They appeared as quite divergent for the first two axes of the Correspondence Analysis (Fig. 1). As previously found (Tang and Knapp, 2003), the two historic US lines HA89 and RHA274 appeared to be

the most divergent ones within this set of oilseed lines. The group of "R" lines – i.e. restoring the male sterility on PET1 cytoplasm - seemed to be divided into two classes (R1, R2). Some "B" lines – i.e. maintaining the PET1 cytoplasmic sterility, such as 2603 or 92B6 which includes some *Helianthus argophyllus* background, seemed to be related to the "R2" group. The lines XRQ and PSC8, FU and PAZ2, RHA266 and PAC2 are parental lines of RIL populations developed by F. Vear at INRA Clermont-Ferrand; together, these RIL populations should account for a large part of the genetic variability, as they reflect the polymorphism exhibited when crossing "B" with "R2", "B" with "R1, "R1" with "R2 respectively.

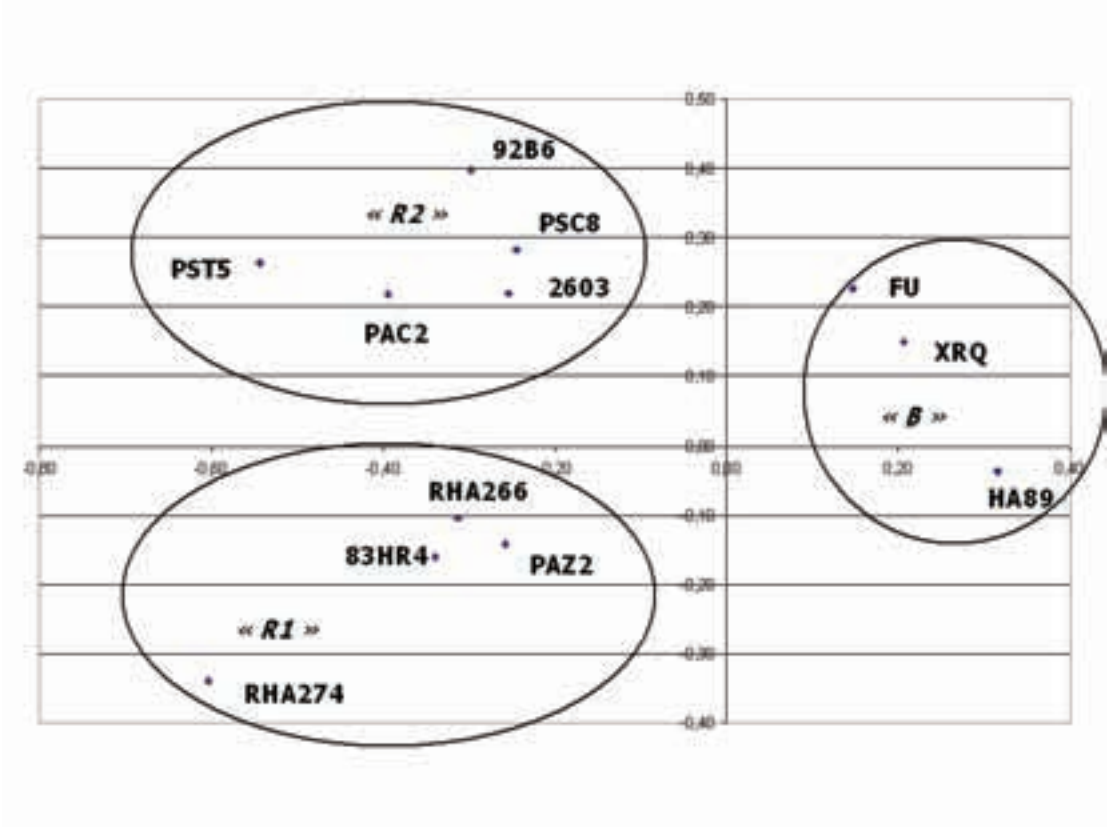

Fig. 1. Representation of the lines included in the "kernel" used to build the core collections with MSTRAT, in the two first axes of the Correspondence Analysis.

Another way to evaluate the effect of the kernel content on the richness of the core collection is to compare the score of the kernel with the score of the best core collection of 12 lines within either the set of public and INRA lines, or the total set of lines. As shown in Table 2, selecting the kernel reduced the richness only by 25-30% compared to the best "12" core collections.

Table 2. Comparison of the relative richness associated with the initial kernel of 12 lines with the score of the best 12 subsets in the set of public or INRA lines, and in the global set.

	Kernel	Best core
Public and INRA	9,0%	11,9%
All	6,1%	8,5%

Core collections

The first core with 48 public lines, including the previously described kernel of 12 entries, accounts for 47% of the total diversity. The core collection of 96 entries, which used the "48" core collection as a kernel, accounts for 59% of the total diversity.

The lists of "48" and "96" core collections are provided at http://lipm-helianthus.toulouse.inra.fr/Web/core/Core_collections_list.html

Allowing lines originated from the private seed companies to be added, led to core collections of 144 and 384 entries that account for 78% and 100% of the total diversity, respectively. Indeed, a 100%

richness of diversity was obtained with only 220 lines. The lines made by the introgression of wild background into sunflower line greatly explain the contribution of the private lines.

When we look at the repartition of the members of the "48" or "96" core collection between the 10 classes defined by STRUCTURE, it appears that only 8 out of the 10 classes are represented. For the "144" core collection, which included more diverse genetic backgrounds, all the 10 classes are represented.

DISCUSSION

As genomic resources are now available for non-model crop plants, the phenotyping activity has become a strong challenge for the workflow efficiency, when linking sequence polymorphisms to the functional evidences. For traits such as yield or adaptation to the abiotic environment, there is no simple criterion to measure, with a high throughput approach, in the field and/or on a large set of genotypes and environment. Building nested core collections of different sizes will make it possible 1) to establish correlations, on a short set of genotypes chosen for their ability to represent a significant part of the polymorphism, between low throughput physiological criteria and higher throughput indirect measurements, 2) to apply these higher throughput indirect measurements for association studies implying a wider genetic diversity.

These core collections certainly need to be improved for the important gap between the 96 and 144 collections and for the type of data used to build them.

The gap in richness between the "96" core collection and the "144" core collection is mainly due to the introgression lines provided by the seed companies. Introgression lines created by H. Serieys at INRA Montpellier from different wild species will be analyzed with the aim of enriching the "96" core collection.

In addition to the marker data, qualitative or quantitative information can be used to build the core collections with the MSTRAT algorithm. The qualification of core collections can be improved in an iterative process where phenotypes recorded on primary core collections are progressively used to check whether there is enough diversity for a particular trait of interest and thus develop *ad hoc* core collections.

ACKNOWLEDGEMENTS

We thank Brigitte GOUESNARD (INRA, UMR DIA-PC, Montpellier) for her helpful comments about MSTRAT.

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Using interspecific hybrids with *Helianthus tuberosus* L. to transfer genes for quantitative traits into cultivated sunflower, *H. annuus* L.

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ABSTRACT

Interspecific hybrids in sunflower have been used historically as sources of disease resistance. Backcrossing for several generations is required to introgress genes from hexaploid species to annual sunflower (*Helianthus annuus* L.). A result of this repeated backcrossing is the rapid loss of genes from the donor parent. This article describes ongoing work to improve the efficiency and efficacy of introgressing genes from the hexaploid perennial *H. tuberosus* L. for quantitative traits. Of particular interest are genes which control host-plant resistance to diseases and insect pests, and perennial habit.

Key words: disease resistance – *Helianthus annuus* L. – *Helianthus tuberosus* L. – insect resistance – interspecific hybrid – perennial sunflower.

INTRODUCTION

Interspecies hybrids are commonly developed to introduce traits of interest, most notably disease resistance, into the domesticated annual sunflower (*Helianthus annuus* L.). Of the perennial sunflowers, the Jerusalem artichoke (*H. tuberosus* L.) has been a commonly utilized donor species historically. This is most likely due to its world-wide presence as a naturalized species and its ability to form hybrids easily with *H. annuus*. There are several notable discoveries of disease resistance introgressed from *H. tuberosus*. Resistance to downy mildew (caused by *Plasmopara halstedii*) has been transferred from *H. tuberosus* to *H. annuus* by Pustovoit et al. (1976). This germplasm was the source of the *Pl5* downy mildew resistance gene (Miller and Gulya, 1987). Resistance to Phomopsis stem canker (caused by *Phomopsis helianthi*) has also been discovered in *H. tuberosus*, and germplasms have been developed in Europe that have a high degree of resistance to Phomopsis (Škorić, 1985). Resistance to Phomopsis is important to American sunflower breeders as this disease has caused notable losses in recent years in the USA. Resistance to Sclerotinia head rot (caused by *Sclerotinia sclerotiorum* (Lib.) De Bary) was transferred from *H. tuberosus* into interspecific populations in Russia (Pustovoit et al., 1976), and has been an important source of resistance. Additionally, *H. tuberosus* has been shown in trials to produce antixenosis to oviposition and antibiosis to larva of the banded sunflower moth (*Cochylis hospes* Walsingham), an insect which damages the heads of sunflower (Charlet and Brewer, 1995). This insect can cause significant damage, and resistance is of particular interest to sunflower breeders in the northern production area in the USA.

There is also interest by some U.S. researchers to develop perennial crops for use in a permaculture system, particularly in areas where soil erosion and leaching or runoff of fertilizers has caused environmental damage. Perennial crops provide living ground cover for longer periods during the growing year because the plants often emerge from dormancy long before annual crops are planted. This characteristic of perennial crops has been linked to reduced tile drainage flow and concurrent leaching of nitrogen from the soil (Randall et al., 1997). Perennialization of domesticated sunflower was first proposed by Ščibria (1936), who noted that populations of *H. tuberosus* X *H. annuus* could be candidates for development of a perennial sunflower. The investigator also noted the tendency for the F_1 hybrids to show hybrid vigor or heterosis. While hybrid populations have been formed by breeders in the past, no one has published specifically on the mode of inheritance of perennial habit in such populations. It is currently assumed to be under the control of many genes, and as such, may act as a quantitative trait. There has been mention of rhizome/tuber production in some populations, while in other populations there was no production of rhizomes or tubers (Cedeno et al., 1985; Kräuter et al., 1991). The populations with tuber production tend to have *H. tuberosus* as the female, indicating that perennial habit may have, at least, partial cytoplasmic inheritance.

Previous investigators have found that the F_1 hybrid plants are tetraploid ($2n=4x=68$) and the BC_1F_1 plants are triploid ($2n=3x=51$) (Cedeno et al., 1985). Low fertility was found in the BC_1F_1 plants, which was likely due to the triploid condition. Further backcrossing in populations like these is expected to result in progeny with greater meiotic stability through progressive loss of chromosomes.

The objective of this work is to determine the feasibility of introducing quantitative traits into domesticated sunflower using *H. tuberosus* as the genetic source of those traits. Traits of interest include resistance to diseases and insect pests, as well as perennial habit and traits of agronomic interest.

MATERIALS AND METHODS

Germplasm. Tuber stock of 18 *H. tuberosus* plants were collected in 2001 from UMORE Park, Rosemount, MN, USA. Nine of the plants were collected from cultivated areas and were named JA1 to JA9. Nine more plants were collected from undisturbed areas approximately 8 km from the first site of collection. These were named JA10 to JA18. All plants were transplanted to plots at the St. Paul Agricultural Experiment Station, St. Paul, MN, USA, where they still exist as a living plant collection. All plants are believed to be native to the area in which they were collected in east central Minnesota. In addition, seed of *H. annuus* inbred lines RHA 265, RHA 274, CMS HA 89, HA 89, CMS HA 434, and HA 434 were obtained from Gerald Seiler and Jerry Miller, USDA-ARS, Fargo, ND, USA, over the course of the project.

Experiment 1. This experiment was initiated to generate and study a maintainer (B-line) interspecific population. During the summer of 2003, seed of RHA 265, RHA 274, and CMS HA 89 were grown in the field in St. Paul, MN. Crosses were made in the following manner: JAx/RHA 265, JAx/RHA 274, and CMS HA 89/JAx, where 'x' = 1 to 18. Each pairwise cross was maintained as a separate pedigree, and grown in a greenhouse during the winter of 2003-2004. Sib-mating among the plants within each full-sib family was attempted. During the summer of 2004, the F_1 plants of the crosses JAx/RHA 265 were transplanted to the field and pollen from HA 434 was used to pollinate the F_1 plants. Backcrosses were made with HA 434 as the pollen parent for three more generations, resulting in the equivalent of a BC_4F_1 population. This population was grown at the St. Paul station during the summer of 2006, and plants were self-pollinated.

Experiment 2. This experiment was initiated to generate and study a fertility-restorer (R-line) interspecific population. The F_1 progenies of the crosses JAx/RHA 265 were backcrossed in the greenhouse during the winter of 2004-2005 with RHA 265 as the pollen source. A second backcross was made on the BC_1F_1 plants during the summer of 2005. The BC_2F_1 plants were grown in the greenhouse during the winter of 2005-2006 and backcrossed again with RHA 265 as the pollen parent. The BC_3F_1 plants were grown in the field during the summer of 2006 for observation.

Experiment 3. This experiment was initiated to generate and study two sets of reciprocal maintainer populations of sunflower. The initial crosses were as follows: JAx/HA 89, CMS HA 89/JAx, JAx/HA 434, and CMS HA 434/JAx. The CMS lines contribute the French male-sterile cytoplasm of Leclercq (1969). The JA lines contribute *H. tuberosus* cytoplasm. At the same time, tetraploid derivatives of HA 89 and HA 434 were obtained by treating seedlings of the two inbred lines with colchicine, according to the protocol of Jan and Chandler (1989). Successfully doubled plants were pollen donors for a backcross with the F_1 plants. This procedure was adopted to avoid a sterility barrier observed in the BC_1F_1 plants in the previous experiments, believed to be due in part to the triploid nature of the BC_1F_1 plants. By crossing tetraploid F_1 plants with tetraploid inbred lines, we could obtain tetraploid BC_1F_1 plants, which we distinguished from standard BC_1F_1 plants using the notation $BC_1F_1^{(4x)}$. The $BC_1F_1^{(4x)}$ plants were grown in the field in 2006, and attempts were made at self pollination, sib-mating within full-sib families, and backcrossing with tetraploid HA 89 or HA 434 as the pollen donor.

RESULTS

Experiment 1. F_1 hybrids between the JA plants and the inbred lines were highly successful, as previously reported. Pollen production was observed on some of the F_1 plants. Sib-mated F_1 plants did not produce many seeds, and the seeds that were obtained produced plants with little vigor. Despite the lack of vigor, the progeny were perennial as determined by regrowth in the field after the winter of 2005-2006.

Backcrosses with the inbred line HA 434 as the pollen parent resulted in viable offspring. These BC_1F_1 plants were triploid and male sterile in appearance. Recurrent backcrossing was performed on the BC_1, BC_2, and BC_3 plants because the plants were male sterile and possessed considerable female sterility. A single BC_4F_1 plant survived to maturity in the field in 2006. The head was bagged and selfed seed was produced, indicating that the plant produced viable pollen. Most of the harvested BC_4F_2 seed was not filled, indicating that some sterility due to cytogenetic imbalance still exists in the population. The restoration of male fertility in the BC_4F_1 generation is common in hexaploid X diploid *Helianthus* populations (C.C. Jan, personal communication). None of the backcross generations beyond the F_1 appeared to produce perennial organs (rhizomes or tubers), and the BC_1F_1 and BC_2F_1 plants failed to recover from the winter of 2005-2006 in the field, indicating loss of perennial habit in the population. By comparison, the *H. tuberosus* parents and F_1 population had nearly 100% survival after every winter since 2004. The population will be maintained for phenotypic evaluation of other traits of interest.

Experiment 2. The BC_1F_1 plants of the R-line population were grown in the field in 2005. The plants were generally short in stature and lacked vigor. During the time in which backcrosses were being made, it was observed that some plants within the JA9/RHA 265//RHA 265 population produced pollen. These plants were sib-mated, but produced no viable seed. The BC_1F_1 plants did not survive the winter of 2005-2006, a result similar to that in Experiment 1, which indicates that the plants were not perennial. The BC_2F_1 and BC_3F_1 generations were obtained by backcrossing with RHA 265 pollen, and neither generation had pollen production. No additional work has been performed on this population since 2006, due to lack of different results. It is likely that fertility will be restored if the BC_4F_1 generation is produced.

Experiment 3. The F_1 plants of the reciprocal populations were grown in the greenhouse, and perennial organs were observed both in plants with the *H. tuberosus* cytoplasm and plants with the *H. petiolaris* (French) cytoplasm. Both sets of F_1 plants were transplanted to the field in 2006, and almost 100% of the plants survived the winter of 2006-2007. The $BC_1F_1^{(4x)}$ generation was grown in the field in 2006. The estimated number of $BC_1F_1^{(4x)}$ plants was around 1000, which was, by far, the largest BC_1F_1 nursery obtained in our experiments. The plants possessed considerable vigor, except for one pedigree JA13/HA 434//HA $434^{(4x)}$, which seemed to be shorter in stature and lacking vigor. The height of the plants of the other pedigrees was similar to that of the annual inbred lines (Fig. 1). Pollen production was observed on many of the plants. Self-pollination and sib-mating within full-sib families was attempted on most of the plants. A small subset of the plants had evidence of rhizome production, especially in the HA 89-derived populations. These plants were backcrossed using pollen from the tetraploid annual inbred line. Despite the vigor of these lines, very few seeds were produced. The viability of this seed is currently being investigated. Eight plants from these populations were perennial, because they were able to regrow after the winter of 2006-2007. These plants have been transplanted to a permanent collection.

Fig. 1. Appearance of $BC_1F_1^{(4x)}$ plants in the 2006 field nursery. The annual inbred parents appear on the left alf of the photo and the $BC_1F_1^{(4x)}$ plants on the right half of the photo.

DISCUSSION

Experiments 1 and 2 represent a backcrossing procedure that has been used in the past to introgress genes of interest from perennial hexaploid species to annual breeding germplasm. This procedure has been used repeatedly worldwide to introgress genes from *H. tuberosus* and other perennial species into *H. annuus*. Most of the traits introgressed by this method are disease resistance traits, although Pustovoit et al. (1976) noted heterosis for yield in hybrids with *H. tuberosus* genes.

The more genes involved in producing a trait, the more difficult it becomes to transfer the trait to a finished line in a backcross program. Most of the disease resistance traits are controlled by one or a few genes or gene blocks. The genetics for perennial habit are likely to be more complex. Hu et al. (2003) discovered that two dominant genes caused production of rhizomes in a rice interspecific hybrid population, with 14 other QTL modifying the characteristics of the rhizomes, including the number, branching habit, length, and size. Because backcrossing rapidly causes loss of genes from the donor species (*H. tuberosus*, in our case) and we have not successfully carried perennial habit through even one standard backcross in Experiments 1 and 2, it is likely that the genetics for perennial habit are at least as complex as that of rice. Until we develop a population with a stable genome and segregation for perennial habit, it will be impossible to perform a similar study in sunflower. In the meantime, the population produced from Experiment 1 will be useful in finding at least partial resistance to diseases and insect pests common to the USA.

The findings of Experiment 3 were more promising for studying perennial habit. First, it appears that perennial cytoplasm is not required to produce perennial plants, because those plants with the French cytoplasm were also perennial. Second, using a tetraploid version of the annual inbreds as the pollen donor in a backcross resulted in a larger BC_1 population that was more vigorous and more closely resembled the annual parent in appearance. It also resulted in 8 out of approximately 1000 plants that were successful perennials, thus indicating that this modified backcross procedure helped to better retain the required genes for perennial habit. This could be due to the fact that a larger population was obtained from these crosses, thus providing more observations. It could also be due to the fact that the tetraploid genome was more stable than the triploid genome of a standard BC_1 population, resulting in less gene silencing. Gene silencing via DNA methylation in interspecific sunflower populations is a documented event (Natali et al., 1998). A discouraging finding is the lack of seed formation in the $BC_1F_1^{(4x)}$ populations. This may be explained by our method of sib-mating, which was to intermate only those plants within a full-sib family. Despite introducing genes for self fertility, it is possible that self-incompatibility genes are still active in almost all of the plants in these populations. If this is true, pollen from full-sib individuals in the population would have been rejected because they would share the same

self-incompatibility alleles. Intermating half-sibs with different *H. tuberosus* parents may improve seed production, and will be attempted on the perennial $BC_1F_1^{(4x)}$ plants. The reason for failure of a second backcross with the tetraploid inbred lines is unknown, but is likely due to mispairing of the chromosomes in the $BC_1F_1^{(4x)}$ plants. Based on chromosome pairing in F_1 interspecies hybrids, Kostoff (1939) determined that the genomic formula of *H. tuberosus* is At_1At_2Bt, where the Bt genome is closely related to the *H. annuus* genome. Thus, the F_1 would have nearly normal pairing, but the $BC_1F_1^{(4x)}$ plants would have abnormal pairing because the At genome is only present in a haploid form. More attempts at backcrossing the $BC_1F_1^{(4x)}$ plants will be made in the future, possibly in combination with embryo rescue.

Rieseberg et al. (1995) theorized that sib-mating interspecific sunflower populations before backcrossing may allow for introgression of more and smaller blocks of genes from the wild parent. In our situation, this may mean that more of the necessary perennial genes may be transferred with greater ease and less linkage drag if backcrossing is attempted after sib-mating the F_1 population for a few generations. More genes for disease, insect, and agronomic traits may be introduced into the *H. annuus* genome, as well. The process of sib-mating half-sib families has already begun, with the first generation of sib-mated progeny already produced. Information from our current and ongoing backcrossing experiments will allow us to perfect a backcross procedure for use after sib-mating to maintain high levels of genetic variability in the population.

ACKNOWLEDGEMENTS

The authors wish to thank Kevin Betts for his assistance in making crosses, maintaining field plots, and supervising student workers. This work has been made possible by graduate student grants from The Land Institute, Salina, KS, USA; and North Central Region – Sustainable Agriculture Research and Education (NCR–SARE).

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2008 update: The USDA sunflower collection at the north central regional plant introduction station, Ames, IA, USA

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ABSTRACT

An update on the status of the *Helianthus* L. germplasm collection in the National Plant Germplasm System of the United States is presented. The collection is held at the North Central Regional Plant Introduction Station in Ames, IA administered by the USDA-ARS Plant Introduction Research Unit in cooperation with the Agricultural Experiment Station at Iowa State University. Sunflower germplasm is acquired, maintained, characterized and distributed as well as used to conduct and support germplasm-related research. Overall, the *Helianthus* collection is 86% available and is composed of accessions of the domesticated species *Helianthus annuus* and its wild relative taxa. Sunflower germplasm and associated information are freely available to scientists and educators world-wide for research, crop improvement and product development.

Key words: cultivated – germplasm collection – *Helianthus* – wild.

INTRODUCTION

The sunflower (*Helianthus* L.) germplasm collection in the National Plant Germplasm System (NPGS) of the United States is held at the North Central Regional Plant Introduction Station (NCRPIS) in Ames, IA. The collection contains accessions for all but one of the 63 taxa recognized in the recent Flora of North America (FNA) treatment of *Helianthus* (Schilling, 2006) as well as accessions of *Helianthus niveus* ssp *canescens* and *Helianthus* x *multiflorus* (neither are considered in the Flora at this time). Forty-two of the taxa are perennial and 21 are considered annual, including the cultivated species, *Helianthus annuus*. The genus *Helianthus* originated in North America and is well distributed across the continent although a number of *Helianthus* taxa have restricted ranges. Two species are on the U.S. Fish and Wildlife Threatened and Endangered Species List. This article describes the sunflower collection at the NCRPIS and briefly outlines some of the recent research we have conducted using this germplasm.

MATERIALS AND METHODS

In 2007, seed was received for 96 wild sunflower accessions collected during two NPGS Plant Exchange Office (PEO) sponsored field trips by Gerald Seiler and Thomas Gulya (USDA Sunflower Research Unit, Fargo, ND) in Australia and by Gerald Seiler and Laura Fredrick Marek (NCRPIS, Ames, IA) in the southwestern U.S. Since the previously published collection summary (Marek et al., 2004), five PEO sponsored collection trips and four independently funded forays have resulted in the addition of 288 accessions of wild populations to the collection. Thirty-one cultivated accessions have been received in the active collection, primarily expired Crop Science Registry (CSR) materials.

We continue to experiment with methods to improve germination of wild sunflower accessions and recently modified our protocol so that seeds are rinsed in cool, running tap water for seven days instead of 24 hours before transfer to germination paper (wild *H. annuus*) or germination boxes (all other wild taxa). Seeds are incubated for up to 8 weeks at 4 ºC before being moved to germinators (20/30 ºC, 12/12 hr light/dark cycles or 15/25 ºC, 14/10 hr light/dark cycles). Seedlings are transplanted to flats and established in the greenhouse before being transplanted to the field. Wild accessions are caged and screened before flowering to maintain the genetic integrity of each accession and honey bees are introduced into the cages for pollination. Increase plantings of cultivated *H. annuus* accessions are direct seeded except accessions with low quantity or low quality seed are started in incubators, transplanted in the greenhouse, and moved to the field as for wild sunflower accessions. Most cultivated accessions are hand pollinated after bagging the primary inflorescence and efforts are made during hand pollination to

mix the pollen within each population. In 2007, several cultivated accessions with significant branching were successfully increased in cages using insect pollinators. After harvesting, drying and cleaning, seeds are stored at 4C and 35% humidity.

A number of wild taxa require a longer growing season than reliably occurs in central Iowa. In 2004, we began a significant partnership with the NPGS Parlier, CA location in the San Joaquin Valley (USDA-ARS National Arid Land Plant Genetic Resource Unit, NALPGRU). Average first frost at this location occurs in early December; average first frost in Ames, IA occurs during the first week in October. Seedlings are shipped to Parlier where they are transplanted and managed through harvest, including caging and use of introduced pollinators. Harvested material is shipped to Ames for processing.

Plasmopara halstedii (Farlow) Berl and de Toni is the causal agent of downy mildew and is a major phytosanitary issue for seed exported from the United States. A two-part disease management plan is employed to ensure production of *Plasmopara halstedii*-free seed (Marek et al., 2004). First, seeds intended for direct-seeding in the field are treated with Allegiance (metalaxyl) fungicide. Secondly, all plants in the field are visually inspected for systemic downy mildew infection a minimum of two times before flowering, initially at the V6-V8 growth stage. It is relatively easy to inspect every plant because seed production fields at the NCRPIS are not large. Infected plants are rare, but if encountered are physically removed from the field. Follow-up inspections are done at 4-7 day intervals until it is apparent that no infected plants are present. Plants are also inspected for symptoms of bacterial and viral diseases. Field inspections have been routinely conducted since 1990. Seed lots produced before 1990 are treated with metalaxyl fungicide prior to overseas shipment if the destination country accepts chemically-treated seed.

RESULTS

The NCRPIS collection currently contains 3,838 accessions representing 64 *Helianthus* taxa (Tables 1 and 2). The only sunflower taxa not present in the collection are *H. nuttallii* ssp *parishii* (likely extinct) and *H. niveus* ssp *niveus* (endemic to Baja California, Mexico and to our knowledge not currently available for distribution by non-Mexican genebanks). The largest proportion of the collection is made up of wild (24%) and cultivated (44%) *Helianthus annuus* accessions of which 94% are available for distribution. Non-*Helianthus annuus* wild annual accessions account for 12% of the collection and are 86% available. Perennial wild accessions, representing 20% of the collection, are 59% available. This information is summarized in Table 3. Approximately the same percentage of *H. annuus* accessions is available as reported in 2004, the non-annuus wild annual accession availability has increased 15%, and availability of the wild perennial accessions has more than tripled from the 18% available four years ago to 59% currently. Accessions are available for 25 taxa which had no distributable germplasm in 2004 (Tables 1 and 2). The absolute number of accessions in the sunflower collection reflects a balance between acquisitions from collections and donations and inactivations. Nearly 350 non-viable sunflower accessions have been inactivated since 2004, primarily accessions collected prior to 1980 which did not germinate during one to several increase attempts. The percentage of available germplasm increases as a result of successful production in Ames and in Parlier.

The partnership with the NALPGRU staff at Parlier has resulted in increased availability of a number of taxa including *H. agrestis, H. argophyllus, H. exilis*, and *H. radula*. The *H. argophyllus* collection is expected to be fully available after the 2008 growing season. To complement the versatility that Parlier provides, protocols have been tested in Ames to manipulate daylength and induce earlier flowering in selected taxa (Marek 2008). In 2007, black landscape fabric was used within the confines of a 20 x 50 screened hoop house to increase the dark period for an accession of *H. argophyllus*. From mid-July until mid-August plants were covered daily, increasing the dark period from about 9 hours to 16 hours. As a result, treated plants were flowering at the time treatment ended; control plants did not begin to flower for six more weeks and only covered plants produced significant seed quantities before the Ames area received its first killing frost. This protocol will be tested during 2008 with additional late flowering taxa.

Table 1. Annual *Helianthus* taxa in the NCRPIS collection. Taxa unavailable in 2004 are shown in boldface type.

Annual taxa	# accns	# avail accns
H. agrestis	5	2
H. annuus, cultivated	1660	1584
H. annuus, cultivated, CSR*	42	0
H. annuus, wild	931	894
H. anomalus	8	4
H. argophyllus	44	30
H. bolanderi	8	4
H. debilis ssp *cucumerifolius*	11	11
H. debilis ssp *debilis*	13	11
H. debilis ssp *silvestris*	22	22
H. debilis ssp *tardiflorus*	5	4
H. debilis ssp *vestitus*	3	3
H. deserticola	22	15
H. exilis	31	25
H. neglectus	28	28
H. niveus	1	1
H. niveus ssp *canescens*	19	13
H. niveus ssp *tephrodes*	12	6
*H. paradoxus***	10	0
H. petiolaris	15	13
H. petiolaris ssp *fallax*	30	30
H. petiolaris ssp petiolaris	94	94
H. porteri	8	8
H. praecox	2	2
H. praecox ssp *hirtus*	7	7
H. praecox ssp *praecox*	8	8
H. praecox ssp *runyonii*	24	24
H. sp.	11	10
H. hybrid	14	13
total	3088	2866

*CSR: accessions covered by Crop Science Registry protection. Distributable after protection expires or with the authorization of the inventor.
**Species covered by the U.S. Fish and Wildlife Threatened and Endangered Species Act

Morphological and phenological observations are collected during the growing season. Descriptor information is also contributed by collaborators at the USDA Sunflower Research Group in Fargo, ND. We travel to Parlier annually to record descriptor data for accessions increased at the NALPGRU. Information is entered into the Germplasm Resources Information Network (GRIN) database maintained by the USDA-ARS Database Management Unit of the NPGS GRIN can be accessed at http://www.ars-grin.gov/npgs/searchgrin.html. Descriptor information is available through the 'Research Crops and Descriptor/Evaluation Data Queries' option. Select "SUNFLOWER"; select "List of Descriptors."Descriptor information can also be viewed under "OBSERVATIONS" when querying GRIN for specific accessions under "Accession Area Queries" at the "search grin" url given above. During 2007 we began loading flower, plant and seed images to GRIN for all accessions increased in Ames and Parlier. "Image" is considered an "uncharacterized descriptor" on the sunflower descriptor page in GRIN and can also be accessed under "OBSERVATIONS" when viewing specific accessions.

Table 2. Perennial *Helianthus* taxa in the NCRPIS collection. Taxa unavailable in 2004 are shown in boldface type.

Perennial taxa	# accns	# avail accns
H. angustifolius	19	6
H. arizonensis	5	2
H. atrorubens	14	8
H. californicus	21	15
H. carnosus	3	2
H. ciliaris	27	15
H. cusickii	21	12
H. decapetalus	31	23
H. divaricatus	32	9
H. eggertii	15	10
H. floridanus	4	2
H. giganteus	29	16
H. glaucophyllus	1	1
H. gracilentus	7	4
H. grosseserratus	44	40
H. heterophyllus	8	0
H. hirsutus	18	5
H. laciniatus	8	7
H. xlaetiflorus	7	2
H. laevigatus	6	2
H. longifolius	3	2
H. maximiliani	65	50
H. microcephalus	11	4
H. mollis	25	11
H. xmultiflorus	1	1
H. nuttallii	8	8
H. nuttallii ssp *nuttallii*	22	19
H. nuttallii ssp *rydbergii*	12	12
H. occidentalis	2	0
H. occidentalis ssp *occidentalis*	1	1
H. occidentalis ssp *plantagineus*	11	9
H. pauciflorus	11	6
H. pauciflorus* ssp *pauciflorus	6	5
H. pauciflorus ssp *subrhomboideus*	15	13
H. pumilus	51	44
H. radula	14	3
H. resinosus	16	11
H. salicifolius	1	0
*H. schweinitzii**	1	1
H. silphioides	2	1
H. simulans	4	1
H. smithii	5	4
H. strumosus	34	17
H. tuberosus	107	35
H. verticillatus	2	2
total	750	441

*Species covered by the U.S. Fish and Wildlife Threatened and Endangered Species Act

Table 3. Summary of the USDA-NPGS *Helianthus* collection.

germplasm category	# of accessionsns	% of collection	% available, 2008
cultivated *Helianthus annuus* *	1702	44	93
wild *H. annuus*	931	24	96
wild annual non-*H. annuus*	455	12	85
wild perennial *Helianthus*	750	20	59
totals	3838	-	86

In order to develop molecular methods to assess within and between population variation, a collaborative effort is underway to associate molecular marker information with *Helianthus* accessions. Genotyping was initiated using *H. pumilus*, a perennial species sampled across its entire geographic range during a 2005 PEO sponsored collection trip to Colorado and Wyoming. This germplasm provides a broad sample of the species genetic diversity and makes it a good choice with which to begin molecular based diversity analyses. Data are being handled as molecular descriptors and following validation, will be available through GRIN.

DISCUSSION

Previous seed increase efforts at NCRPIS focused on ensuring high availability of cultivated *H. annuus* accessions. Discounting accessions not distributable due to Crop Science Registry restrictions, that portion of the collection is now greater than 95% available. Since 2004, efforts have focused on making wild germplasm more available for distribution both by increasing germplasm already in the collection and by organizing and participating in collection trips to obtain more complete genetic representation of the wild taxa in this native North American genus. Since 2004, availability of wild annual non-*Helianthus annuus* germplasm has increased 15% and wild perennial availability has more than tripled to 59%. Seed from wild populations can have varied dormancy requirements that affect germination. A protocol has been adopted that has enhanced our success with wild material, presuming adequate initial seed viability. Perennial species from the southern and southwestern United States and Mexico do not over-winter well in Ames; additionally, the growing season in Ames is too short to allow flowering and mature seed development for some of the wild taxa from these regions. To address some of these regeneration issues, we have developed a strong cooperative program with the NPGS site personnel in Parlier, CA with its longer growing season for the regeneration of up to 40 *Helianthus* accessions each year. Photoperiod control methods have been developed for use in Ames, IA.

In addition to routine field inspections to ensure quality seed increases, we also participate in select projects evaluating germplasm for disease resistance. One of the major diseases affecting sunflower in the United States is basal stalk rot, caused by *Sclerotinia sclerotiourum*. In 2007, screening of wild germplasm for resistance to Sclerotinia was begun as part of a joint effort with the USDA Sunflower Research Unit in Fargo, ND. Accessions of *H. resinosus* were notable in their resistance to infection both in greenhouse tests (greater than 99% for four accessions) and in the field. When greenhouse survivors were transplanted to the field and re-inoculated, survival remained high: 87-96% compared with approximately 20% for the resistant check hybrid. A long term goal is the incorporation of useful traits into cultivated germplasm.

The sunflower collection maintained by the NPGS is a very diverse collection. Sunflower germplasm is available for research and educational purposes at no charge. Samples may be obtained by ordering through GRIN or by contacting the senior author at lmarek@iastate.edu. A caveat for international requesters: NCRPIS must be able to meet any import order requirements stated by the requesting country. There are times when this is not possible and requests cannot be fulfilled.

ACKNOWLEDGEMENTS

We gratefully acknowledge the dedicated efforts of Irvin Larsen, Oil Seeds Research Technician and the cooperation of our collaborators at the USDA Sunflower Research Unit in Fargo, ND and at the USDA NPGS National Arid Land Plant Genetic Resources Unit in Parlier, CA.

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Helianthus annuus natural populations to increase the whole genetic diversity of domesticated sunflower: the concept of neodomestication

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ABSTRACT

To broaden genetic basis of cultivated crop, seventy-seven *Helianthus annuus* natural ecotypes from North America were grown in Mauguio near Montpellier and crossed with three domesticated sunflower lines. The crossed F_1 progenies were conducted in maternal lineage to maintain 10 plants per lineage in isolation for pollination and the intermixing was continued till G4 generation under low to high selection pressure for some domestication traits. The G0 generation enabled to phenotype thirteen morphologic descriptors. Some traits: phenology, plant height, branching, oil content, seed size and yield were evaluated though the G1, G2, G3 and G4 cycles. The studies of the first generations enabled us to release some main trends which are promising for the future of sunflower breeding. Indeed, we showed that the neo-domesticated materials gather adaptative traits from the natural populations. The first immediate effect of the intraspecific crossing between wild *H. annuus* and cultivated lines (G0 -> G1) was to increase 1000 seeds weight (1000SW) by 3.6 (9.7 g to 35.2 g), oil content by 20% (26.8 % to 32.3 %), and to reduce apical, intermediate and basal branching, respectively, by 53%, 40% and 13%. In the last G3 and G4 cycles, strong selection pressures were applied on the combined (1000SW*oil content) trait. Oil content increased by 3% in G3 and continued by 11% in G4. In the same time 1000SW, after small decrease, exhibited strong seed size increase (13%) in G4 cycle. The other visible effect was a clear evolution towards domestication traits, i.e., decrease in the different types of branching, reaching 76% non branchy plants in G4. Qualitatively, the different types of branching declined in the same way in G4 cycle: 14% plants were apical branched, 11% intermediate and 17% basal branched.

Key words: domestication – genetics resources – neodomestication– wild sunflower.

INTRODUCTION

Wild sunflowers spread naturally in North America where they thrive as populations covering most of the environments from sea to mountain. Heiser (1947) reported that wild sunflowers were found throughout most of North America on disturbed, mesic, heavy soils that are wet in the spring but dry out by midsummer. The *H. annuus* natural populations reveal a particular interest for adaptation to biotic and abiotic environments (drought, cold tolerance). Rust, Alternaria and downy mildew resistances are frequently observed in that wild compartment. In screening downy mildew tests performed at INRA we observe that most of the wild accessions (61/72) carried resistance factors to downy mildew (race 710).

Broadly, wild sunflowers did not exhibit meiotic abnormalities in crosses with cultivars like other annual *Helianthus* species that carry genomic rearrangements in comparison to sunflower. Consequently, one can expect efficient intermingling of the cultivated and wild genomes along recurrent crosses. Moreover, the wild sunflower is strictly self-incompatible, whereas crop lines are self-compatible, which also favours intermingling of genomes.

Sunflower is an oil crop and breeding has dealt mainly with increasing oil yield. Oil yield has been increased drastically in Russia (Vrânceanu, 2000) and oil sunflower spread worldwide. In Russia, oil improvement has been accompanied by broomrape resistance since broomrape was prevalent in this country (Vrânceanu, 2000). To become an industrial crop sunflower diversity has been reduced for crop adaptation to hybrid seed production through a CMS (Leclercq, 1971) that infers splitting lines in A (male sterile), B (maintainer) and R (restorer) classes, to diseases, mainly downy mildew (Leclercq, 1971) and after Phomopsis spread (Škorić, 1985). Recently, the introduction of Pervenets mutation in the classic sunflower to obtain seed oil with high oleic acid content has revealed the narrowness of genetic diversity in the crop (Tang et al., 2003).

Until now, the method used to enlarge the genetic diversity for agronomic traits in sunflower crop was to cross sunflower with a wild sunflower carrying the useful trait and to evaluate progenies for the

trait under breeding. Similarly, other *Helianthus* annual species have been used in this way to introgress useful traits in sunflower such as Rf genes: (Jan and Seiler, 2007); Phomopsis (Škorić, 1985; Griveau et al., 1992; Serieys et al., 1998), and fertility (Quillet et al., 1992). This method has, as a main advantage, the limitation of the material to be examined in the field, but it also has as a main defect that only one main trait can be considered in each cross. Moreover, introgression lines keep only a few of the foreign genomes, which are eliminated to restore male fertility required in agreement with cultivation. We therefore conceived a method to enlarge genetic diversity of sunflower crop by accumulating, in a series of crosses between wild sunflower and crop lines, neutral diversity from seventy-seven wild sunflower accessions and the main already domesticated traits from three sunflower elite lines. During four successive cycles, we applied selection pressures both to improve the agronomical value of the population and maintain at its highest level the genetic diversity coming from the wild *H. annuus* compartment. After four generations of mixing in isolated plots, but always keeping separated the female progenies, we evaluated the diversity. We present here the experimental design, the characterization of the main agro-morphological traits at each cycle and the analysis of response of important phenotypical and phenological traits in the HAS population as a consequence of the breeding constraints exerted on that genetic material.

MATERIALS AND METHODS

Plant Material

Seventy-seven *H. annuus* populations (HAS) were chosen among the set of 350 maintained at INRA Breeding station (Mauguio). They were screened for their spreading in the USA by covering most media under different climates and by the diversity of the morphological traits (Table 1). Between 1 to 8 populations were sampled in each of the following 15 US states.

Table 1. Distribution, by states, of the 77 collected accessions in North America

US State	*H. annuus* accessions with INRA CODE
ARIZONA	383; 386; 519; 654
CALIFORNIA	410; 421; 435; 437; 446; 458; 468; 833
COLORADO	363; 660; 980; 989; 996; 1147; 1148
ILLINOIS	211
IOWA	665; 829
KANSAS	733; 997; 998; 999; 1000
MISSOURI	351
MONTANA	943; 945; 948; 954; 1150
NORTH DAKOTA	928; 929; 931; 933; 939
NEW MEXICO	461; 463; 649; 661
OKLAHOMA	646; 651; 662
SOUTH DAKOTA	378; 388; 970; 1042; 1047; 1055; 1136
TEXAS	209; 509; 511; 647; 648; 650; 652; 734
UTAH	658; 774; 775; 822; 826; 1149
WYOMING	358; 361; 955; 963; 966; 974; 975

Construction of an intraspecific H. annuus genepool with wide genetic background

The wild and cultivated *H. annuus* were grown in the field at the INRA breeding station of Montpellier. Sowing dates were on 1 April 1st 1996, in greenhouse with transfer in the field April 15 for G0, April 1st and 11 April 1997 for G1, April 1998 G2, May 17, 2006 G3, April 17 2007 G4. Five plants per population were grown in our nursery (G0) and each of the plants received separately pollen from the 3 testers, (branching allowed different crosses on the same plant). Testers were oil sunflower lines 89HR2, 90R19 and RT1B11. The two first are top branched (CMS-PET1 restorers) and the third is single headed (CMS-PET1maintainer)

We grew 10 G1 plants per progeny (77 accessions x 3 testers x 10 individuals) the next year in a field isolated from gene flow from other sunflowers and we left the plants intercrossing. Each set of 10 plants was harvested separately and the seeds were bulked. At the next generation (G2) we grew up to 52 plants per progeny to enable a smooth elimination of too many branched individuals before they contributed to pollination. At the end of the G2 cycle, an average of 8 plants with reduced or no branching was harvested in each of the cultivated 228 progenies (3 combinations were missing). In order to induce fast increase for the domesticated traits and the agronomical value of the population, the following G3 and G4

generations were obtained after choosing the best individual in each progeny for maximum (OIL * 1000SW) combination value. Like in previous cycles, 5 plants per progeny were grown.

The measured variables
Each G0 plant was phenotyped for 13 morphological and phenological traits reported in Table 2.

Table 2. History of selection pressures applied on the wild *H. annuus* Pool (HAS), built on 1996

Cycle /year	Operation *at Melgueil*	Size of populations	Traits examined	Selection pressure
Cycle 0 (G0) 1996	F1 construction in field HAS= female parent 5 plants from each accession	77 HAS pollinated by 3 sunflower lines	Description origin, agromorphology + phenology + kernel size	None Harvest; only F1 hybrid kernels,
Cycle 1 (G1) 1997	Intermixing in isolation (=77 ecotypes x 3 testers x 10 plants)	228 F1 hybrid combinations with 10 plants per progeny *2280 F1 plants*	Branching type intensity, leaf shape, pollen fertility, head diameter, earliness; kernel yield, oil content, TSW, seed yield.	Harvest: Whole plants[1] * H1 (=10 plants separately) * H2, H3 (= 10 plants in bulk) G1 to G2: weak selection (24 % plant strongly branched were discarded for H1). None for H2 and H3
Cycle 2 (G2) 1998	Intermixing in isolation 228 maternal progenies (776 X 3 testers	228 maternal progenies *11800 plants*	Height + blooming date (start, medium, end) On harvested plants: Branching, high, TSW, oil content, Total W	Before blossom Elimination of most branched plants (bush form). At harvest: choice of 8 plants less branched. On the average, 84 % plants were eliminated in G2
Cycle 3 (G3-BL) 2006.	Intermixing in isolation (77 x 3 testers)	228 maternal progenies with 5 plants *1140 plants*	Notations: blooming date, height, branching, TSW, oil content, seed yield	For each G2 progeny: the best OIL content * TSW plants were selected for G3. 80 % plants eliminated
Cycle 3 (G4-BL) 2007	Intermixing in isolation (77 x 3 testers)	228 maternal progenies with 5 plants *1140 plants*	Notations: blossoming date, height, branching, TSW, oil content, seed yield	For each G3 progeny: the best OIL content * TSW plants were selected for G4. 80 % eliminated plants.

[1]H1=89HR2, H2=90R19, H3= RT1B11

In the G1, G2, G3 and G4 generations the measured variables mainly concerned flowering period, branching, plant height, seed size, yield and oil content. Branching was defined according to two components (I) types of branching architecture including, basal, medium, apical, and (II) branching intensity scale related to the size of the branches. We used the grid shown in Fig. 1 to note the plants. Analyses were performed according to qualitative and quantitative notation grid presented in Table 3.

Table 3. Quantitative and qualitative characters measured on the HAS population through G0 to G4 generations

ACC	INRA code	Units / notation scale	HAS				
			G0	G1	G2	G3-BL	G4-BL
SOWFLO	Sow-flowering duration	days	X	X	X	x	X
HEIGHT	Plant Height	cm	X	X	X	x	X
BRAPIC	Basal branching	0: absent, 1: present	X	X	X	x	x
BRMED	Medial branching	0: absent, 1: present	X	X	X	x	X
BRBAS	Apical branching	0: absent, 1: present	X	X	X	x	x
NR	Unbranched plants	0: branched, 1: unbranched	X	X	X	X	X
INT_BR	Branching Intensity	0 (null) to 4 (high)	X	X	X	X	X
SW	Seed weight	g	X	x	X	x	X
TSW	1000 seeds weight	g	x	x	X	x	x
OIL	Oil content	%	x	x	x	x	x

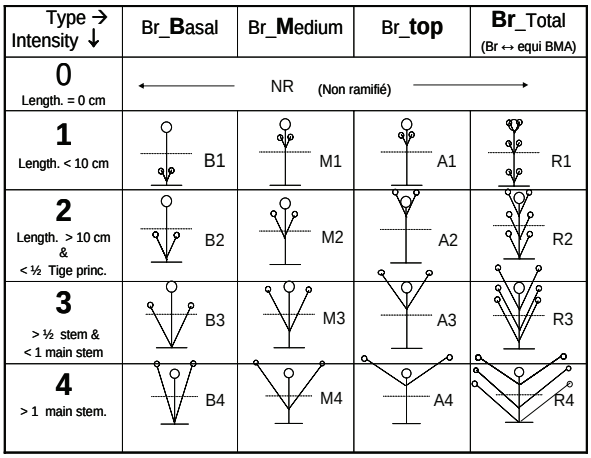

Fig. 1. Notation grid mixing 'Type' and 'Intensity' for branching of *HAS* and sunflower.

RESULTS

Construction and phenotypic variability in the HAS population

Our principle was to use enough HAS ecotypes to handle a genetic diversity representative of the HAS compartment in North America, to catch from the wild sunflower most of its favourable genes to enhance stress resistance and adaptation in sunflower and to drive the population to decrease the unfavourable traits such as branching, low oil content and seed size. As indicated in Table 4, an important variability is observed for the sow-flowering period among the plant population (more than 2 months), and plant height (from 71 to 308 cm).The branching was also characterised and all the wild *Helianthus* observed were extremely branched at all levels (apical, intermediate, basal), associated with high branching intensity, Average seed weight (TSW) was estimated at 9.9 g with a range of between 5.7 and 29 g. (close to cultivated types). On the other hand, average oil content of the 77 HAS was low (26.6%) and varied between 20.4 and 32.0%. Seed yield / head in open pollination varied between 0.1 and 9.5 g.

Table 4. Agro-morphological characteristics of the selected 77 accessions, in the G0 HAS population

Trait	Mean value	Std	Max	Min
Sow-flowering duration (SOWFLO), days	70.27	13.25	120.60	50.78
Plant Height (HEIGHT), cm	180.99	52.58	308.00	71.11
Max length of lateral branch (LAXL), cm	117.84	28.95	179.00	51.11
Basal branching (BRAPIC)	0.97	0.08	1.00	0.60
Medial branching (BRMED)	0.99	0.06	1.00	0.60
Apical branching (BRBAS)	0.97	0.11	1.00	0.20
Branching Intensity (INT_BR)	3.00	0.53	4.00	0.98
Total length lamina + petiole (LONGLEAF), cm	39.86	12.89	59.00	14.33
Petiole length (LONGPET), cm	17.70	7.26	4.22	4.22
Seed weight (SW), g	3.21	1.34	9.51	0.12
1000 seeds weight (TSW)	9.71	3.64	29.09	5.69
Head diameter (DHE), mm	29.24	6.23	68.64	17.81
Seed length (SLEN), mm	5.04	0.62	7.61	3.95
Seed width, (SWID), mm	2.47	0.30	4.04	1.99
Oil content (OIL), %	26.80	2.15	32.05	20.44

The G0 plants were evaluated for 15 traits in the Mauguio environment although they originated from different locations and climates in the USA. A PCA analysis (data not shown) gives the relations between measured phenotypic traits. Plotting of the geographical origin (State) on the graph provides identification of large geographical areas (northern, eastern, south –southwest US), grouping wild *H. annuus* with similar characteristics. Correlation analysis between main strengths of the climate

characteristics and morphology of the accessions showed that temperature was highly correlated (r>50%) with height, leaf and petiole lengths.

Cycle length and plant height were significantly linked to precipitation (r=0.4) and temperature levels (r= 0.28 and 0.57 respectively). Additionally, plant height is connected to important rainfalls in short periods (coastal Texas climate). The apical and intermediate branching also seems to be affected by rainfall and to a lower number of rain days. Increased temperature range (maxi-mini) enhanced architectures with intermediate branching. The native accessions from geographical areas with a low rainfall (New Mexico, Arizona) were more branched. On the contrary, the different types of branching did not clearly respond to temperature.

Changes observed from G0 to G4 for main traits (Fig 2):

Changes in sowing-flowering period and plant height, plant branching, in the average oil content for seed size, seed yield were plotted. Although the experiments were not done the same year, we observed some general trends.

DISCUSSION

Systematic series of crosses between HAS and elite lines of domesticated sunflower have never been performed. We did not have any problems in the intercrosses, except some male sterility that segregated in some HAS. Here we reported several main features that tended to show that the material could be used for breeding sunflower and we tried to quantify our conclusions as much as possible. Some traits not retained in our notations were strongly selected such as seed dormancy, which was rapidly eliminated. Although HAS all displayed strong seed dormancy, we did not observe any problems in our progenies. This means that the trait is probably monofactorial and the locus efficiently eliminated. Branching behaved as a complex trait: we distinguished branching at 3 levels: basal, medium and top (Fig. 2). Basal and medium levels are unfavourable in the crop whereas top branching is used in most restorer lines. We surmised that the length of the branches was not inherited but due to the environment; the later the sowing, the shorter the branches for some lines and ecotypes. Non branching plants appeared in the G1 and due to selection pressure for the traits, it increased continuously. We used the grid (Fig. 1) to note the plants. We observed all the combinations of the elementary traits, which suggested that they are controlled by different genetic factors mostly independent. Seed weight was much lower in HAS (4-15g for 100 seeds) than in the crop (50-70g). However, when we chose the HAS we retained some with higher seed weight up to 30g. Two explanations are possible: 1) this is the range of variation for seed weight in HAS or, alternatively, 2) they have already been introgressed by domesticated sunflower. Due to the origin of the HAS with a large seed size in areas where the sunflower is cropped, we favoured the second hypothesis. However, in our experiment we used a combination of seed weight and oil content to avoid screening progenies with large seeds and poor oil content. Seed oil content did not increase rapidly, but we did not exert any strong selection pressure on the trait. Plant height decreased rapidly without any selection pressure. The variability is wide and height reduction will probably be rapidly feasible.

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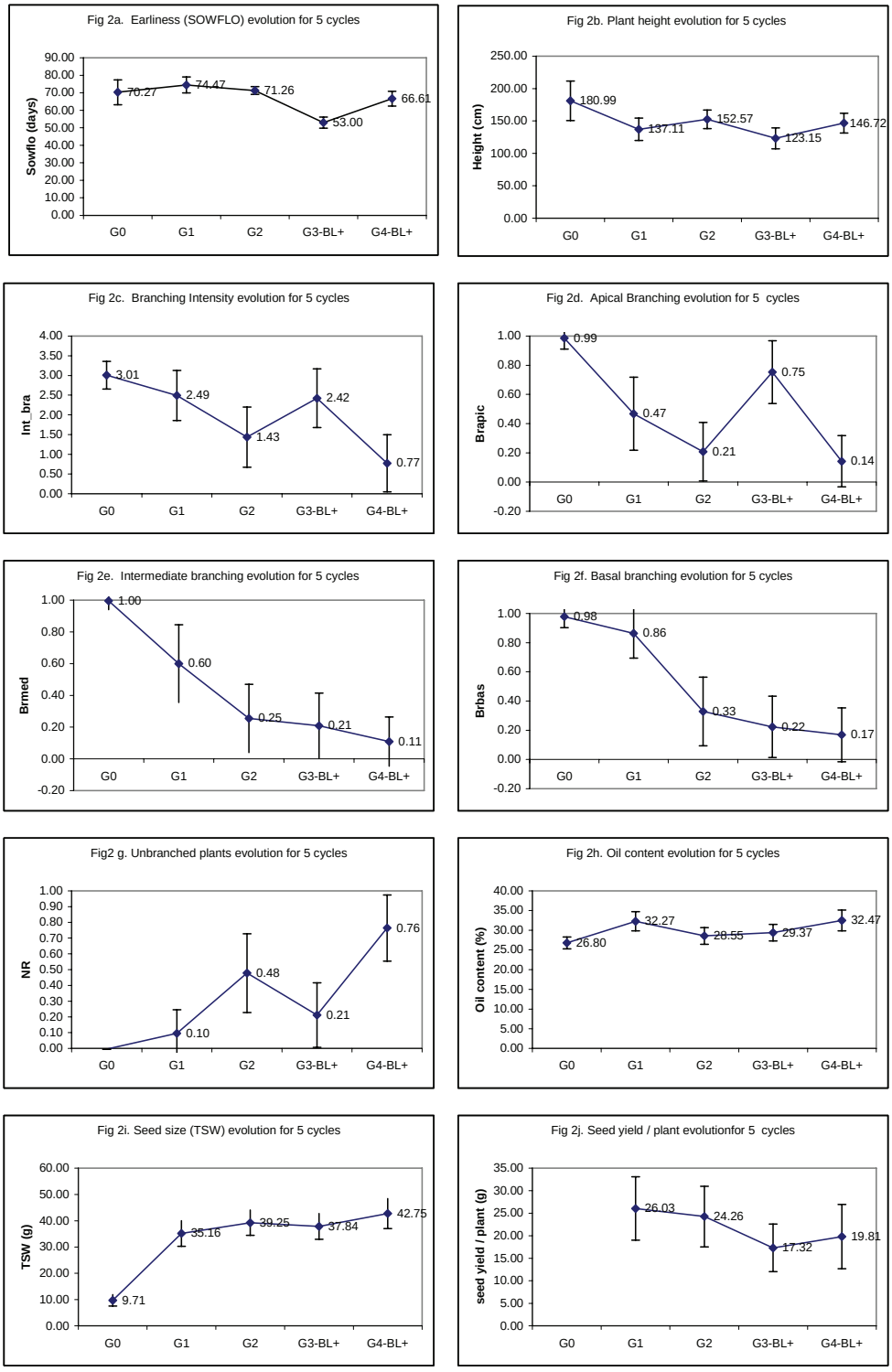

Fig. 2. Changes in phonological and morphological parameters along five generations in the neo-domesticated progenies. SOBLO, sowing – blossoming stages in days. NR: non branching. Two G3 progenies were obtained in two different fields: One with ten plants per family with all 228 families (G3BL+), which has also led to G4BL+; and one with only 100 families (G3-Syspro)

Effect of the environment on the chemical composition and some other parameters of sunflower seed quality

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ABSTRACT

Considering the area of sunflower cultivation in the world, the profit made by selling the seed varieties and sunflower hybrids, the number of companies whose business activity is related to production and selling of sunflower seed, the tendency of the struggle for taking the greatest market share is bigger and tougher. This type of competition conditions all seed companies to encourage as many producers as they can with their choice of hybrids and seed quality, all aimed at making bigger profits. The common argument of seed companies for attracting producers is the chemical composition of seed. Chemical composition of sunflower seed consists of great number of different organic compounds, the oil content and proteins being the most important. The objective this research has been to evaluate the differences appearing in seed quality parameters in two localities.

Key words: linoleic acid – oil content – oleic acid – seed germination – sunflower seed – tocopherols.

INTRODUCTION

Seed is the beginning of a plant's new life, a complex biological system and as such it is the first and basic factor of successful plant production (Milošević et al., 1996).

Seed quality is a complex category, determined by great number of factors (purity, germination, moisture, weight of 1000 seeds and others), which are under the influence of different environmental factors (Karagić et al., 2001). One of the most important characteristics of a seed is the germination energy and germination of a seed itself (Radić, 2003). The same author further mentions that great number of factors influence the germination such as climate conditions in time of production, pollination, fertility, harvest, factors that appear during the seed drying and seed cleaning (transport from a plot, method of drying, storage of natural seed, cleaning, seed treatment) as well as factors of storage of the seed (damage possibility by various warehouse insects as well as damage during the seed usage). Škorić et al., (1996) conclude that environmental factors can influence the chemical composition of a seed as well.

Sunflower is produced in more than 40 countries of the world (Putt, 1997). Considering the area that sunflower covers in the world, the profit made from selling the seed varieties and sunflower hybrids, the number of seed companies whose business activity is related to the production and selling of sunflower seed, the tendency of a struggle for taking the greatest market share is becoming bigger and tougher. This type of competition conditions all seed companies to encourage as many producers as they can in their choice of hybrids and seed quality, all aimed at making bigger profits.

The common argument of seed companies for encouraging producers is the chemical composition of seed and its use. Chemical composition of sunflower seed consists of great number of different organic compounds, but for sunflower, oil content and composition and protein content in seed are the most important (Marinković et al., 2003).

The same authors further mention that beside fatty acids, sterols, carotenoids, phosphates and some other compounds, the quality of oil is determined by the total amount of tocopherols, as well as the content of its respective forms.

During the process of breeding, breeders are helped by information about correlations that appear between the oil content in the seed and other characteristics of a plant and seed, since they make it possible to find connections that help or prevent the successful selection for these characteristics. Most authors have dealt with studying the relation of oil content and seed yield, plant height, weight of 1000 seeds, head diameter, number of leaves, dry matter obtained during the vegetation and others .

However, some authors have dealt with problems related to the influence of the environment on oil composition and content in sunflower seed. According to Škorić (1988) and Krizmanić et al. (1992), who take into account the environmental factors that have an effect on oil content, the average daily temperatures and the amount of moisture in the soil are the factors that have the biggest effect. Marinković et al., (2003) believe that the content of oil in seed is lower if the lack of soil moisture

appears in the period of flowering-maturation. Dušanić (1994; 1998) and De la Vega and Hall (2002) believe that the main cause of variations in content and composition of oil in sunflower seed, as well as for oil yield is the influence of the production year, locality and the sowing deadline.

The aim of the research described in this paper was to determine if the content of oil, α-tocopherol, linoleic and oleic acid in sunflower seed depends on the place of production, and if there are factors that can affect the sunflower seed germination.

MATERIALS AND METHODS

The research was made on seeds produced in two localities, Argentina and Serbia, in quite similar conditions of production. Two genotypes were used: HA-26-OL (high oleic type) and HA-48 (standard type). Complete research was carried out at the Insitute of Field and Vegetable Crops, Oil Crops Department.

During the research, the following parameters were used:

1. Germination – Examination of seed germination of both genotypes was repeated 6 times. Each time 50 seeds were used. Germination was determined after 10 days. Only naturally formed germed seeds were used for determination of this parameter. Germination was expressed in percentage.
2. Oil content – Determined by classical method and expressed in percentage.
3. Tocopherols content – The content of α-tocopherol was determined by liquid chromatography method (HPLC) and expressed in mk/kg oil.
4. Linoleic and oleic acid content – Determined by gas chromatography method. The content of these two acids is expressed percentage (% of the total fatty acids).

Computer programme GENSTAT was used for the analysis of variance of two factor experiment and interdependence of the observed parameters.

RESULTS AND DISCUSSION

The results of the research show that the percentage of seed germination of the two genotypes produced in a locality in Serbia was 64% and 71% respectively (Table 1), while for those produced in a locality in Argentina germination percentage of HA-26-OL was 97% and that of of HA-48, 96% (Table 2).

As for seed germination, lower values were determined for oil content for seed produced in a locality in Serbia (Table 1) than for seed produced in a locality in Argentina (Table 2). A percentage of 31.34% of oil content was determined for HA-26-OL genotype for seed produced in a locality in Serbia and 36.42% for seed produced in a locality in Argentina. For HA-48 genotype those values were 36.82% in Serbia and 48.90% in Argentina.

Table 1. Results on a locality in Serbia

Genotype	Seed germination (%)	Oil content and composition			
		Oil content (%)	α-tocopherol (mg/kg oil)	Linoleic acid (%)	Oleic acid (%)
HA-26-OL	64	31.34	425.67	19.63	72.73
HA-48	71	36.82	393.40	62.23	26.57

Similar situation was found during the observation of tocopherol content. For the seed obtained in a locality in Serbia a total tocopherol content of 425.67mg/kg oil was determined in HA-26-OL and 393.0 mg/kg oil in HA-48 genotype (Table 1). These values were higher than those obsserved for the seed obtained in Argentina for HA-26-OL (585.30 mg/kg oil) and for HA-48 genotype (569.45 mg/kg) (Table 2).

Considering that for this study a high oleic acid and a standard type hybrid were used, the content of linoleic and oleic acid was different between observed genotypes. Nevertheless, HA-26-OL had lower linoleic acid content (19.63%) in a locality in Serbia (Table 1) in comparison to the value observed in a locality in Argentina (35.83%) (Table 2). The same situation was observed for the HA-48 (standard type) genotype. In Serbia the result was 62.33% while in Argentina it was 67.23%.

In contrast to all observed characteristics, the content of oleic acid had a higher value in a locality in Serbia than in a locality in Argentina, in both genotypes observed (Tables 1 and 2).

Oleic acid content was 72.73% for HA-26-OL in a locality in Serbia, in comparison to 57.30% in Argentina. HA-48 had a oleic acid content of 26.57% in Serbia was 26.57% while in Argentina that value was 30.73%.

Table 2. Results in a locality in Argentina

Genotype	Seed germination (%)	Oil content and composition			
		Oil content (%)	α-tocopherol (mg/kg oil)	Linoleic acid (%)	Oleic acid (%)
HA-26-OL	97	36.42	585.43	35.83	57.20
HA-48	96	48.90	569.45	67.23	30.73

The calculation of simple coefficients of correlation, showed a highly significant positive correlation between seed germination and tocopherol content (Table 3). A highly significant positive correlation between oil content and linoleic acid content was also observed (Table 3). Škorić (1982) and Petakov et al. (1983) reached similar conclusions. A highly significant negative correlation between linoleic and oleic acid content was identified (Table 3).These values are in agreement with the results of Seiler (1994), while Gonzales et al. (2000) determined the existence of negative correlation between both fatty acids, even though the authors found that the correlation was not statistically significant.

A significant positive correlation was observed between seed germination and oil content. Correlation between oil content and oleic acid was significantly negative (Table 3). In their research, Álvarez et al. (1992) detected the existence of a highly significant positive correlation of oleic acid with and seed oil content and weight of 1000 seeds, while the correlation coefficient between linoleic acid content and seed yield was negative but not statistically significant.

A positive but not significant correlation was detected between seed germination and linoleic acid content, between oil content and tocopherol content, as well as between linoleic acid content and tocopherol content. A weak and negative correlation was observed between seed germination and oleic acid content , and between oleic acid content and tocopherol content (Table 3).

Table 3. Coefficients of correlation of the observed parameters

Parameters	Oil content	Linoleic acid	Oleic acid	Tocopherols
Germination	0.675*	0.392	-0.289	0.766**
Oil content		0.791**	-0.681*	0.433
Linoleic acid			-0.985**	0.136
Oleic acid				-0.025

Studying the relationship between oil content, linoleic and oleic acid with tocopherol content, Demurin (1986) determined that there is no significant correlation between these parameters.

The results of seed germination point to a great possibility of utilizing other localities for the purpose of getting high quality seed. It is obvious that Serbia, in the case of these two genotypes, is characterized as a locality that gives weak results. The results obtained showed that the influence of the environmental factors is important, even highly significant for certain observed parameters. In contrast to this statement, Balalić et al. (2006) considered that for the interaction between the hybrids and the year there is no significant difference, while in all variation sources (relation of oil content and yield, locality, year, sowing deadline and plant density) there are significant differences. Miklič (2001) concluded that the external factors do not have a great effect on oil content in the seeds, but the influence of the genotype prevails.

Seed produced in Serbia showed poorer results than the seed produced in Argentina. On the basis of given results it can be concluded that different environmental factors affect the content and chemical composition of oil in sunflower seed with certain genotypes.

In conclusion, oleic acid content was higher in seed produced in Serbia in comparison with the same seed produced in Argentina. Differences between both locations were highly significant, except for tocopherol content. Considering the great difference in seed germination, this experiment provided information about the possibility of successful seed production in other localities. Correlations were in agreement with previous results. Considering the results obtained in this research, further research should be directed towards observation of the relationships between seed germination, oil content, tocopherol content and oleic acid content in sunflower seeds.

ACKNOWLEDGEMENTS

This work was supported by Ministry of Science of Republic of Serbia.

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Variability and genetic analysis of sterols content in sunflower seeds

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ABSTRACT

Phytosterols are triterpenoid molecules naturally present in plants that are involved in the functioning of cell membranes and embryogenesis. These bioactive molecules are of interest due principally to their natural properties decreasing the Low Density Lipoprotein cholesterol, but other properties have also been highlighted. These findings have greatly increased industrial demands for sterols but present extraction methods imply toxic chemical products. Therefore, increased plant sterol contents could not only help to meet industrial demands but also to develop environmentally friendly extraction methods. The aims of this study were to evaluate genotypic variability and to analyze genetic determination of sterol content in sunflower seed. Seventeen genotypes and a population of 200 recombinant inbred lines (RIL) derived from a cross XRQ x PSC8 were grown at INRA Clermont Ferrand (France) in 2005. The seed harvested was used for sterol analyses in the Lipochemistry platform, INRA Toulouse (France). Results showed a large variability among the genotypes studied, which was confirmed within the RIL population. Values of total sterols varied twofold between extreme genotypes. QTL analyses showed several zones detected for all traits. One or two QTL were detected for the most abundant sterols. For example, one QTL was identified in LG 1 explaining 10% of the total variability. Possible further investigations and use of the results to start breeding programs aiming at improvement in sterol content are discussed.

Key words: bioaccumulation – genotypic variability – phytosterols – QTL - sunflower.

INTRODUCTION

Sterols, minor compounds, are naturally present in plants. They are involved in membrane fluidity and permeability (Schaller, 2003). The role of sterols in embryogenesis has also been demonstrated (Clouse, 2000). During the last decade, there has been interest in sterols due to their potential benefits for human health. They may reduce Low Density Lipoprotein cholesterol (LDL) (Miettinen et al., 1990; Bosner et al., 1999; Ostlund, 2007) and several studies have highlighted other interesting properties such as anti-cancer (Awad et al., 2003), anti-inflammatory (Bouic, 2001) and anti-oxidation activities (Van Rensburget et al., 2000). Consequently, these bioactive molecules are now used for various industrial applications. They are used in nutrition as functional food, in particular in enriched margarines (Moreau et al., 2002). By chemical modification, phytosterols could also be used as raw materials in the production of pharmaceuticals as a source of steroids (Van Dansik, 2000); or in cosmetics (Folmer, 2003). Sterols have more recently been used in liquid crystals in the optics industry (Zhang et al., 2005).

The wide uses of these molecules require a specific composition of sterols in oil depending on the applications which are used in native statement or chemically modified. Phytosterols are commercialized mostly as by-products from seed oil processing industries and deodoriser distillates from industrial seed oil refiningl (Daguet and Coïc, 1999). This source of production suffers from two major problems. The first concerns the traceability of seeds samples whose origin is not well known. The second, linked to the methods of extraction, results from the use of chemical substances damaging to human health and to the environment.

Phytosterols are present in different plant parts and mostly in seeds. Their level depends on species and sunflower seeds contain quite a high concentration (Mouloungui et al., 2006). Nevertheless, these minor compounds are present in low thresholds which considerably limit their extraction. The improvement in phytosterol concentration could improve accessibility of molecules and, therefore, could help the development of extraction methods. Moreover, the diversification of sterol uses has led to an increase in industrial demands. Levels of seed sterol contents can be maximized by crop management (Roche et al., 2006). Whereas the effects of the genotype on seed oil and protein contents are widely reported, studies of genotypic effects on sterol content in sunflower seeds are lacking. Similarly, genetic

determination of oil content has been investigated but, information on genomic regions governing sterols in sunflower seeds is not available.

The aim of this study was focused on the determination of genotype effect on sterol accumulation and also on the genetic analysis of this trait in sunflower seeds.

MATERIALS AND METHODS

Sunflower genotypes: Seventeen diverse cultivated genotypes were used in this study. These genotypes (from INRA Clermont-Ferrand, France) are contrasted for their seed yield and oil content (Table 1). Two hundred recombinant inbred lines (RIL), from a population obtained by single seed descent from a cross of INRA lines XRQ (bred from a cross of USDA line HA89 and the Russian open pollinated variety Progress) and PSC8 (bred from a populations under recurrent selection for *Sclerotinia* resistance) were used for the genetic study. These RIL were genotyped with SSR and RFLP for construction of a genetic linkage map (Vear et al., 2008).

Table 1. Characteristics of seventeen genotypes studied (inbred lines and hybrids)

Genotype	Characteristics	Origin	Genotype	Characteristics	Origin
OF	High C18:1	France	OEG	High C18:1	Spain
83HR4	Standard	France	HA382.LS2	Low C18:0[1]	USA
VHQ	Standard	France	OPA3	High C18:1	France
OSQ	High C18:1	France	59259	Dwarf	France
XRQ	Standard	France	Trisun (hybrid)	High C18:1	USA
PSC8	Standard	France	HA300	High sterol content	USA
83HR4OL	High C18:1	France	R105	High sterolcontent	France
RHA345	High C18:1	USA	Olbaril (hybrid)	High oleic	France
HA821.LP1	Low C16:0[1]	USA			

[1]C18:1 standard

Field crop conditions: The inbred lines and RIL were grown in a breeding nursery at INRA Clermont-Ferrand (45°46'59'' N, 3°4'56'' E latitude) 2005. For each genotype, there was 1 row of 13 plants, 5 to 10 of which were bagged to obtain seed by self-pollination. Local climate data (mean temperatures, rainfall and evapotranspiration) were checked at the weather station at INRA Clermont-Ferrand. The weather data are summarized in Table 2.

Table 2. Weather conditions during plant cycle of 17 contrasted genotypes and 200 RIL of sunflower cultivated in INRA station of Clermont-Ferrand in 2005.

Months	June	July	August	September (1-10)	Mean or sum
Mean temperatures (°C)	19.8	20.9	18.3	20.5	19.9
Rainfall (mm)	56.6	29.6	17.0	15.8	62.4
Evapotranspiration (TP) (mm)	140.5	159.0	125.7	33.1	317.8
Rainfall / TP	0.40	0.19	0.14	0.48	0.20

Climate conditions prevailing during the cropping season were very stressing for plant development. The evaporative demand during the plant cycle greatly exceeded the rainfall. In contrast, the grain filling stage (August) coincided with a drought period (Table 2) as shown by the low rainfall /ETP ratio noticed in *August 2005.*

Determination of sterol contents and composition using a small-scale sample extraction method: Biochemical analyses were performed at the Lipid platform of the Agro-Industrial Chemistry laboratory. A small-scale sample extraction method was developed for reliable and economic analysis of sterols in sunflower seeds. Cholestanol (Dihydrocholesterol, Aldrich Chem. CO.) was used as an internal standard. Sunflower seed samples were saponified with ethanolic KOH (1M) (Titrinorm™, Prolabo). The non-saponifiable fraction was extracted with iso-hexane (Merck). Sterols were silylated by 1ml of N-methyl-N-trimethylsilyl-heptafluorobutyramide (MSHFBA, Macherey-Nagel) mixed with 50µl of 1-methyl imidazole (Sigma) called silylation reagent. 1 µl of sterol trimethylsilyl ether derivatives were injected in a Perkin-Elmer GC equipped with a CPSIL 5CB 30m (D: 0.25mm), FID detector. The thermal regime

was the following: 160°C (0.5min), 10°C/min until 260°C, 2.5°C/min until 300°C, 25°C/min until 350°C, and 350°C (1.5min) for the oven temperatures, 55°C (0.5min), 200°C/min until 320°C, 30°C/min until 350°C, and 350°C (2.5min) for the injector temperatures and 365°C for the detector temperature. Total phytosterols detected included desmethylsterols (β-sitosterol, stigmasterol, campesterol, Δ7-stigmastenol, Δ5-anenasterol, Δ7-avenasterol), methylsterols (24-ethyldiene lophenol, 24-methylen lophenol) and dimethylsterol (cycloartenol and methylencycloartanol).

Statistical data analysis: Phytosterol data are expressed as a weight percentage of seed dry matter (mg of sterol per 100g of seed dry matter). Analysis of variance and Student-Newman & Keuls test were applied to the experiment results to determine the significance between (General Linear Models Procedure, SAS Institute, 1988); genotypes and for the different measured traits. The map built for QTL detection included 39 RFLP, 162 SSR, and 4 mendelien traits (P12, P15, Rf1, and b1). It was developed with CARTHAGENE software (de Givry et al., 2005) with the commands [group 0.4 4], then [builtdfw 3 3 {} 0] to build a framework for each of the groups identified, and, finally, [build] to add the remnant markers. It spans over 1666 cM, with an average of 12.2 markers per linkage group. QTL detection was performed with the software MCQTL (Jourjon et al., 2005) under the "forward" algorithm and with the iQTL" option (Charcosset et al., 2001). The level of significance was determined through 3000 permutations for each trait. As several phytosterols were recorded, we used the software BIOMERCATOR ® (Arcade et al., 2004) to map the different QTLs and to check the hypothesis of a single QTL associated with different related traits.

RESULTS

Genotypic variability for sterol content in sunflower seeds: A large genotypic variability was shown for the traits measured (Fig 1). The mean value of total seed sterols within the collection studied was more than 280 μmol.g^{-1}DM. The difference between the extreme genotypes for this trait was nearly two-fold (Fig. 1). The most abundant sterols (70% of total) were desmethysterols which were mostly constituted by β-sitosterol (Fig. 1a and 1d). Methyl and dimethylsterols represented only 19% (Fig. 1b and 1c). The highest values of the total and desmethylsterols were obtained for XRQ which is one of the parents of the recombinant inbred lines used for the QTL study. The lowest values were noted for Trisun, a high oleic hybrid variety and, as expected for 59259, a dwarf genotype. PSC8 the second parent of RIL population showed an intermediate values for sterol content. Although, they present the same sterol profile, the two parents were contrasted for the seed sterol content.

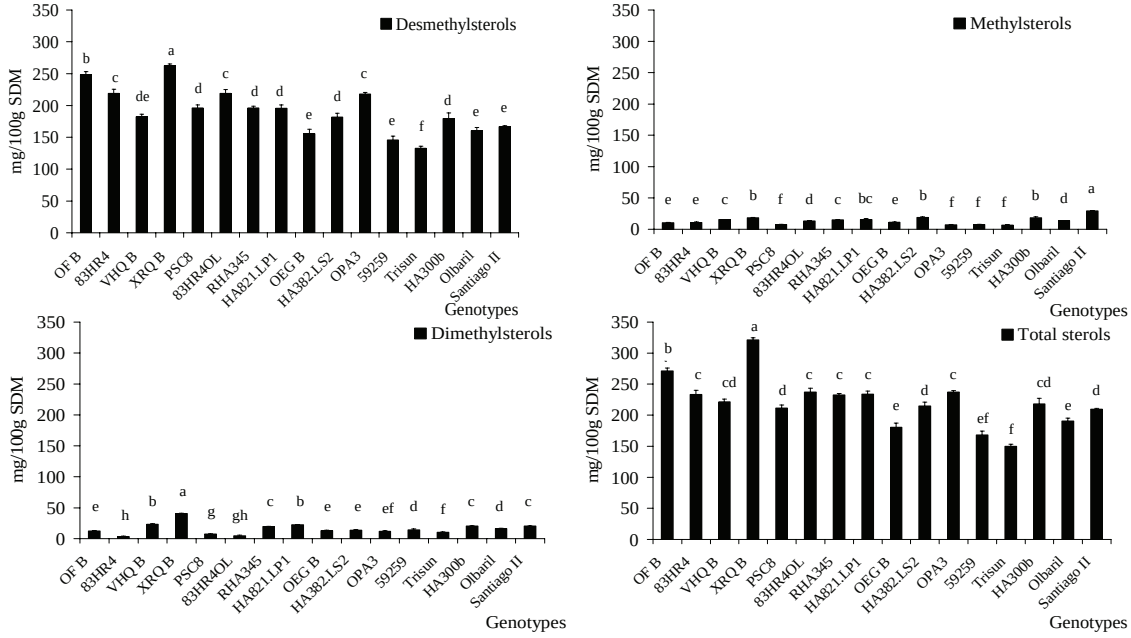

Fig. 1. Variability for total sterol (a), desmethylsterol (b), methylsterol (c) and dimethylsterol contents (d) among seventeen genotypes cultivated under rainfed conditions in 2005. Within each figure, means followed by a different letter are significantly different at P = 0.05.

Genetic analysis of sunflower seed sterol content

- Variability within the RIL population: The two parental lines were different for most traits (Fig. 1). Wide phenotypic variability was observed within the RIL population. Extreme RIL values showed both positive and negative transgression compared with parents (Fig. 2).

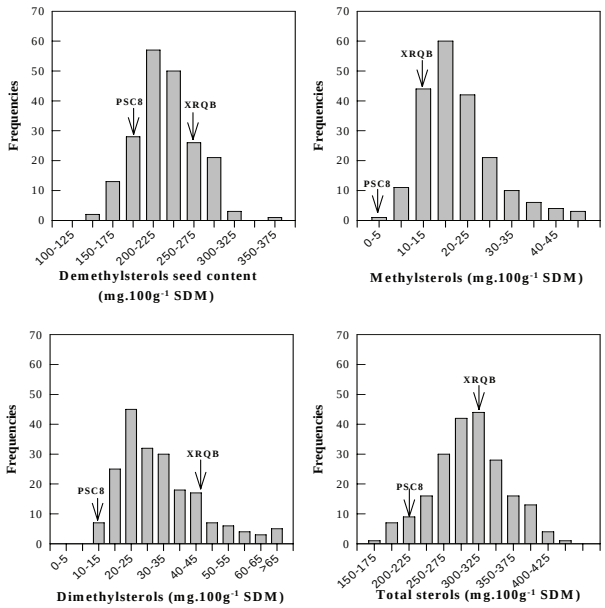

Fig. 2. Distribution of total sterol (a), desmethylsterol (b), methylsterol (c) and dimethylsterol contents (d) within a population of 200 RIL grown under rainfed conditions in 2005. Arrows indicate the parental values.

--QTL detection

Table 3 presents QTLs detected, their additive effect of alleles of each parent and the LOD likelihood confidence interval for the most important sterols traits. 24 QTLs were detected for all measured traits, at the level 1% for each trait (LOD>2.88). For some ones two regions have been identified while for others only one has been noted. One QTL was detected for sitosterol on LG1 explaining more than 10% of the variability of this trait. In the same LG QTLs were detected for desmethylsterols and total sterols content. Three QTLs were found for campesterol on LG7 and LG4 (nearly 20%) and stigmasterol on LG10 (12%).

Table 3. QTLs detected for the most important sterol seed content in sunflower.

Trait	Linkage group	R^2 (%)	LOD
β-Sitosterol	LG1	10.4	4.99
Campesterol	LG7	10.1	4.83
	LG4	9.6	4.57
Stigmasterol	LG10	12.3	7.08
Demethylsterols	LG1	13.9	6.98
Total sterols	LG1	14.3	7.21

DISCUSSSION

Genotypes were chosen in order to give a large range of sterol variability. Variance analyses show a very highly significant effect of the factor genotype for all traits measured (Fig. 1). Generally, high oleic genotypes produce lower content of phytosterols than conventional lines. Highest values were obtained for the genotype XRQ and the lowest ones for Trisun and for the dwarf genotype 59259. The values

observed of sterol content in sunflower seed are similar to those reported by Anastasi et al. (2000) but lower than the results obtained by Roche et al. (2006) in our laboratory. This difference is partly due to the germplasm used in the two studies but mostly due to the climate conditions during the cropping season. Mean temperatures which have prevailed during 2005 in Clermont-Ferrand were 2 to 4 °C lower than those reported by Roche et al. (2006) in their study in Toulouse in 2002 and 2003, respectively. Delayed sowing in the latter region may have increased sterols content.

Similarly, the variability observed within the RIL population was greater than the difference observed within the genotype collection. Moreover, the extreme values of some RIL were higher than the highest parent value or were lower than the lowest parent value, indicating transgressive segregation.

Studies on genetic determination of seed sterol content in sunflower have not so far been published. Several QTLs were revealed for the traits measured. Our results showed that for most abundant sterols, only one or two QTLs were detected. For β-sitosterol, which constitutes more than 75% of total sterol content, one QTL, explaining 10% of the observed variability, was detected on LG1 (Fig. 3). As checked with BIOMERCATOR, a unique metaQTL was found in this linkage group for β-sitosterol, demethylsterols and total sterols. In the same linkage group 1, another QTL position was detected for citrostadeniol and Δ7-stigmasterol. In linkage group 10, a unique metaQTL was also detected for citrostadenol, Δ7-stigmastenol and gramisterol.

The results show the potentialities existing within cultivated sunflower for sterol accumulation in seeds and this could help breeders for parental choice in order to initiate breeding programmes for sterol content improvement. These data need other investigations in multi-year experiments to assess the stability of QTLs identified across environments and genetic backgrounds in sunflower. Moreover, the genetic map used in this study should be improved by mapping more molecular markers and gene candidates as enzymes involved in sterol biosynthesis in sunflower. For example, starting from Arabidopsis amino acid sequence of cycloartenol-C-24-methyltransferase (EC 2.1.1.41), several potential SNP positions are found across the *Helianthus* EST sequences, and some of them can be used to check candidate co-localizes with some of the QTLs.

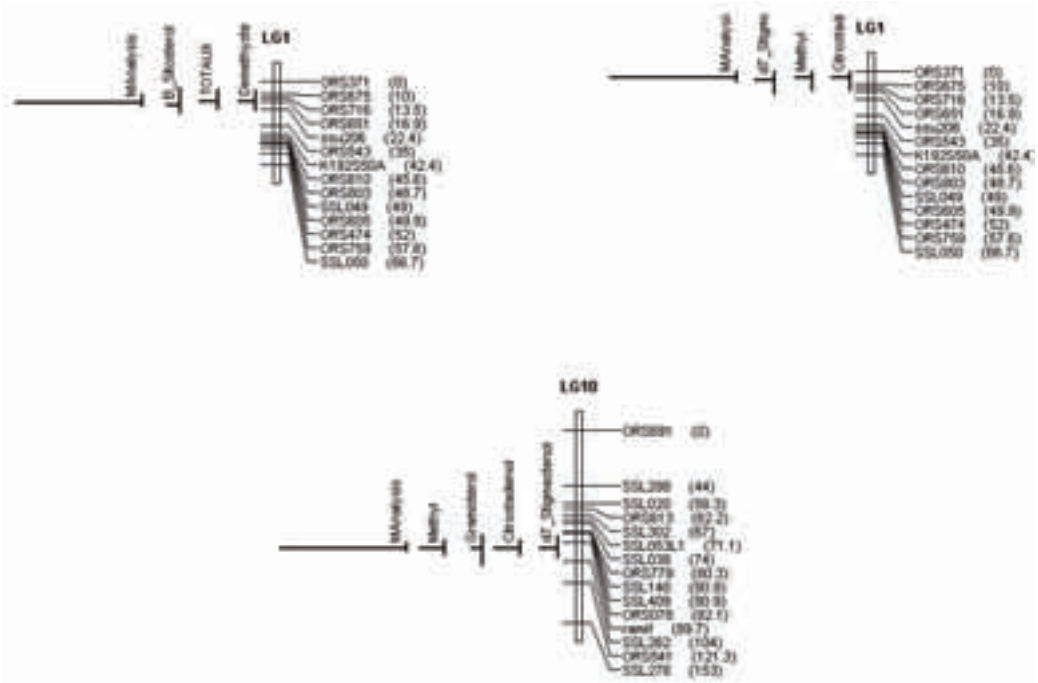

Fig. 3. MetaQTL mapping for some of the phytosterols data recorded on the XRQ x PSC8 RIL population (detailed results on http://lipm-helianthus.toulouse.inra.fr/Web/QTL/synthese-phytosterols.xml).

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Caractérisation par infra-rouge des teneurs en acides gras de la graine entière décortiquée de tournesol

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ABSTRACT

Oil seed market evolution requires the production of seeds with an elevated content of oleic acid instead of linoleic acid. Seeds producers need a rapid, accurate and low cost analytical method to discriminate sunflower's seed by their fatty acids content. This work's objective is to determine the feasibility of utilisation of near infrared reflectance spectrometry (NIRS) to predict fatty acid percentages in one husked sunflower seed. Calibration equations of palmitic, stearic, oleic and linoleic acids were developed with 400 husked seeds and were validated with another 400 independent seeds. The validation parameters showed that NIRS could replace classical and destructive gas chromatography analyse for oleic and linoleic acids in intact sunflower seeds. The correlation coefficient between NIRS prediction and GC reference values were of 0.93 for oleic acid and 0.95 for linoleic acid. Breeders could use NIRS analysis to evaluate the potential of their sunflower pools to obtain more performing hybrids for their fatty acid composition.

Key words: fatty acids – near infrared reflectance spectroscopy – NIRS – seed – sunflower.

INTRODUCTION

La France produit 57% du tournesol européen (FAO, 2007). L'huile de tournesol bénéficie d'une bonne image, en raison de sa composition équilibrée en acides gras insaturés qui représentent environ 90% de l'huile (Bozkurt and Karacal, 2001; Napier, 2006). Sa faible teneur en acide linolénique (0,2%) en fait une huile stable et sa richesse en composés mineurs (tocophérols) sont intéressants pour les actions anti-ranciment de l'huile (Bramley et al., 2000). De plus, l'huile de tournesol présente un intérêt industriel pour la production d'esters d'acides gras (essentiellement pour les biocarburants et les biolubrifiants) (Van Dievoet, 2005; Ballerini, 2006). Ainsi, il est nécessaire de renforcer le potentiel de sélection d'hybrides associant performances agronomiques et compositions vs teneurs en acides gras.

L'industrie semencière a besoin d'un outil analytique rapide, fiable et facile à mettre en œuvre pour sélectionner des lignées et des hybrides de tournesol. La méthode par spectrométrie proche infra rouge (SPIR) s'avère répondre à cette demande (Sato et al., 1995; Pérez-Vich et al., 1998; Moschner and Biskupek-Korell, 2006). C'est une méthode indirecte qui permet d'analyser avec peu de préparation des échantillons. Le principal avantage de cette technique est la facilité et la rapidité d'échantillonnage. D'autre part, cette technique peut être utilisée en laboratoire ou comme système embarqué. Ainsi, la spectrométrie proche infra-rouge permet de déterminer simultanément différents paramètres dans les graines oléagineuses. Cette méthode est largement utilisée car cet outil simple permet d'accélérer le volume d'échantillons à analyser. Cependant, cette analyse est actuellement destructive (broyage des graines) et est utilisé en pied de silo (allotement), ce qui ne répond pas au besoin de la sélection de disposer d'un outil ne dénaturant pas les graines. Actuellement, aucune analyse rapide non destructive (chromatographie gazeuse) et aucune calibration par SPIR des acides gras sur graine entière de tournesol n'a été identifiée dans la littérature avec un niveau adéquat à la sélection.

Dans cette étude, une prédiction des acides gras sur des graines décortiquées de tournesol par spectrométrie proche infrarouge sera développée. Ce projet s'inscrit dans une démarche de discrimination rapide de l'enrichissement en différents profils d'acides gras afin de procéder à une sélection précoce du matériel génétique au cours du processus de création variétale.

MATÉRIELS ET MÉTHODES

Matériel végétal

Une sélection de 800 graines de tournesol dans une gamme de teneur en acide oléique variant entre 15 et 80% a été retenue. Ces lignées et hybrides ont été sélectionnés pour étudier la faisabilité de la prédiction de la composition en acides gras d'une graine par spectrométrie proche infrarouge. Chaque graine a été mise à température ambiante 24h avant un décorticage manuel à l'aide d'un scalpel. Chaque graine décortiquée est analysée par spectrométrie proche-infrarouge puis analysée par la méthode de référence par chromatographie en phase gazeuse.

Analyse par SPIR

Pour l'analyse par spectrométrie proche-infrarouge (SPIR), chaque graine est placée dans un adaptateur en acier inoxydable sur la cellule en quartz et l'ensemble est bloqué avec un joint en silicone noir. Un appareil Foss NIR 6500 (Foss Analytical, Danemark) permet de collecter les spectres. Pour chaque graine, la mesure est réalisée sur les deux faces afin de s'affranchir de la différence spectrale. La moyenne entre les deux spectres s'effectue automatiquement. Les valeurs de réflectance [log (1/R)] de chaque échantillon sont mesurées entre 400 et 2500 nm à intervalles de 2 nm. La calibration des acides gras est réalisée sur 400 échantillons et la validation sur les 400 autres échantillons.

Analyse des acides gras

Chaque graine est broyée manuellement au mortier. Le broyat est placé dans un tube à hémolyse avec 3ml de hexane (qualité HPLC, SDS, France). Le mélange est vortexé, puis installé dans un rotary pour réaliser l'extraction de l'huile pendant 15 min. La saponification de l'huile est réalisée en ajoutant 1ml de NaOH méthanolique à 0,5 M. Les tubes sont chauffés dans un bain marie à 70ºC pendant 30 min sous système réfrigérant. La méthylation est effectuée en ajoutant 0,5 ml de BF_3 méthanolique à 14% (Sigma-Aldrich, France). Les tubes sont chauffés dans un bain chaud pendant 3 min. Les tubes sont ensuite rapidement refroidis et 0,5 ml d'eau et 1 ml d'hexane sont incorporés pour séparer les phases. Après agitation, la phase organique est prélevée et mise dans un vial pour l'analyse par chromatographie en phase gazeuse. 1 µl d'échantillon sont injectés sur une colonne (Z-FFAP 30m, 0,25mm, 0,25µm, Phenomenex, France) en chromatographie en phase gazeuse Fisons (GC 8000 series, TSP, France).

Calibration par SPIR et procédures de validation

Les équations de prédictions sont calculées à partir d'une régression modifiée des moindres carrés partiels après 4 passages d'éliminations d'échantillons déviants (WINISI 1.02 ; Infrasoft International LLC). Les traitements mathématiques sont décrits dans Ayerdi Gotor et al. (2007). La validation de la méthode par spectrométrie proche-infrarouge (SPIR) pour la prédiction des acides gras sur une graine décortiquée de tournesol est déterminée par les paramètres suivants: l'erreur standard de calibration (SEC), le coefficient multiple de détermination en calibration (RSQ), l'erreur standard de validation croisée (SECV), le coefficient multiple de détermination de validation croisée (1–VR ou R^2) et l'erreur standard de prédiction (SEP).

RÉSULTATS

Valeurs de référence

Les valeurs de référence utilisées dans cette étude pour réaliser les calibrations sont résumées dans le Tableau 1. Ils ont été obtenus par chromatographie gazeuse à partir d'une seule graine décortiquée de tournesol. Les pourcentages en acides oléique et linoléique ont des gammes significativement différentes, par contre la gamme des pourcentages en acides palmitique et stéarique varient dans une moindre mesure.

Spectre infrarouge

Le spectre infrarouge s'une graine entière décortiquée de tournesol est présenté sur la Fig. 1. Les pics caractéristiques de l'eau à 1970 nm et les 2 bandes carbonyles vers 1700-1800 et 2300-2400 sont apparentes.

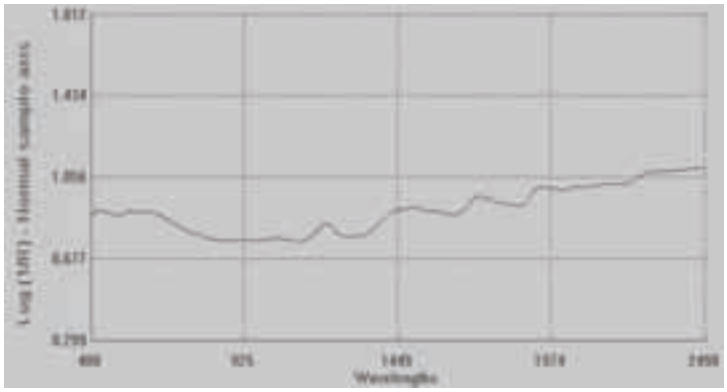

Fig. 1. Spectre infrarouge d'une graine décortiquée de tournesol de 400 à 2500 nm exprimé en log (1/Réflectance)

NIRS calibration

Les valeurs de référence des échantillons ont été introduites dans le modèle mathématique pour déterminer les équations de prédiction. Chaque valeur de référence est comparée à une valeur prédite. Les graphiques de validation de la calibration sont regroupés sur la Fig. 2. Les coefficients de corrélation obtenus sont satisfaisants pour les acides oléique (0,969) et linoléique (0,970) mais moins performants pour les autres acides: acide palmitique (0,782) et acide stéarique (0,329). La distribution des populations des acides gras révèle que les acides palmitique et stéarique ont une courbe normale mais une gamme de variabilité des teneurs faibles. La distribution des acides oléique et linoléique montre deux populations normales bien distinctes (fruit de la sélection pour ce caractère oléique).

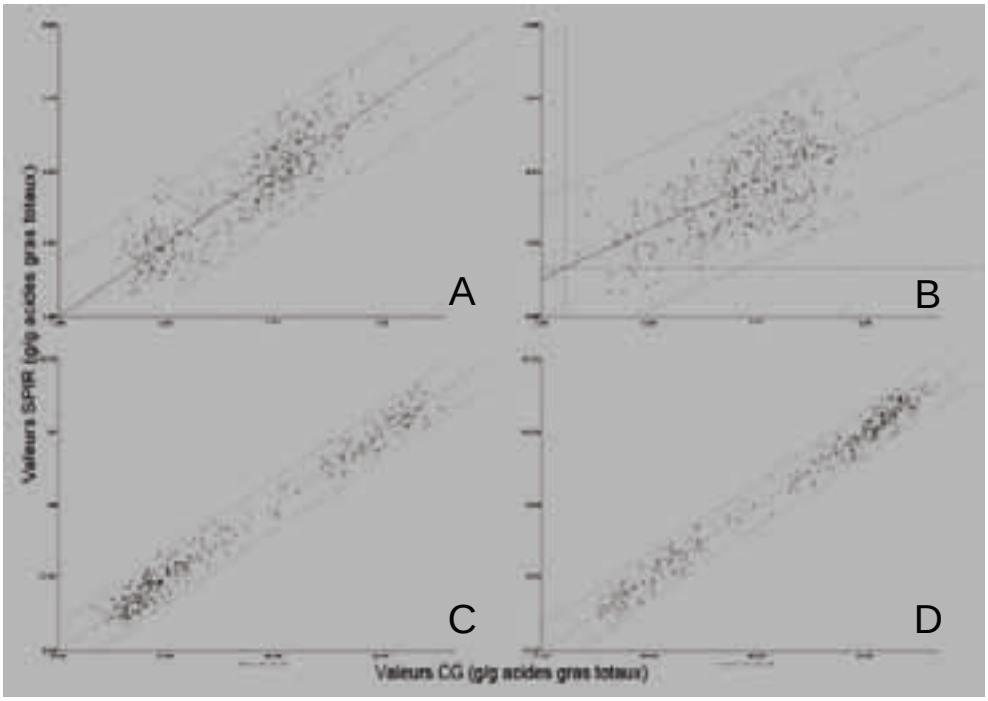

Fig. 2. Référence vs. Valeurs prédites pour: A – acide palmitique; B – acide stéarique; C – acide oléique, et; D – acide linoléique exprimés en g d'acide/g d'acides totaux des échantillons pour la calibration.

Le ratio DS/SECV est de 2,13 pour l'acide palmitique, de 1,19 pour le stéarique, de 5,68 pour l'oléique et de 5,84 pour l'acide linoléique. Ces valeurs de ration sont considérées comme correctes pour une prédiction sur des produits végétaux dès qu'ils dépassent 3 selon la littérature (Moschner and Biskupek-Korell, 2006). Les équations pourraient être utilisées pour les acides C18:1 et C18:2 mais il faudrait apporter une variabilité plus importante dans les teneurs an acide palmitique et stéarique.

NIRS validation

La validation des équations de prédiction est faite sur 400 échantillons non utilisés pour la calibration (Tableau 1). En calculant avec les équations le pourcentage d'acide gras on obtient les valeurs prédits. Ces valeurs vont être comparés aux valeurs de référence. Le coefficient de corrélation et l'erreur standard de prédiction (SEP) entre ce deux valeurs sont montré en Fig. 3. Le coefficient de corrélation (R^2) et le SEP ont un ordre de grandeur égal à ces des 1–VR et SECV de calibration. En conclusion, les équations de prédiction par spectrométrie proche infrarouge de l'acide oléique et de l'acide linoléique peuvent être utilisées pour déterminer leurs teneurs. Les acides palmitique et stéarique ne peuvent actuellement pas être prédits par spectrométrie proche infrarouge sur la base des équations actuelles.

Tableau 1. Résultats de la calibration et de la validation croisée des quatre acides gras présents dans une graine décortiquée de tournesol.

Ac. gras	Calibration sets				Calibration		Cross-validation	
	NE[1]	Range	Moyenne	DS[1]	SEC	RSQ	SECV	1 – VR
Palmitique (C16)	345	2,08 -8,12	4,78	1,30	0,572	0,805	0,609	0,782
Stéarique (C18:0)	342	0,79 – 7,88	3,91	1,22	0,942	0,404	1,022	0,329
Oléique (C18:1)	319	15,49 – 77,35	37,71	19,13	2,836	0,978	3,370	0,969
Linoléique (C18:2)	313	16,93 – 74,94	53,19	17,46	2,318	0,982	2,991	0,970

[1]NE: Nombre d'échantillons; DS: Déviation standard.

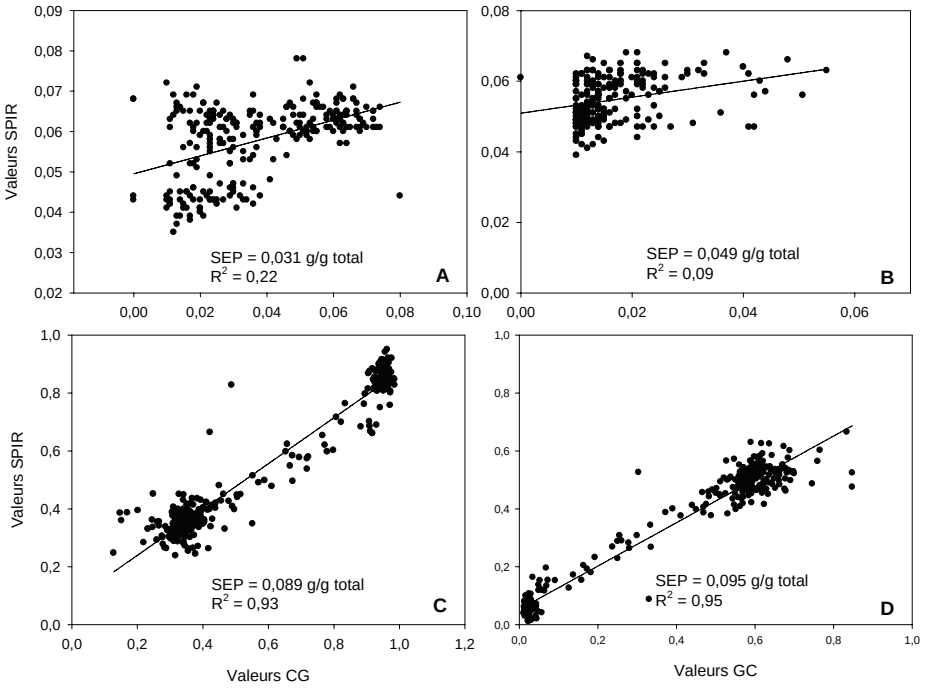

Fig. 3. Valeurs estimées par SPIR vs. valeurs de référence de: A – acide palmitique; B – acide stéarique; C – acide oléique, et; D – acide linoléique exprimées en g d'acide/g d'acides totaux des échantillons de validation avec le coefficient de corrélation (R^2) et l'erreur standard de prédiction (SEP)

DISCUSSION

La discrimination des teneurs en acide oléique et linoléique peut être effectuée par spectrométrie proche infrarouge sur une graine décortiquée de tournesol. Pour les autres acides gras, il est nécessaire d'augmenter la variabilité de leurs teneurs afin d'améliorer les coefficients de corrélation. D'autres études vont être menées pour améliorer la fiabilité des équations: développement d'outils mathématiques, tests interlaboratoire.

REMERCIEMENTS

Les auteurs souhaitent remercier SOFIPROTEOL pour son soutien financier (Fonds de Soutien à la Recherche Semencière Oléagineuse).

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Near infrared spectrometry (NIRS) prediction of minor components in sunflower seeds

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ABSTRACT

Minor components such as tocopherol and phytosterol present in sunflower seed are becoming interesting to consumers for their health properties, and to the food industry and sunflower breeders to obtain an added value to their products. The near infrared spectrometry (NIRS) technique could be a rapid and accurate method to determine the content of these useful molecules. The total tocopherol content reference values used to make calibration by NIRS varied from 62.8 to 451.9 mg/kg of dry matter (DM) and total phytosterol content values ranged from 53.9 to 189.0 mg/100g DM. The calibration equations obtained by NIRS showed a relatively good correlation coefficient between reference values and predicted values R^2 = 0.58 for the tocopherol content and of 0.61 for the phytosterol content. These encouraging results showed that NIRS could be employed to estimate minor components such as tocopherols and phytosterol rapidly in sunflower seeds. Nevertheless, further investigations are required to improve calibration equations to permit an accurate selection with this method.

Key words: minor components – near-infrared spectrometry – oil – phytosterols – sunflower seeds - tocopherols.

INTRODUCTION

Sunflower contains minor components with interesting properties for human health. Tocopherols and specially α-tocopherol (the main homologue present in sunflower oil) are good antioxidants, and they protect against some cancers and reduce cardiovascular disease problems (Bramley et al., 2000; Niki, 2004). Furthermore, sunflower contains phytosterols which reduce cholesterol levels in blood (Patel and Thompson, 2006). The concept of "food-medicine" or healthy foods is starting to be introduced to consumer philosophy. The food industry is looking for these compounds in current food products and seed producers are becoming interested in this added value for their seeds. Unfortunately, the available methods of analysis of these compounds takes a long time and are expensive, requiring a specialised person to perform it. Therefore, it has become necessary to develop new techniques for the analyses. Near infrared spectrometry (NIRS) is nowadays used by food industry and breeders to determine multiple parameters such as moisture, proteins and oil content or fatty acid composition in a large variety of matrices (Pérez-Vich et al. 1998; Velasco and Becker, 1998; Biskupek-Korel and Moschner, 2007). Few studies have focused their interest on the analysis of minor component by NIRS (González-Martín et al., 2006; Ayerdi Gotor et al., 2007). The objective of this work was to improve the values in our previous work (Ayerdi Gotor et al., 2007) especially in the prediction of total phytosterol content in sunflower seeds.

MATERIALS AND METHODS

Plant material

From a collection of nearly 2000 sunflower mature seeds from 4 growing seasons between 2003 and 2006 in different places all over France and Chile, 600 samples were selected as having the greatest variability for the parameters investigated, and the highest analytical accuracy and repeatability. These samples were used to elaborate the NIRS calibration. Each sample used for NIR spectrometry analysis had at least two replicates of tocopherols and phytosterols determined by classical methods (HPLC and GC respectively). The mean of these two replicates was considered as being the reference value.

NIRS analysis
In this work, 40 g of sunflower seeds per sample were ground in a Knifetec Mill (1975, Foss Tecator, Höganäs, Sweden) three times for 10 s. No sample material adhered to the walls of the mill because it was mixed at certain intervals. A FOSS NIR System 6500 (Foss Analytical, Denmark) was used to collect spectra from the milled sunflower seed samples (around 30 g) using a small round cup with a quartz window. The reflectance values as [log (1/R)] of each sample were measured from 400 to 2500 nm at 2 nm intervals. For each sample, a screening of 32 measures was carried out and compared with the 32 measures of a ceramic reference. For tocopherol prediction, an 860 spectra database was used for the calibration set, and for the phytosterol prediction, a 660 spectra database was used. For the validation set, around 200 samples for the tocopherols analysis and about 260 for the phytosterol content were used

Chemicals
For analysis, hexane, methanol, ethanol, acetone and diethyl ether had an HPLC grade from SDS (France). The trimethyl silyl ether (TMS) derivatives of all sterols were prepared using 1-methyl imidazol and N-methyl-N(trimethylsilyl)-heptafluorobutyramide reagent (Sigma, France). All sterol standards: β-sitosterol, stigmasterol and campesterol and betulin were purchased from Sigma (Paris, France). The four α-, β-, δ- and γ-tocopherol standards (99% minimum purity) were purchased in a Chromadex kit (USA).

Solvent extraction of lipids
The analysis of the total oil content was performed by hexane (n-hexane, Prolabo/Subra, Toulouse, France) extraction using a soxhlet extractor apparatus for a 4 extraction of 15g of the ground seeds (NF EN ISO 659, 1998) or with an accelerated solvent extractor apparatus (ASE 200, Dionex, France) with an isopropanol/hexane mixture (5:95 v/v) during 20min. Then, the solvent was removed from the extracts under low pressure evaporation (Rotavapor, Bioblock Scientific HS 40 HUBER, Heildorph, Germany). Lipid extracts were weighed and conserved at – 18°C.

Tocopherol determination
The complete separation of all native tocopherols was achieved using a high-performance liquid chromatography (HPLC) (SpectraPhysics, Thermo Separation Products, USA) with a normal phase LiChrorosorb Si60 column - 250cm, 4mm, 5µm (CIL Cluzeau, France) (ISO 9936, 1997, Ayerdi Gotor et al., 2006). The mobile phase was a mixture of hexane/isopropanol (99.7:0.3 v/v) at 1mL/min flow rate. One gram of oil sample was diluted in 25 mL of hexane and injected directly into the HPLC. Detection was performed with a fluorescence detector (excitation wavelength = 298 nm and emission wavelength = 344 nm; Waters 2475 multi λ). Tocopherols were identified by comparison of retention times with respective standards. Total tocopherol content was calculated as the sum of α- , β-, γ- and δ-tocopherol contents.

Sterol determination
The total and individual sterol content was analyzed by gas chromatography (GC) after saponification and a preparation with trimethylsylil (TMS) ether derivatives (NF EN ISO 12228, 1999). 1µl of the TMS solutions were injected into a fused silica capillary (ZB-5) column (Phenomenex, Paris, France) in a Fisons gas chromatograph (GC 8000 series MMFC 800 Multi-function controller, Italy) fitted with a flame ionization detector. Sterols were identified using the ratio obtained between betulin (Internal standard, Sigma-Aldrich, France) and sterol standards.

NIRS calibration and validation procedures
Prediction equations were calculated with a modified partial least-squares regression (MPLS) model after 4 outlier elimination passes (WINISI 1.02 - Infrasoft International LLC). With the MPLS regression method, factors are extracted in decreasing order of reliance measured by covariance with the response variable. To prevent overfitting in calibration, the number of factors is optimized by cross-validation in calibration sample. Previous mathematical treatment was applied on each spectrum as described in Ayerdi Gotor, et al. (2007). The equation with the highest coefficient of determination (R^2) and the lowest standard error (SE) in the calibration was used to predict the tocopherol and the phytosterol values of the validation set.

The validation of this NIRS method, for the estimation of tocopherols and phytosterols, was determined by the following parameters: the standard error of calibration (SEC), the multiple coefficient of determination in calibration (RSQ), the standard error of cross-validation (SECV), the multiple coefficient of determination of cross-validation (1–VR) and the standard error of prediction (SEP).

RESULTS

Reference values were calculated for tocopherol and phytosterol content expressed as a function of oil weight but in order to improve NIRS's calibration, data were converted with the oil yield (g/g of dry matter) into other units referring to dry matter (seeds at maturity stage). Data set used for the calibrations and the statistical parameters employed to make calibrations and cross-calibration are shown in Table 1.

Total tocopherol content represented the sum of the four homologues α, β, γ and δ-tocopherol present in the sunflower oil. Total phytosterol content represented the sum of seven sterols: Campesterol, Stigmasterol, Δ7- campesterol, β-sitosterol, Δ5-avenasterol, Δ7-stigmasterol and Δ7-avenasterol.

Table 1. Data set samples used to make calibration equations and statistical results. The standard error of calibration (SEC), the multiple coefficient of determination in calibration (RSQ), the standard error of cross-validation (SECV), the multiple coefficient of determination of cross-validation (1–VR) and the standard error of prediction (SEP).

	Calibration sets				Calibration		Cross-validation	
	SN[1]	Range	Mean	SD[1]	SEC	RSQ	SECV	1- VR
Oil (g/gDM[1])	513	16.4 – 54.4	36.38	8.57	2.41	0.92	2,57	0.91
Total tocopherol (mg/Kg DM)	511	62.8 – 451.9	191.90	70.14	41,93	0.64	45.73	0.58
Total phytosterol (mg/100gDM)	489	53.9 – 189.0	117.81	26.89	15.72	0.66	17.19	0.61

[1]DM: Dry matter ; SN: Sample number ; SD: Standard deviation.

The high correlation level of oil content (g/g DM) between the reference and predicted values confirmed that the change in units would not affect the estimation of minor components by NIRS, because it was higher than 0.9 (Fig. 1 A). The calibration for the total phytosterol content (mg/100g DM) (Fig. 1 B) showed that samples were uniformly distributed. The correlation between reference and predicted values for the calibration set of phytosterol was 1–VR = 0.61, better results than those obtained in the previous work (1–VR = 0.27) (Ayerdi Gotor et al., 2007). For the total tocopherol content (mg/kg DM) the results found were not better than those in the previous work.

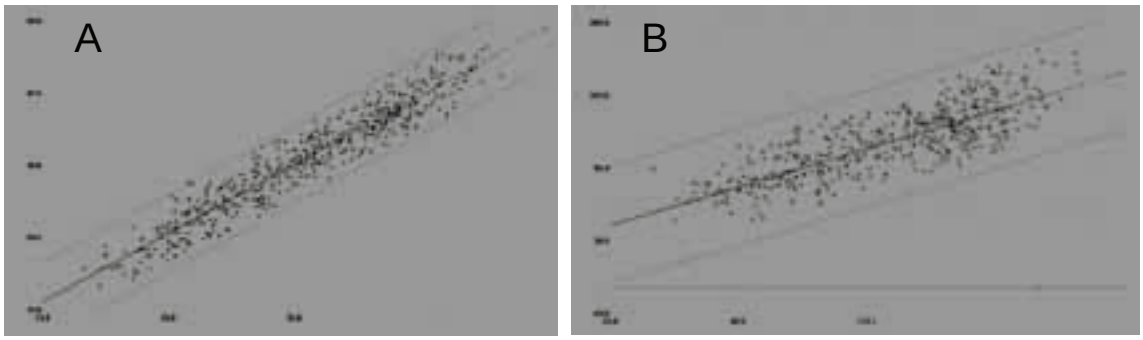

Fig. 1. Reference values *vs*. predicted values of: A- Oil (g/gDM); B- Total phytosterol content (mg/100 g DM) for the calibration group of samples.

The comparison of the two total phytosterol populations of reference values and predicted values (Fig. 2) showed that the reference values were a normal population, contrary to the predicted values that showed clearly two different populations, a fact that could explain the problems experienced for obtaining an accurate equation for this parameter with the modified partial least-square mathematical treatment.

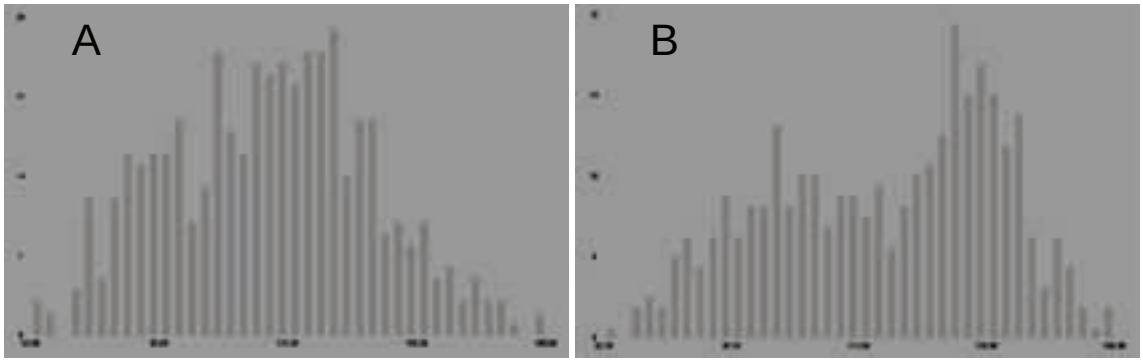

Fig. 2. Distribution of Total phytosterol population (mg/100g DM) and the sample number from each group of: A– Reference values used as calibration set; B– Predicted values for the calibration set.

The ratio SD/SECV for oil content was 3:3, for the total tocopherol content 4:1 and for the total phytosterol content 1:6. Values over 3 are considered as being good for a NIRS calibration for agricultural raw materials in the literature (Moschner and Biskupek-Korell, 2006). But, taking into account that tocopherols and sterols in sunflower represented less than 1% of the total dry matter, these values are promising.

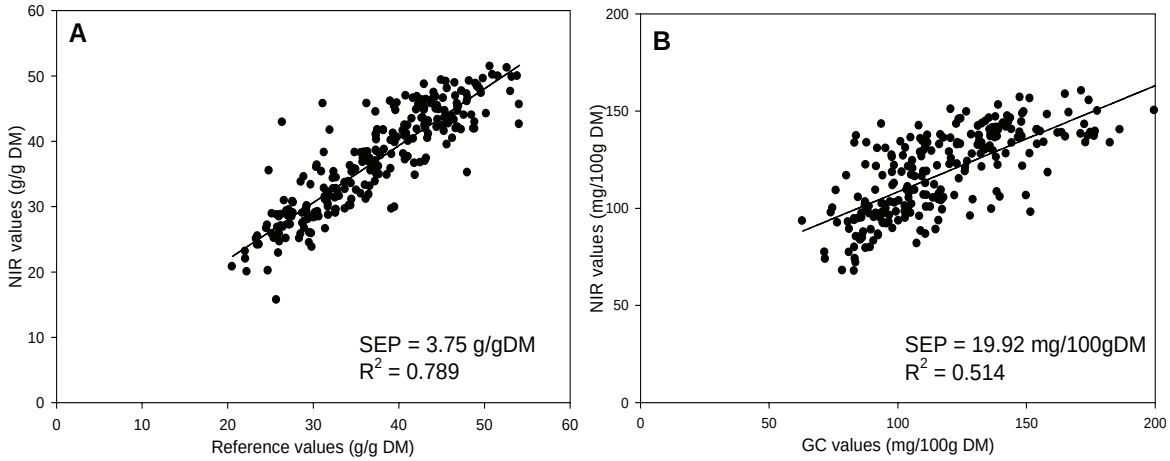

Fig. 3. Scatter plots of reference values vs NIRS predicted values for: A– Oil (g/g DM); B– Total phytosterol content (mg/100g DM).

The validation of the prediction equations was made with 260 independent samples. The comparison of the reference values with the predicted values was performed with these equations (Fig. 3). The SEP values are similar to the SECV values obtained in the cross-validation (Table 1), R^2 values were lower than the 1–VR values of cross-validation. The lower R^2 values for the oil content could be explained by the fact that inside the selected samples there were some parental lines with poor oil extraction results that could affect the validation, but that they were necessary to the calibration of tocopherols and phytosterol.

DISCUSSION

The analysis of minor components by near infrared spectrometry is starting to be used because of its potential: multi-parameter analysis, rapidity and low cost. But the establishment of this methodology requires accurate measurements to obtain reference values and powerful mathematical treatments to calculate prediction equations.

The selection of total tocopherol and phytosterol content based on NIR spectra for plant breeding or food industry allotment could be possible by this method. But better results could be obtained with a selection of a better mathematical treatment to generate equations.

ACKNOWLEDGEMENTS

The authors thank Caussade Semences, Maïsadour Semences, Monsanto Dekalb SAS, RAGT-R2n, Soltis, Syngenta Seeds for field experiments and for providing sunflower plant material. Financial support was provided by ASEDIS-SO, ONIDOL (French oilseeds organization) and Midi-Pyrenees Region. We also thank Cognis-France for their technical support.

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Expansion of sunflower crop production in Brazil: a survey of future trends

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ABSTRACT

The sunflower-cropped area in Brazil has been showing potential possibilities for being increased within a short period of time for biofuel production. Planning the activities is one of the requirements for the success of future cropped area expansion. This requires a previous survey that identifies future trends in the transformation and rearrangement of the sunflower agro-industry sector and also identifies technological needs that may affect this process. With the objectives of identifying future trends and technological needs, a value production chain was built and a questionnaire was distributed to agents of all the sectors participating at the V National Brazilian Symposium of Sunflower and at the XVII Sunflower National Research Meeting Network. The results pointed to a strong tendency for area expansion in the next two to five years (75%); this being as a secondary follow-up crop (83%), especially after soybean, and to be used for biofuel (77%). The main research needs were linked to disease control, crop zoning and varietal improvement for disease resistance and high oleic oil content. Also, when considering the vision of and concerns regarding the future expansion and transformation of the sunflower production complex, it is believed that this expansion is a consolidated trend, requiring a strategic sector planning associated with an economic and technological policy for its success within Brazilian agribusiness.

Key-words: agroenergy – biodiesel – Brazil – *Helianthus annuus* L. – planning.

INTRODUCTION

The sunflower (*Helianthus annuus* L.) cropped area in Brazil is incipient (110 thousand ha) when compared to the main world producers such as Russia, the European Union, Ukraine and Argentina, which sum up 19.9 million hectares of the world's cropped area. As it is incipient, the Brazilian yield production represents only 0.4% of the world production when compared with those countries which represent 72% (FAO, 2007). However, there is an enormous potential for expansion if sunflower is planted in rotation after soybean, which occupies an area of 21 million hectares (CONAB, 2007). The potential for area expansion is also driven by the Brazilian government's demand for biofuel and for high oleic oil for human consumption.

The use of vegetable oil, as a biofuel or for energy generation, has been known for long time in Brazil (França, 2005). However, there is a current debate in relation to its viability for being used as a biofuel or for human consumption.

The sunflower crop has shown advantages for possible biofuel use such as: high oil content (40%), also allowing cold extraction (Gazzoni, 2005), low production costs and a high positive energy balance (unit of energy produced as biodiesel/unit of energy used for crop production) when compared with other oil crops (Ungaro, 2006), particularly when it is used as a secondary follow-up crop after soybean (Lazzarotto et al., 2005). These facts reduce the demand for fossil fuel and optimize the use of fertilizer, water, land and other inputs, therefore producing environmental benefits due to the reduction of fertilizer and the maintenance of the soil productivity capacity.

Considering the fact that the economic and environmental benefits (except for fossil fuel use in reducing greenhouse gas emissions) of the sunflower for biofuel production are exactly the same as those for food production, there is a current debate regarding its destiny. Some authors (Mandarino, 2005) advocate that such a high quality and noble oil, with a high content of unsaturated fatty acids, the presence of vitamin E, β-caroten and phospholipids should be used for human consumption rather than for biodiesel production. Moreover, there is an increased demand in South America for sunflower genotypes with high oleic oil for frying purposes.

Despite the debate, the demand for an increase in Brazilian sunflower production on a short and medium term is evident. However, there are many doubts about the future, since on many occasions in the past, the market signaled an increase in the demand for sunflower production, but this did not happen.

These facts decreased the confidence of the producers in investing capital, financial and human resources in the sunflower crop area expansion.

In this context, the use of strategic planning tools is appropriate in order to minimize future risks and opportunities to the sunflower production complex. Identifying consolidated future trends and uncertainties, and rapid diagnoses involving all the agents of a given sector are necessary to the construction of future scenarios that could support a more rational decision-making process (Godet, 2000). So, the objective of this study was to identify future trends and technological needs of the sunflower value chain in order to subsidize the formulation of future public policies.

MATERIALS AND METHODS

This research was done in two phases; first a bibliographical review allowed to identify agents of the sunflower agro-industry value chain and to gather sunflower production data from Brazil and from the world since 1997. The second phase was the elaboration of a semi-structured questionnaire directed towards the future expansion, transformation and technological needs of the sunflower value chain. This questionnaire was distributed to agents of all the sectors participating in the V National Brazilian Symposium of Sunflower and in the XVII Sunflower National Research Meeting Network in Uberlândia/MG/Brazil in October 2007. These meetings are very important for the Brazilian sunflower agro-industry complex, due to the intense participation of the representatives of the whole value chain. It is also a forum to show the latest research achievements, identifying research needs, exchanging experience, practical knowledge and identifying policy needs.

The questionnaire was given to 89 participants, with a return of 59 respondents (55%). At the moment of the delivery the research objectives, the filling-in process and the importance of the participation of each respondent were explained. Also expressed was the commitment of the researchers to return the compiled results to the respondents.

The Gil´s (1995) recommendations were used in the drafting, application and analysis of the questionnaire. Statistical descriptive analyses (frequency distribution) were made following Pimentel-Gomes (1985).

RESULTS AND DISCUSSION

Analysing the past 10 years of world sunflower production (Fig. 1A), it could be seen that there had been a relatively constant volume of seed and oil production, 27.148 and 9.211 thousand tons year^{-1}, respectively, reflecting a stable market, except for in 2001, when very low volumes of seed and oil production were observed. However, the Brazilian data (Fig. 1B) did not follow this tendency. They showed an increasing tendency from 1997 to 2000, then leveling off and peaking in 2003, followed by a period of accentuated decrease 2004-2005, and showing another increasing tendency in 2006. It is expected that this trend will continue in 2007, with a higher increment in 2008 due to the Brazilian requirement of a 2% mixture of biodiesel in common diesel (Law 11.0097, January 13th, 2005).

Vieira (2001) also observed a great variability during the 1980s and concluded that this was due to the low yielding varieties, the scant volume of technologies available to farmers, the lack of tradition and knowledge of the farmers for planting sunflower, besides the inexistence of defined market and commercialization channels. This situation seems to have stayed the same up to today.

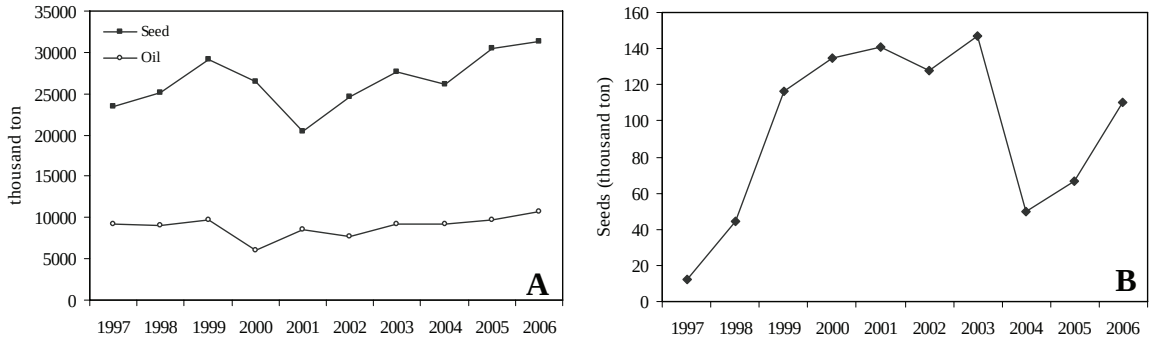

Fig 1. Historical series of world sunflower seed and oil production (A), Brazilian seed production (B). *Source*: FAO (2007).

The increasing demand for biofuels has been causing a drastic transformation in the Brazilian oil seed processing sector. Approximately 13 new plants have come into operation, i.e, 33% from the 45 constructed in 2007 for processing biodiesel (http//www.biodieselbr.com.br). Also, traditional plant mills have changed their focus into processing high oleic sunflower genotypes. Therefore, biodiesel and high oleic are playing an important role in motivating the expansion and transformation of the sunflower value chain.

Lazzaroto et al. (2005) built a schema of this new sunflower volume chain involving the main players, from the input suppliers to the final consumer (Fig. 2). It can be seen that each segment is responsible for a great number of activities generating employment and income, and that that segment is interconnected to the others, forming a long, complex value producing chain.

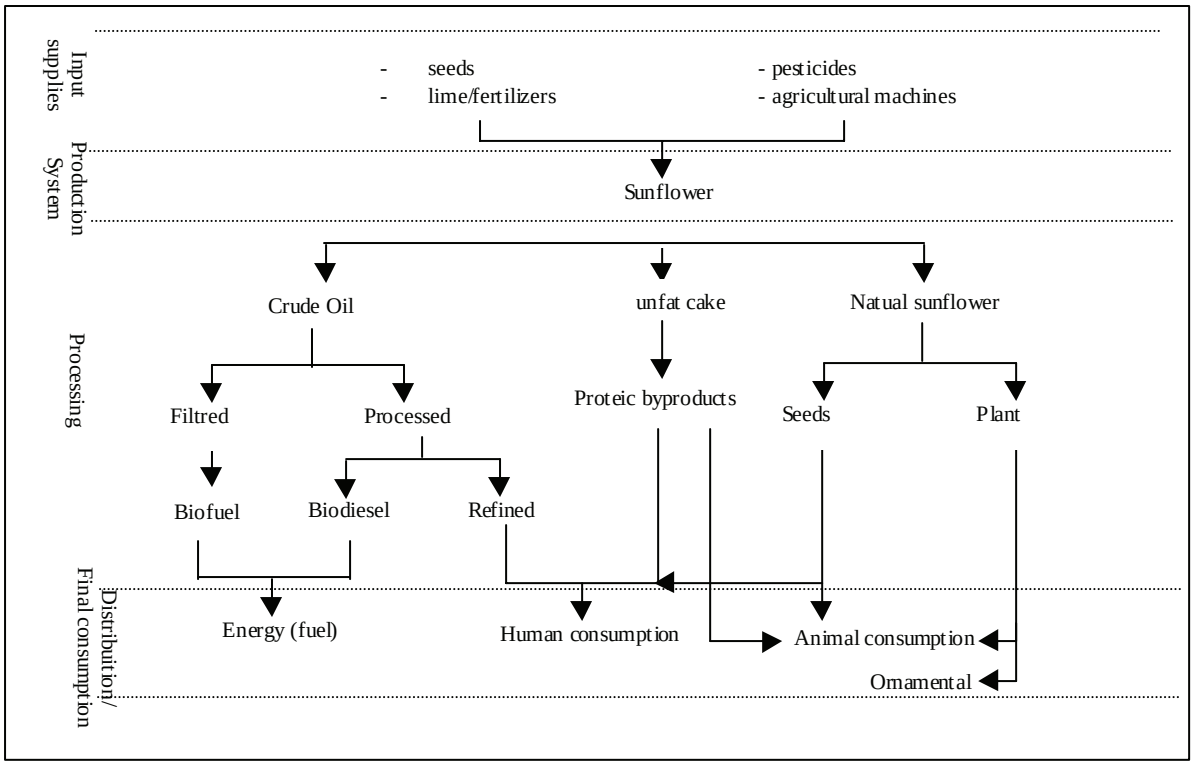

Fig 2. Schematic representation of the Brazilian sunflower agro-industy chain.
(*Adapted from* Lazzarotto et al., 2005)

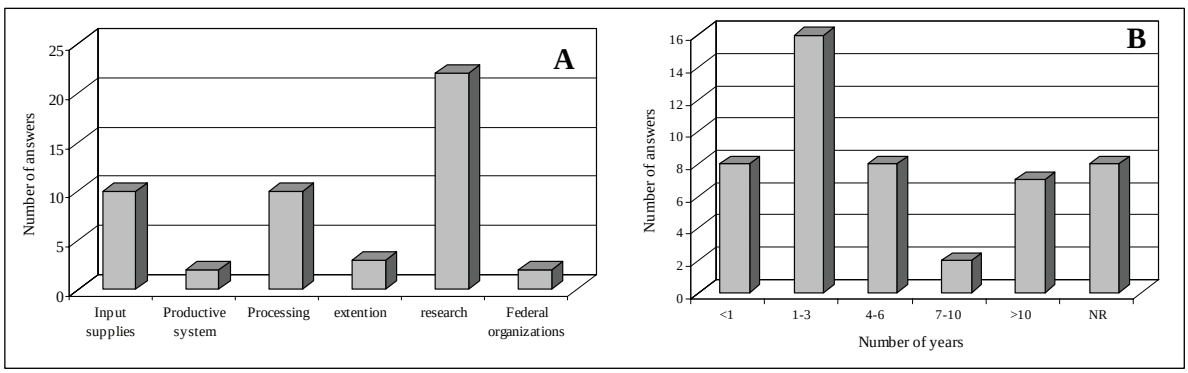

Fig. 3. Number of representatives per segment of the sunflower value chain (A) and period of time dealing with the crop (B). (Obs: NR – blank answers).

The experience in dealing with the sunflower crop was an important point for the persons surveyed. Thus, the time period in dealing with the sunflower was the parameter selected and it is shown in Fig. 3B. It can be observed that 16% had less than 1 year of sunflower management, 33% had from 1 to 3 years, 16% from 4 to 6 years and 16% had more than 10 years of experience with the crop.

The fact that 51% of the respondents had been working with sunflower for more than 4 years, besides ensuring the knowledge of the sector in the answers, also indicated that sunflower has been commercially produced for more than a decade in Brazil, although on a small scale. On the other hand, the high percentage (49%) of professionals with less than three years' experience coincides with the time period in which the Brazilian Government officially initiated the biodiesel national program (2005). This indicated a tendency of a growing crescent sunflower sector, with the interest of trained and specialized professionals. This information corroborated the expectations of crop expansion (Fig. 4A), to which 75% answered that a large expansion was expected in the next 2 to 5 years. In this way, the professionals, at the moment with less than 3 years' sunflower management experience, would be adequately trained for the expected period of high and rapid expansion.

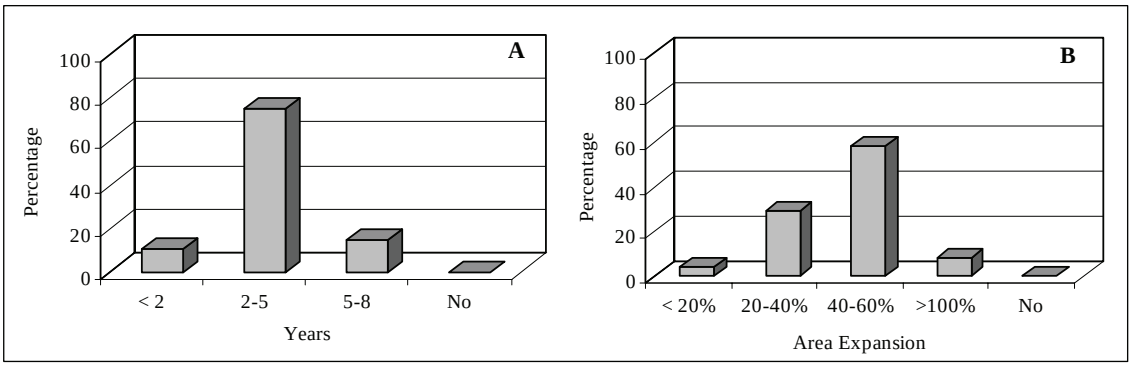

Fig. 4. Period of time (A) and percentage of cropped area expected for sunflower expansion (B). (No – agents who did not expect expansion)

In relation to the expected increment in the cropped area expansion (Fig. 4B), 58% of the professionals estimated that this increment would be between 40 to 60%. So, in the next 2 to 5 years the planted area of 110 thousands ha (2005) would be in a range of 154-176 thousand ha. It becomes clear that, even with this expansion, Brazilian participation in the international sunflower market will be insignificant. However, it represented a decrease in the importation of sunflower flour, crude and refined oil that reached in 2004 2.000, 10.065 and 7.454 tons, respectively (Lazzarotto et al., 2005). In addition, 29% of the professionals expected an area expansion ranging between 20-40% and the most optimistic ones (8%) had an expectation of 100% or more.

The survey also indicated that 83% of the professionals believed that increments varying from 40 to 100% are expected for sunflower as a secondary follow-up crop, especially after soybean, mainly concentrated in the Brazilian Savannas, which have the highest percentage of the soybean 21 million ha planted area (CONAB, 2007). This tendency indicated that the sector seems to be focused towards a more rational use of the areas under no-till. It permits the lowering of production costs due to the use of residual fertilizer and less energy while also maintaining the soil production potential by increasing the water holding capacity and infiltration rate, the organic matter content and by reducing compaction.

Considering that sunflower is one of the oil seed crops included in the Brazilian National Program for Production and Use of Biodiesel (PNPB) launched in 2005, a question on the expectations of the use of sunflower for biodiesel production within the PNPB was asked (Fig. 5A). As a result, 75% of the respondents answered positively. However, this expectation level depended on the degree of success of the PNPB expected by the agents, since 49% believed in total success, 47% in moderate success and 4% in failure (Fig. 5B).

The PNPB was drawn up with the purpose of substituting fossil fuel by renewable and efficient sources of biofuel, in order to achieve the sustainability of the energy matrix in the economic, social, and environmental spheres (MAPA, 2006).

In order to identify technological gaps and other barriers that may affect the expected area expansion rates, open questions were asked in which the professionals had to cite the five most relevant needs. However, some confusion was shown up in their answers and only two main research needs could be identified.

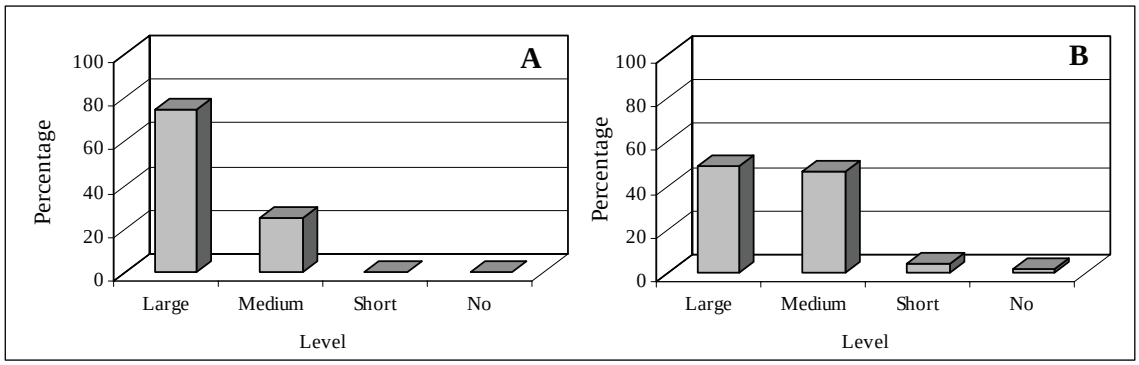

Fig. 5. Expectation of sunflower use as a source for biodiesel production (A) and of success of PNPB (B).

In the open questions, resistant/tolerant high yielding sunflower genotypes and disease control methods and products were cited by 61% of the specialists. In relation to diseases, the sector´s main concern was with *Sclerotinia sclerotiorum*, one of the worldwide sunflower's most devastating pathogen, although still with a very low incidence in Brazil (Gulya et al., 1997). A second research need was the crop agro-climatic zoning (39%). Besides focussing on the determination of the best planting date for maximizing yields and reducing the incidence of diseases, agro-climatic zoning is the official instrument required by the government to be used for rural credit and crop insurance purposes.

Another concern was related to the official recommendation and registration of fungicides, insecticides and herbicides for the crop (35%). Although some experimental tests were required, this subject could be seen to be more of a policy need. The sector was concerned with the small number of agrochemical products, registered by the Brazilian Ministry of Agriculture to be used in the sunflower cropping system. As an example, alachlor and trifluralin are the only two herbicides registered (Briguenti et al., 2005). Thus, the sector requires registration measures in emergency cases.

With regard to public policies, an agricultural policy was clearly necessary including price guarantee, rural credit, crop insurance and commercialization measures for the sunflower sector. According to CEPEA, 2007, the whole Brazilian agribusiness has been suffering from the inexistence of a public policy directed towards favoring the competitiveness and development of this sector, which represents 29% of the Brazilian Gross Product.

CONCLUSIONS

When considering the vision of and concerns regarding the future expansion and transformation of the sunflower production complex, it is believed that the expansion is a consolidated trend, requiring a strategic sector planning associated with economic and technological policies for its success within the Brazilian agribusiness

ACKNOWLEDGEMENTS

To the organizers of the V National Brazilian Symposium of Sunflower and at the XVII Sunflower National Research Meeting Network, as well as to all the participants in the survey who kindly answered the questionnaire.

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Breeding of sunflower as a biogas substrate

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ABSTRACT

The acreage for energy crops will increase during the next years. Biogas offers the possibility of producing a high amount of energy per hectare. To enhance the methane yield of sunflower as a biogas substrate, we investigated the biomass yield, oil content, oil yield and ash content of total sunflower plants. In the mean, biogas hybrids showed greater biomass production, oil content and oil yield than oil hybrids. Some recently developed experimental biogas hybrids derived from non adapted germplasm and genetic resources exceeded the best biogas hybrids in biomass and oil yield. Ash content was high for all investigated sunflower types.

Key-words: ash content – biogas – biomass yield – energy – methane yield – oil yield.

INTRODUCTION

Biogas is a product of anaerobic digestion or fermentation of biodegradable materials such as manure or sewage, municipal waste, and energy crops. It is comprised primarily of methane and carbon dioxide. Biogas can be used for electricity production, space-, water- and process heating and in local gas distribution networks. If compressed, it can replace natural gas for use in vehicles, where it can fuel an internal combustion engine or fuel cells. According to a recent study (Trend: research, 2007) the number of biogas plants will be increasing during the coming years, not only in Germany, but also in many European countries especially France, Italy, and Spain. To feed these biogas plants, the acreage and efficiency of energy crops for biogas production has to be raised.

Sunflower is a well-known oil crop. However, the high genetic variation in habit type, rapid growth, and seed oil content offers the possibility of breeding for biogas types to be used as entire sunflower plants in biogas reactors for the production of methane. In general, the methane yield per hectare depends on the biomass yield, the amount of biogas per kg organic dry matter and the methane content in the biogas. Here sunflower offers an advantage as its oil produces a higher methane content in biogas than other biogas crops.

Only a few results are available on the prospects of sunflower as biogas substrate. Therefore, we investigated sunflower varieties and newly established biogas hybrids for their total biomass yield, oil yield and ash content.

MATERIALS AND METHODS

In 2007 we conducted three experiments to investigate the biogas potential of sunflower. In experiment 1 we tested 22 oil hybrids, four biogas hybrids, and four populations (OPV) at the German locations Eckartsweier (South West), Hohenheim (South), Bonn (West) and Rostock (North East) in a 6x5 alpha design with two replications for their plant height, biomass yield and dry matter content. Oil content was investigated from samples of Eckartsweier, Hohenheim, and Rostock. In experiments 2 and 3, one oil hybrid, three biogas hybrids and 46 or 26 experimental hybrids (EH), respectively, were investigated at Eckartsweier and Rostock for biomass yield and dry matter content in a 10x5 or 6x5 alpha design, respectively. For experiment 2, additionally, oil content and plant height were measured at Eckartsweier. Ash content was examined from the samples of Eckartsweier and Rostock for experiment 1 and Rostock for experiment 2. In experiment 1 the plots consisted of four rows with harvesting of the two center rows. In experiments 2 and 3 the plots consisted of two rows. All rows were 5 m long with row spacing of 0.75 m. The plots were harvested with choppers. Samples of about 2 kg fresh weight were taken and dried to quantify the water content. The dried samples were milled. Oil content was determined by investigation of five subsamples of about one gram with a Bruker minispec. One gram of the samples was reduced to ashes in an oven by heating to 1,000°C.

The experimental hybrids of experiment 2 were developed by crossing a biogas sterile female line with pollen from single F_3 plants derived from crosses between non adapted or elite oil lines. The

experimental hybrids from experiment 3 were developed by crossing the same female line with pollen from single F_3 plants derived from crosses between elite oil lines and genetic resources.

RESULTS

In experiment 1, the biogas hybrids showed the highest mean biomass yield (Table 1), lowest dry matter content, and tallest plants; the oil hybrids showed the highest oil content and the lowest ash content. The populations showed the lowest biomass yield, lowest oil content and highest ash content. In experiments 2 and 3 the biogas hybrids again showed the highest mean biomass yield. However, in both experiments, some experimental hybrids produced more biomass than the best biogas hybrids, especially when F_3 plants with a genetic resource background were used.

Table 1. Results of the field trials[1].

Trait		E 1			E 2			E 3		
		Oil hybrids	Biogas hybrids	OPV*	Oil hybrids	Biogas hybrids	EH*	Oil hybrids	Biogas hybrids	EH
	n	22	4	4	1	3	46	3	3	26
Biomass yield (dt/ha)	Mean	110	146	89	94	133	119	98	156	147
	Min	86	134	72		131	72		140	109
	Max	138	153	137		135	179		170	231
	LSD*		30,7			32,7			58,2	
Dry matter (%)	Mean	29.8	25.3	29.0	52.7	25.3	27.6	49.2	32.1	30.9
	Min	25.7	24.0	23.8		23.3	22.2		27.8	32.3
	Max	35.8	25.8	33.3		27.9	40.0		36.9	39.7
	LSD		5.0			6.7			10.3	
Plant height (cm)	Mean	195	217	207	185	209	200	183	208	204
	Min	167	210	157		205	175		195	178
	Max	249	222	280		215	238		215	225
	LSD		30,2			16,6			20,8	
Oil content (%)	Mean	10.3	10.1	6.7	11.0	9.3	9.2	11.5	12.2	9.7
	Min	1.2	9.2	4.1		8.7	6.3		10.5	6.4
	Max	13.0	10.8	9.7		9.7	11.4		13.9	15.2
	LSD		3.4			3.3			3.0	
Oil yield (dt/ha)	Mean	10.9	14.1	5.2	10.3	12.4	11.0	8.0	13.8	10.6
	Min	1.3	11.5	3.2		11.6	6.6		11.8	5.0
	Max	16.5	15.7	8.1		12.8	17.2		15.4	14.7
	LSD		5.2							
Ash content (%)	Mean	8.6	9.2	10.3	7.3	8.0	8.1			
	Min	7.9	8.1	8.5		7.7	5.9			
	Max	10.7	11.0	11.5		8.4	11.1			
	LSD		2.6			2.6				

[1]E = Experiment, OPV = open pollinated variety, EH = experimental hybrid, LSD = LSD 5%

DISCUSSION

A high biomass yield is a prerequisite for a high methane yield of a crop. In this research project we selected sunflower lines which showed a superior biomass production in hybrid combinations. Those biogas hybrids were compared with hybrids used for oil production. Most of these oil hybrids produced significantly less biomass than our newly developed biogas hybrids. For a further increase of the biomass, we selected tall lines with high lodging resistance, derived from either non adapted germplasm or genetic resources and crossed them with our elite biogas lines. To get an idea about the potential of this material we produced experimental hybrids. Although, on average the experimental hybrids produced less biomass than the biogas hybrids, some of them showed a pronounced biomass yield. Thus, using non adapted germplasm or genetic resources allows a further increase in biomass yield.

A high methane yield also depends on the specific methane yield of a substrate. As oil achieves high methane rates, we investigated the oil content of the tested hybrids. The highest mean and maximum oil content were found for the oil hybrids. But the oil yields of the biogas and experimental hybrids were comparable with the oil yields of the oil hybrids. This means that a high biomass yield can be achieved without loss of oil yield.

High ash content decreases the specific methane yield of a substrate. In our primary investigations we generally found a relatively high ash content in the biomass of sunflower. But, as there are differences between genotypes, selection for lower ash content seems possible.

Currently, biogas production from energy crops is mainly based on the anaerobic digestion of maize. Sustainable biogas production must include a whole system of sustainable and environmentally friendly crop rotations. Sunflower has shown to have the potential to be one partner of a biogas crop rotation.

ACKNOWLEDGEMENTS

We thank the NCRPIS, USDA, USA for seeds of the genetic resources and the Federal Ministry of Food, Agriculture, and Consumer Protection of Germany for financial support (Project number FNR22013104).

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Veinte años de ensayos de girasol en Andalucía: evolución del rendimiento de semilla y riqueza grasa

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ABSTRACT

Using 20 years (1987-2007) of annual data from the Andalusian Network of Agrarian Trials – sunflower subnetwork -, a study on the evolution of yield and oil content of sunflower varieties was carried out in three locations very representative of the areas of Andalusia (south of Spain), where sunflower is widely cropped. The results show that in two locations (Córdoba campiña and the Carmona Valley), both seed production and oil content have a descending trend. This may have been due to the sudden appearance of the sunflower broomrape (*Orobanche cumana*) in these areas, which has obliged seed obtainers to quickly breed for varieties resistant to broomrape as a first objective, letting yield and oil content stand at a lower priority level. However, in the Jerez campiña, seed yield has shown a moderately increasing trend. In this area, sunflower broomrape has not appeared yet, which perhaps has allowed the new conventional sunflower varieties to better express their yield potential.

Key words: broomrape – oil content – *Orobanche cumana* – seed yield – sunflower.

RESUMEN

Se ha realizado un estudio sobre la evolución de los rendimientos en producción de semilla y contenido en aceite, obtenidos de las parcelas de ensayos de la subred de girasol perteneciente a la RAEA (Red Andaluza de Experimentación Agraria), desde el año 1987 hasta el año 2007. Se han seleccionado tres zonas para el estudio: la campiña de Córdoba (Córdoba), la vega de Carmona (Sevilla) y la campiña de Jerez de la Frontera (Cádiz). Los resultados muestran que tanto en la campiña de Córdoba como en la vega de Carmona la recta de tendencia de los rendimientos tanto en producción como en riqueza grasa tiene una pendiente negativa, posiblemente debido a la aparición del jopo en zonas tradicionales de cultivo, lo que ha provocado una retirada del mercado de antiguas variedades muy productivas pero muy sensibles al parásito. Los mejoradores han buscado variedades resistentes a las nuevas razas de jopo, aunque no sean tan productivas como las variedades antiguas. Por el contrario, la pendiente de la recta ajustada a los rendimientos obtenidos en la campiña de Jerez es positiva, lo que pudiera deberse a la ausencia del parásito en esta zona unido a unas temperaturas mas suaves en el periodo de maduración del girasol.

Palabras clave: Girasol, ensayos, jopo, rendimientos, riqueza grasa

INTRODUCCIÓN

La Red Andaluza de Experimentación Agraria (RAEA), comenzó sus actividades en el año 1987 y desde entonces la subred de ensayos de variedades de girasol de primavera, incluida dentro del Programa de Cultivos Herbáceos, ha proporcionando resultados anualmente, convirtiéndose en una referencia para el sector de las semillas oleaginosas (agricultores, empresas privadas de semillas, cooperativas agrícolas, asociaciones agrarias, etc.) en la región, cumpliendo con su objetivo de proporcionar al agricultor información generada por una experimentación en condiciones de cultivo similares a las de sus explotaciones.

Los datos utilizados para este trabajo son parte de los resultados obtenidos en 20 años de experimentación, de los cuales cabe destacar:

- El numero total de ensayos de variedades asciende a 271, distribuidos en las siguientes provincias: Sevilla (98), Córdoba (61), Cádiz (39), Huelva (27), Málaga (25), Granada (16), y Jaén (5).
- Los datos, tanto de producción como de riqueza grasa, han sido generados por alrededor de 350 variedades (Resultados ensayos de Girasol RAEA 1987 a 2007).

- El material vegetal utilizado en los ensayos ha sido proporcionado anualmente por las empresas obtentores y productoras de semilla con sede en España.
- El número de localidades donde se han realizado los ensayos ascienden a más de 30, repitiéndose la mayoría de ellas a lo largo de estos años, lo que ha permitido realizar diferentes estudios de adaptabilidad de variedades a distintos medios ambientes (RAEA Variedades de girasol. Campaña 91/92 y 93/94), y la publicación de " Listas de variedades recomendadas por localidades" (RAEA, Campaña 90/91).

MATERIALES Y METODOS

Se ha estimado la producción y la riqueza grasa de todas las variedades de girasol ensayadas a lo largo de estos 20 años (1987-2007), excepto el año 1995 (extremadamente seco). Todos los ensayos corresponden a cultivo de secano.

La parcela elemental siempre tuvo una superficie de 28 m^2,y estuvo formada por 4 líneas de 10 m de longitud y 0.70 m de separación entre las líneas, de las cuales se cosecharon sólo las dos centrales.

Los porcentajes de aceite de las distintas variedades se obtuvieron en muestras de semilla de cada unidad experimental, desecadas en estufa a 80 ºC durante 24 horas, a 0% de humedad, y sin impurezas, utilizando un analizador de Resonancia Nuclear Magnética (NMR).

Los diseños experimentales de los ensayos fueron lattices cuadrados o rectangulares con 3 repeticiones.

Para analizar la evolución de los rendimientos y contenido graso durante este período de tiempo se eligieron tres zonas de Andalucía Occidental muy representativas de las zonas de cultivo de girasol, bien diferenciadas: la Campiña de Córdoba, la Vega de Carmona en Sevilla, y la Campiña de Jerez de la Frontera en la provincia de Cádiz.

Se ha ajustado un modelo sencillo a los datos de producción anual media de semilla y de contenido de aceite, para estimar su evolución en el período.

La Fig. 1 muestra la variación anual de temperaturas y precipitaciones en las tres zonas, usando la información de la red agroclimática andaluza (Gavilán et al., 2006) que dispone de estaciones climatológicas situadas en las fincas de Tomejil, (Carmona-Sevilla) y el Rancho de la Merced, (Cádiz), en donde están dos de los campos de ensayo, y en Santaella (Córdoba). Los datos medios diarios pueden ser consultados en la pagina web del IFAPA: www.juntadeandalucia.es/innovacioncienciayempresa/ifapa/ria

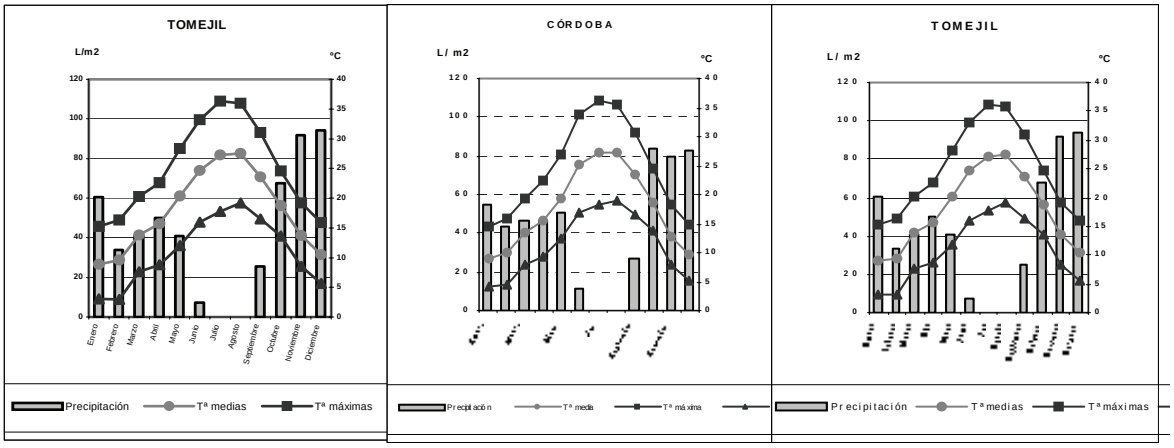

Fig 1. Climogramas de Jerez de la Frontera (Cádiz), de Santaella (Córdoba) y Carmona (Sevilla) desde el año 1987 al 2007

En la Fig. 1, se observa que los periodos de sequía, caracterizados por los meses en los que las precipitaciones están por debajo de las curvas de las temperaturas, son prácticamente los mismos en las tres zonas extendiéndose desde Mayo a Septiembre.

En cuanto a la distribución de temperaturas (máximas, medias y mínimas) son muy semejantes en las tres zonas, oscilando entre los 3-4ºC de mínima en los meses de invierno y los 35-36ºC para los meses de verano (Julio y Agosto) en la Campiña de Córdoba y la Vega de Carmona, mientras que este intervalo se suaviza en Jerez, por la proximidad a la costa, con mínimas de 5ºC en enero y febrero y máximas alrededor de 32ºC en los meses de verano.

La precipitación se concentra en los meses de otoño-invierno en las tres zonas. En la Campiña de Córdoba la media registrada de los veinte años es 80 mm en cada uno de los meses de Octubre, Noviembre y Diciembre, algo superiores son los 90 mm registrados en la Vega de Carmona en Noviembre y Diciembre, y 80 y 100 mm para los meses de Noviembre y Diciembre respectivamente, en Jerez.

RESULTADOS Y DISCUSIÓN

La Fig. 2 muestra las precipitaciones anuales de las tres zonas de estudio entre los años 1989 al 2007. Hay que destacar que la campiña de Córdoba el 55% de estos años registra precipitaciones inferiores a los 500 mm. Este porcentaje asciende al 66% en la vega de Carmona y se sitúa en el 50% en la campiña de Jerez.

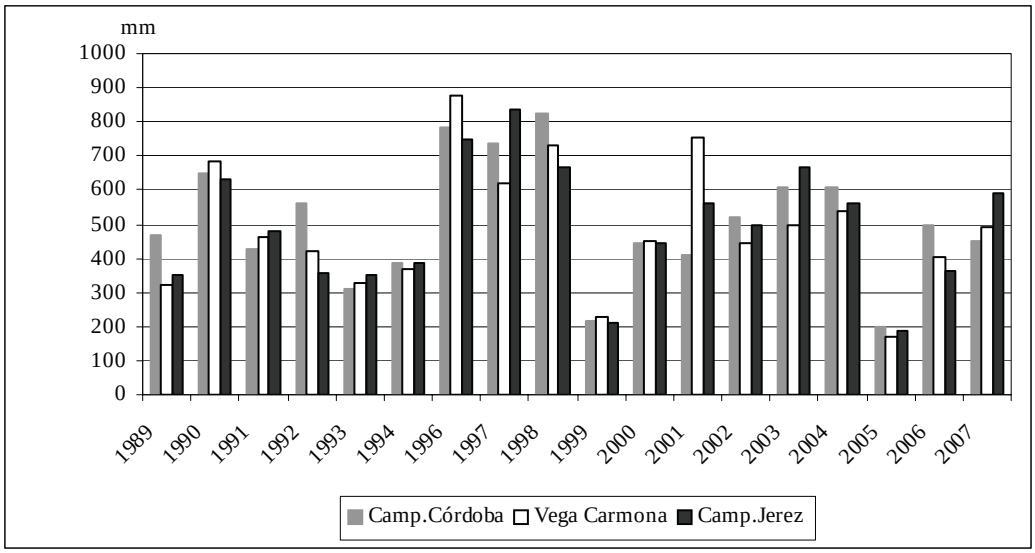

Fig. 2. Precipitaciones anuales en la Campiña de Córdoba, la Vega de Carmona y la Campiña de Jerez desde el año 1989 al año 2007

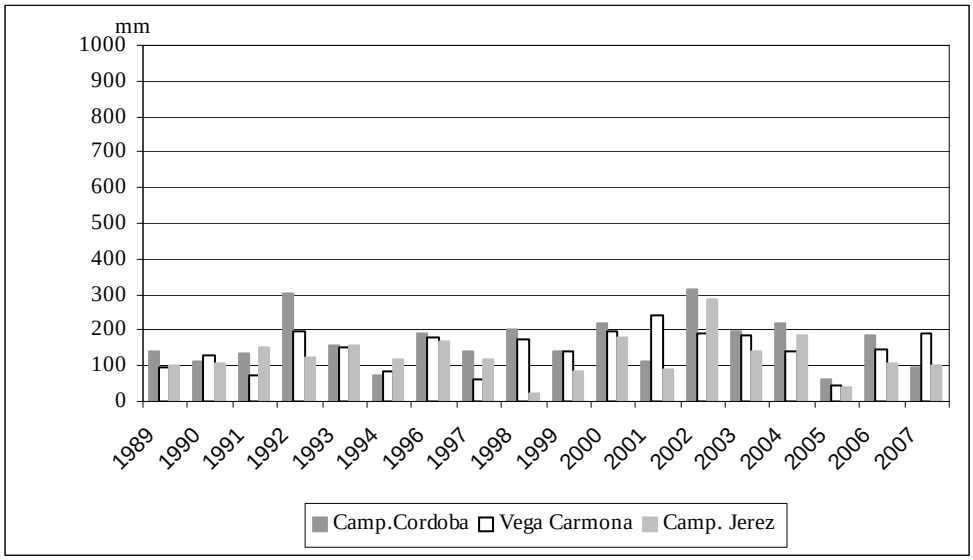

Fig. 3. Precipitaciones registradas en la campiña de Córdoba, la vega de Carmona y la campiña de Jerez desde siembra a recolección del girasol.

Se puede observar cómo las precipitaciones recogidas durante el ciclo del cultivo en la campiña de Córdoba son menores de 200 mm el 72% de los años en estudio y este valor asciende al 94% de los años en la vega de Carmona y en la campiña de Jerez.

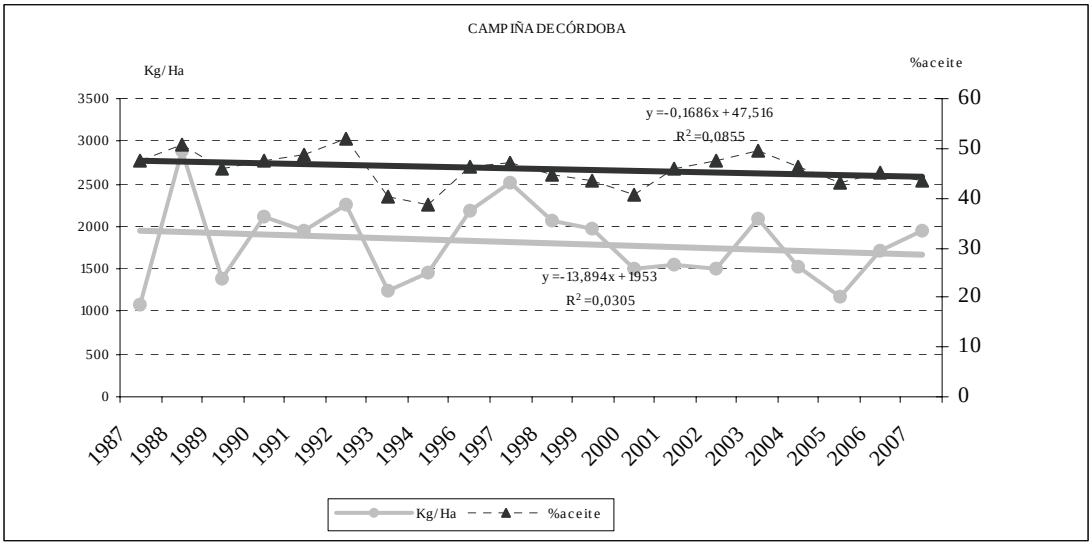

Fig. 4. Evolución de la producción y de la riqueza grasa desde el año 1987 al 2007 en la campiña de Córdoba.

En la Fig. 4 se representan los rendimientos medios de todas las variedades tanto en producción como en riqueza grasa en la Campiña de Córdoba. Tanto la producción de semilla (b= - 13. 9) como el contenido graso (b= -0.168), muestran una tendencia descendente, dentro de la fluctuación interanual, entre 1.100 y 3.000 kg/ha. Estas subidas y bajadas del rendimiento pueden estar directamente relacionadas con la precipitación anual, y mas concretamente con la precipitación registrada durante los meses de otoño e invierno (octubre a febrero), período que se debe considerar de recarga del suelo, y que es fundamental para el desarrollo posterior de los cultivos de invierno y primavera (Perea et al., 2006). El coeficiente de correlación, entre la precipitación invernal y el rendimiento del girasol, para los 20 años del estudio en la campiña de Córdoba es del 0.6316, lo que podría explicar que una parte importante de los rendimientos del girasol están relacionados con las lluvias invernales. Estos resultados parecen estar en consonancia con los obtenidos por Merrien (1992), quien afirma que más importante que la cantidad total de agua recibida por el cultivo es su distribución en cada fase del mismo.

A partir del año 1997, los rendimientos medios obtenidos sufren una bajada continúa salvo un fuerte rebote en el año 2003 (coincidiendo con una gran pluviometría recogida en los meses de otoño e invierno) y una subida más suave en el año 2006 y 2007. Una posible explicación de este descenso de los rendimientos a partir de 1997 podría deberse a la aparición de la raza F de jopo (*Orobanche cumana* Wallr.), que empezó a detectarse por aquellos años y que atacaba a la mayoría de las variedades ensayadas. En años sucesivos se produjo una gran expansión de este parásito, lo que provoco una disminución importante de los rendimientos. La subida de los rendimientos en los dos últimos años de estudio, podría ser debida a la inclusión en los ensayos de la red de material vegetal resistente a las nuevas razas de jopo.

El contenido en aceite es un parámetro que además de depender de las características genéticas de la variedad es muy influenciado por las temperaturas ambientales en el período de maduración, y por cualquier accidente que provoque un estrés fisiológico en la planta, impidiendo un buen llenado del grano.

En el período 1997-2007 el contenido medio en aceite permanece casi inalterable, con unas ligeras bajadas y subidas, aunque la pendiente de la recta es negativa (b= -0.168), posiblemente sea debido a la abundante presencia de jopo en el campo a partir del año 1997, que han impedido una correcta maduración de las variedades susceptibles.

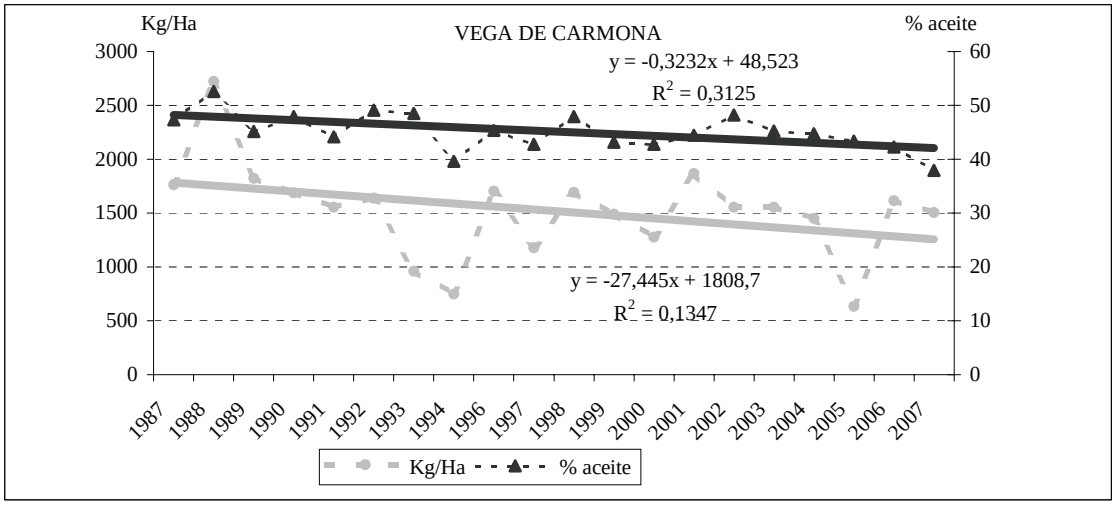

Fig. 5. Evolución de la producción y de la riqueza grasa desde el año 1987 al 2007 en la Vega de Carmona.

En la Vega de Carmona (Tomejil), se observa también esta tendencia decreciente en el mismo período de tiempo, rectas con pendientes negativas mucho más pronunciadas (b=-27.4 y b=-0.32) que en el caso de la campiña de Córdoba.

El coeficiente de correlación obtenido entre el rendimiento y la pluviometría registrada entre los meses de otoño e invierno es de 0.4654, lo que indica que prácticamente un 50% de la producción esta condicionada por la pluviometría otoño-invernal.

Los resultados obtenidos de estas dos zonas, la vega de Carmona y la campiña de Córdoba son semejantes tanto para la tendencia de las producciones como de las riquezas grasas. Las ordenadas en el origen de ambas rectas son muy parecidas, en la campiña de Córdoba es 1.953 kg/ha y en la vega de Carmona 1.808 kg/ha.

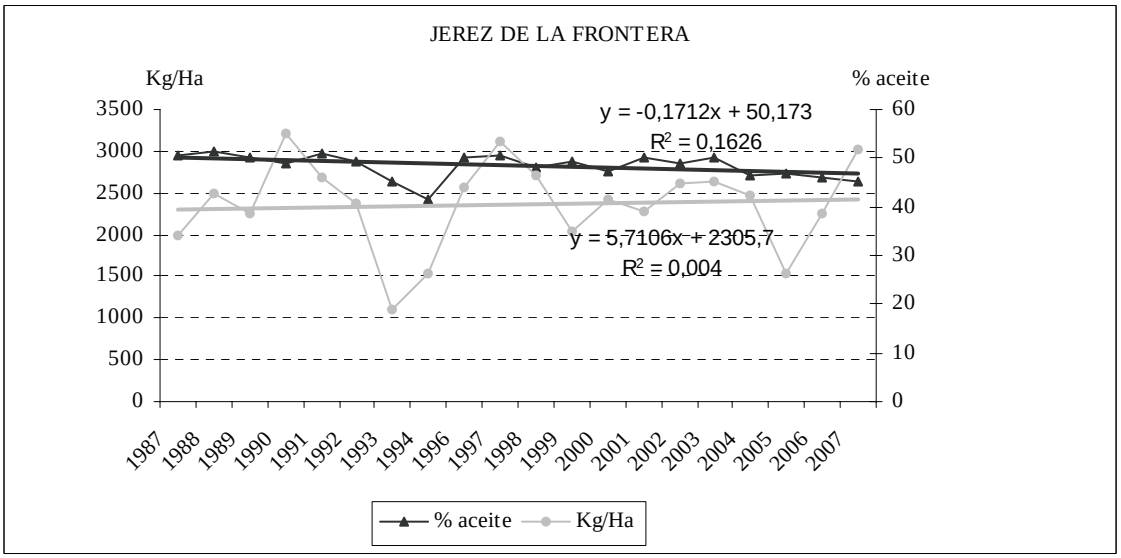

Fig. 6. Evolución de la producción y de la riqueza grasa en el periodo 1987-2007 en Jerez de la Frontera.

En la Fig. 6 se han representado los rendimientos medios tanto en producción como en riqueza grasa en la Campiña de Jerez de la Frontera. En este caso la tendencia de la producción de semilla es positiva (b= 5.71, mientras que el contenido graso desciende (b= -0.17) como en las otras dos zonas estudiadas.

Hay años en que las producciones superan 3,000 kg/ha y raramente algún año desciende por debajo de los 1,500 kg/ha, lo que expresa unas condiciones ambientales superiores, debido posiblemente a la mayor humedad relativa del aire, debido a la influencia de la costa y los vientos del Oeste. El punto de corte de la recta de regresión se sitúa en 2,305 kg/ha muy superior a los obtenidos en las otras dos zonas.

El coeficiente de correlación obtenido para la zona de Jerez entre el rendimiento y la pluviometria registrada en los meses de otoño e invierno es de 0.7025, el más alto de las tres zonas estudiadas. Como se ve es la única de las tres zonas de estudio donde la tendencia de los rendimientos presenta una ligera subida, ello podría ser debido a que gran parte de la provincia de Cádiz y en particular la campiña de Jerez es una zona aún libre de la presencia de jopo. Además la alta correlación entre la pluviometria invernal y la producción final y a las temperaturas mas suaves que se registran durante los meses de maduración del cultivo (Fig. 1) produciría una buena maduración de la mayoría de las variedades del ensayo con el consiguiente aumento del rendimientos respecto a las otras dos zonas estudiadas.

CONCLUSIONES

Los resultados obtenidos en 20 años de experimentación (1987-2007) con variedades de girasol parecen indicar una tendencia negativa de los rendimientos en algunas zonas muy concretas de Andalucía. Posiblemente estos resultados puedan deberse a que en los últimos diez años los esfuerzos de la mejora han ido encaminados de una manera especial a la obtención de material vegetal resistente al jopo, debido a la sistemática aparición de razas nuevas y más virulentas. Se ha preferido, lógicamente, desarrollar variedades de girasol con resistencia a esta planta parásita, variedades que quizás no han tenido un potencial productivo similar a las variedades convencionales, muy mejoradas para este carácter. No obstante se ha podido comprobar cómo en aquellas zonas de cultivo donde el jopo aún no ha hecho su aparición, los rendimientos del girasol han mostrado una tendencia ascendente.

Por otro lado se observa que existe una correlación entre la pluviometria registrada durante los meses invernales y los rendimientos del girasol, por lo que aquellas zonas más favorecidas (Campiña de Jerez) por la precipitación en ese período manifiestan unos rendimientos mayores.

AGRADECIMIENTOS

A todos los agentes de las OCA que a lo largo de estos años han sido responsables de ensayos de la RAEA de girasol. A Francisco Perea Torres y Juan José González Tascón, responsables de los ensayos realizados en las fincas del IFAPA localizadas en Carmona (Tomejil) y en Jerez (Rancho de la Merced), respectivamente.

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The situation and future directions of sunflower production in the Black Sea Region

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ABSTRACT

Sunflower is one of the main crops in the rotation system and the main oil crop in the Black Sea Region (BSR). While Black Sea countries (Russia Federation, Ukraine, Turkey, Bulgaria, Romania, Moldova and Georgia) share only 14% (even including Russia's huge lands aside from Black Sea basin) of world arable land areas, both sunflower harvesting area and production almost cover half of the world. Except Georgia and Moldova, other BSR countries are in top ten countries in sunflower production and planted areas in the world. Because of its great importance in the region, it could easily be assumed that sunflower is a Black Sea-identity plant. Russia and Ukraine are the main sunflower producers (ranking 1st and 2nd, respectively) of the world. While gradually increasing sunflower crushing capacities in Russia and Ukraine impede exportable sunflower supplies, sunflower oil productions and exports are becoming higher year by year. On the other hand, Turkey is the biggest importer in BSR with about 30% market share of world sunflower trade. Nowadays, Ukraine is the biggest and Russia is the third sunflower oil exporter of the world. In the 2007/08 season, Black Sea countries suffered almost over 3.5 million t of crop losses due to a historical drought and observed the highest temperatures of the last 70 – 80 years during summer 2007. Therefore, both sunflower and sunflower oil exports dropped significantly and export prices reached historical heights. Unlike the new trend of oleic type sunflower in other parts of the sunflower world, all sunflower production is linoleic type in BSR.

Key words: Black Sea – planted areas – price – production – sunflower – trade.

INTRODUCTION

The Black Sea Basin region covers a considerable area (2.3 million km^2), and is inhabited by 160 million people (16 million of whom live in coastal areas). BSR is steeped in history and culture and forms a vital trading area linking Europe with Asia (Fig. 1). It is the world's largest locked internal sea with a surface area of 423,000 км^2. The Black Sea with its total area of roughly one-third the size of continental Europe is one of the largest inland water basins in the world. It is almost entirely isolated from the world's oceans but receives river inputs from a large catchment territory, and the second, third and fourth longest rivers in Europe, respectively the Danube, the Dnieper and the Don.

Turkey, a part of the Black Sea region, has a steep, rocky coast with rivers that cascade through the gorges of the coastal ranges. However, the upper and the western coastal part of BSR have large, flat lands which are very suitable for agricultural production (Table 1).

Agriculture in the Black Sea Region has a vast potential. For instance, around 75 percent of vast Ukrainian territory is involved in agricultural production. Moreover, between 90 and 95 percent of land is arable. Some sources claim that Ukraine possesses nearly one third of the world's richest black soils. The total arable land of BSR is twice bigger than that of the EU-25 (about 100 million ha), but the agricultural production is mostly non-irrigated and in dry lands (Table 1). Turkey has the most irrigated area among BSR countries. Because of having large cultivated lands, farmers prefer field crops such as wheat, barley, sunflower, etc. growing under rainfed conditions, especially in the upper part of Black Sea countries. BSR countries have more production and planted areas than EU-25 community of many field and horticultural crops such as wheat, barley, corn, cotton, potato, sugar beet, lentil, total vegetables, cabbage, pepper, pumpkin, cherries, apple, apricot, plum, etc.

Although sunflower is native to and originates from America, it was first cultivated and used in vegetable oil production in Russia starting from the 18th century. In recent years of the 21st century, sunflower became a major crop again in the BSR. After being a major sunflower producer, Argentina has turned more to soybean and corn in recent years, and BSR countries especially Ukraine and Russia, lead both the production and crushing of sunflower in the world. Black Sea sunflower producers comprise Russia Federation, Ukraine, Turkey, Bulgaria, Romania, Moldova and Georgia. However, Georgia may be ignored in most of the surveys due to less sunflower production.

Fig. 1. Black Sea regional map.

Table 1. Land use in BSR countries[1]

Countries	Total Area	Land Area	Agricultural Area	Arable & Permanent Crops	Arable Land	Irrigated Land	Perm. Crops	Perm. Pasture	Non Arable Permanent
	1000 ha	1000 ha	1000 ha	1000 ha	1000 ha	1000 ha	1000 Ha	1000 ha	1000 ha
Bulgaria	11,099	11,063	5,325	3,583	3,355	588	228	1,742	7,480
Georgia	6,970	6,949	3,004	1,064	799	469	265	1,940	5,885
Moldova	3,,384	3288	2,534	2,143	1,843	300	300	391	1,145
Romania	23,839	22,987	14,837	9,899	9,398	3,077	501	4938	13,088
Russia	1,707,540	1,688,850	216,651	125,300	123,465	4,600	1,835	91351	1,563,550
Turkey	77,482	76,963	41,690	28,523	25,938	5,215	2,585	13,167	48,440
Ukraine	60,370	57,935	41,396	33,457	32,544	2,208	913	7,939	24,478
Total	1,890,684	1,868,035	325,437	203,969	197,342	16,457	6,627	121,468	1,664,066
%	14,1	14,3	6,5	13,2	14,1	5,9	4,9	3,5	14,4
World	13,427,880	13,066,880	5,012,266	1,540,708	1,404,130	277,098	136,578	3,471,729	1,152,6172

[1]FAO Statistical Database.

The situation of sunflower-harvested areas and productions in BSR

Sunflower is a vital crop in the Black Sea region and both BSR sunflower harvesting area and production cover almost half of the world (Table 2). Besides, average yields are virtually the same as worldwide levels. In 2007/08 season, when the harvesting area was reduced by 12%, sunflower production nearly plummeted by 1/3 or 3.67 million t due to a historical drought and the highest temperatures of the last 70–80 years during the summer of 2007. For instance, sunflower seed production in Romania was 62 percent lower than estimated in spring 2007, due to these severe drought and extreme hot conditions. Now, Russia and Ukraine are the first and second sunflower seed producers, respectively, amid worldwide ones.

Sunflower production areas in Turkey (sharing over 75% of Turkey's total) are mostly located in the Trakya Region, which is the European part of Turkey. The main sunflower seed producing provinces (oblasts) are Donetsk, Dripropetrovsk, Zaporizhya, Kharkiv, Odesa and Kirovohrad in Ukraine. These oblasts, actually, subsidise a lower domestic price for processing enterprises in the rest of the country with large capacities to process sunflower seeds which are definitely better off. Major regions of

sunflower growing in Russia are North Caucasus, Volga and Central Black Earth; Central Black Earth and North Caucasus are also sugar beet growing regions.

Sunflower growing in Turkey is mostly mechanized (planting with pneumatic planters), applying fertilizer and using hybrid seed (Kaya, 2004). However, in Ukraine, only about half of the country's large agricultural enterprises are profitable and most farms have neither the cash nor access to credit to enable them to purchase the additional inputs (fertilizer, herbicide, etc.). Yield improvement has been focused mainly but not exclusively on the largest enterprises: farms over 10,000 hectares (25,000 acres) in size in Ukraine. With the continuous changing of inefficient farms into large and successful enterprises, overall sunflower and other crop productivity is expected to gradually increase in both Russia and Ukraine.

Table 2. Sunflower harvested area and production with proportions in the world (%) by season in BSR[1]

	Harvesting Area, 1,000 ha					Production, 1,000 t				
	07/08[2]	06/07	05/06	04/05	03/04	07/08[2]	06/07	05/06	04/05	03/04
Russia	5,250	5,900	5,280	4.650	4,875	5,300	6,100	6,440	4,800	4,870
Ukraine	3,400	3,820	3,690	3,425	4,000	4,400	5,550	4,950	3,280	4,480
Romania	830	980	970	975	1,190	550	1,370	1,180	1,220	1,505
Bulgaria	520	680	635	590	660	520	1,030	830	1,030	790
Turkey	475	500	415	380	425	640	820	780	640	560
Moldova	230	290	275	270	350	170	380	330	335	390
Georgia	35	35	40	30	45	25	25	20	20	25
BSR Total	10,740	12,205	11,305	10,320	11,545	11,605	15,275	14,530	11,325	12,620
World Total	22,770	23,910	22,940	21,305	22,835	27,560	30,000	30,300	26,430	26,970
%	47	51	49	48	51	42	51	48	43	47

[1]Oil Word, [2]Estimated

Table 3. Sunflower crushing and sunflower oil production with proportions in the world (%) by seasons in BSR[1, 2]

	Sunflower Crushing, 1000 t					Sunflower oil Production, 1000 t				
	07/08[3]	06/07	05/06	04/05	03/04	07/08[3]	06/07	05/06	04/05	03/04
Russia	4,740	5,750	5,530	4,530	4,070	2,005	2,460	2,380	1,825	1,720
Ukraine	4,150	5,230	4,570	2,955	3,325	1,715	2,255	1,990	1,180	1,400
Romania	480	790	850	910	960	205	335	360	375	385
Bulgaria	400	410	450	475	360	170	175	190	195	145
Turkey	960	1,200	1,130	1,100	1,185	380	495	465	475	510
Moldova	140	265	240	270	270	60	110	100	115	110
Georgia	25	25	25	25	20	10	10	10	10	10
BSR Total	10,895	13,670	12,795	10,265	10,190	4,545	5,840	5,495	4,175	4,280
World Total	24,300	27,320	26,590	23,050	23,470	9,735	11,265	10,995	9,420	9,580
%	45	50	48	45	43	47	52	50	44	45

[1]Oil Word, [2] UkrAgroConsult/Black Sea Grain, [3]Estimated

The application of 17% and 20% sunflower export taxes, in force since 2001 in Ukraine and Russia, respectively, have been promoting higher sunflower crushing / sunflower oil production in Black Sea region (Table 3). The current oilseed crushing capacity in Russia exceeds approximately 6.6 million t per year, including 6.0 million t at industrial plants and 0.6 million from on-farm presses. Based on US FAS Report, these relatively new, large-scale crushing plants with 42 percent of current crushing capacity, compared to outdated and inefficient Soviet-era plants, are 37 percent in Russia. With many of these older plants gradually being modernized, Russia currently has a substantial overcapacity for oilseed crushing which will continue to grow (estimating increase of 50% in next 3 years and reaching 9 million t).

Due to this policy, sunflower crushing and oil production have been decreasing gradually in recent years in Turkey, which has over 4 million t crushing potential, working at less than a 50% capacity.

The crushing factory investments of multinational firms like Bunge, Cargill, Glencore etc also play an important role in the region. Nowadays, Russia and Ukraine are the first and second Sunflower seed crushers/Sunflower oil producers in the world, respectively.

Sunflower trade in BSR

With increasing crushing capacities, both Ukraine and Russia have limited sunflower seed supplies for export. However, Bulgaria and Romania have replaced the earlier positions of Ukraine and Russia. While almost all Black Sea countries are sunflower exporters, on the contrary, Turkey appears to be the biggest (also the second in the world) importer country in the region (Table 4).

Sunflower grows mostly in rainfed areas in BSR as a summer crop so it is easily affected by environmental conditions. Because of that, in 2007/2008 season, with 3.67 million t of crop losses due to a historical drought and the highest temperatures of last 70–80 years during summer 2007, Black Sea Sunflower exports will drop by over 70%. In addition, Turkey will also have some difficulties in providing exportable supplies.

With gradually enlarging crushing capacities, both Ukraine and Russia have started to export more sunflower oil. Now, Ukraine is the biggest worldwide sunflower oil exporter. Russian sunflower oil imports have also been markedly reduced and Russia has gained the third position among the worldwide sunflower oil exporters (Table 5).

Table 4. Sunflower trade and their proportions in the world (%) by seasons in BSR[1,2,3]

	Sunflower Exports, 1000 t					Sunflower Imports, 1000 t				
	07/08[4]	06/07	05/06	04/05	03/04	07/08[P]	06/07	05/06	04/05	03/04
Russia	50	160	380	45	350	5	5	10	10	10
Ukraine	75	340	220	10	930	5	5	5	5	5
Romania*	100	265	100	40	135	70	30	30	30	30
Bulgaria*	100	295	215	370	200	5	5	5	5	10
Turkey	-	-	-	-	-	300	470	400	530	670
Moldova	25	120	70	90	100	10	-	-	-	-
Georgia	-	-	-	-	-	5	5	5	5	-
BSR Total	350	1.180	985	555	1.715	400	520	455	585	725
World Total	1,000	1,920	1,550	1,190	2,310	1,020	1,920	1,515	1,300	2,285
%	35	61	64	47	74	39	27	30	45	32

[1]Oil Word, [2] UkrAgroConsult/Black Sea Grain, TUIK, [3]Intra EU trade is excluded, [4]Estimated.

Table 5. Sunflower oil trade and their proportions in the world (%) by seasons in BSR[1, 2 , 3]

	Sunflower Oil Exports, 1,000 t					Sunflower Oil Imports, 1,000 t				
	07/08[4]	06/07	05/06	04/05	03/04	07/08[4]	06/07	05/06	04/05	03/04
Russia	220	660	620	225	185	100	125	100	135	175
Ukraine	1,290	1,840	1,590	660	970	-	-	-	-	-
Romania*	10	20	25	70	45	25	15	10	10	5
Bulgaria*	10	35	40	30	25	20	5	5	5	5
Turkey	75	80	235	45	15	200	130	455	160	80
Moldova	5	55	55	55	60	15	-	-	-	-
Georgia	-	-	-	-	-	35	35	35	25	20
BSR Total	1,610	2,690	2,565	1,085	1,300	395	310	605	335	285
World Total	3,230	4,350	4,350	2,785	2,785	3,455	4,345	4,290	2,845	2,790
%	50	62	59	39	47	11	7	14	12	10

[1]Oil Word, [2] UkrAgroConsult/Black Sea Grain, TUIK, [3]Intra EU trade is excluded, [4]Estimated.

The same as for sunflower seed, Turkey is once again the main destination for sunflower oil exports of other Black Sea countries. In 05/06 season, Turkey also held the record for sunflower oil exports to mainly Iraq and Syria by using its logistics and neighboring advantage. In 2007/2008 season, with crushing reduced by 20%, Black Sea Sunflower oil exports will be cut by 40% (Table 5).

Sunflower and sunflower oil prices in BSR

Since Turkey is the main buyer in BSR and the major part of Turkish imports are of Bulgarian origin, DAF-Turkey sunflower export prices are taken into account in our table (Table 5). As is clearly seen from the table, DAF prices almost remained the same between January 04 – December 06 and then started to climb. From March 2006 till February 2007, DAF prices skyrocketed by 170% and reached historical heights. Huge reduction in sunflower crop of Bulgaria (also of other Black Sea countries) as well as rises in other oilseed and vegetable oil prices were the main reasons for these record prices.

As Ukraine is the biggest sunflower oil exporter country in the world, FOB (free on board) Ukraine sunflower oil export prices have to be taken into account. Similar to sunflower export prices, sunflower oil export prices also virtually remained unchanged between January 04 and March 07. However, after the relevant month, sunflower export prices peaked by undergoing a 165% increase in 1 year. Record raw material (sunflower) prices promoted such historical prices.

Table 6. Sunflower (DAF) and Sunflower Oil (FOB) export prices ($/t) by months in BSR[1,2,3,4,5]

Sunflower Seed (DAF) export prices ($/t)						Sunflower Oil (FOB) export prices ($/t)					
Months	2004	2005	2006	2007	2008	Months	2004	2005	2006	2007	2008
January	295	335	270	320	735	January	650	640	500	630	1,640
February	305	325	270	325	880	February	675	615	505	615	1,700
March	305	325	275	325		March	660	635	520	640	
April	300	325	280	335		April	645	650	570	675	
May	310	330	295	370		May	630	645	600	755	
June	310	340	300	400		June	575	650	595	845	
July	305	310	295	425		July	545	645	580	910	
August	255	300	260	465		August	555	600	575	1,015	
September	250	280	245	570		September	580	585	580	1,180	
October	260	275	245	660		October	635	550	580	1,290	
November	285	270	270	670		November	670	510	645	1,290	
December	300	270	320	680		December	660	505	675	1,315	

[1]Oil World, [2] MinAg[3], Trakya Birlik, [4]DAF=Delivered at Frontier, [5]FOB=Free on Board.

Future directions of sunflower production in BSR

By February 5th, 2008, Ukraine will have signed an agreement with World Trade Organization (WTO) and will probably be WTO-member by early August 2008. After the accession of Ukraine to WTO, sunflower export duty of a current 17% will be lowered by 1% for each year till 10%. Additionally, Ukraine will be properly involved in the worldwide trade system. Russia's negotiations with WTO are also in progress and it is Russia's turn after Ukraine's accession. This may also result in a reduction in the Russian sunflower export duty, currently 20%.

As it is known, Bulgaria and Romania have been European Union members since January 2007. In addition, Turkey is an EU member-candidate and is negotiating its entry. If Turkey becomes EU-member, all borders will be abolished and sunflower oil trades will be totally free without import duties.

In Black Sea, rapeseed production is getting higher year by year, so some sunflower acreage may be captured. However, upon gradually increasing sunflower crushing capacities, any significant reduction in plantings is not likely. However, sunflower yields are expected to be higher due to better input (fertilizer, herbicide, irrigation etc.) conditions year by year in upper part of BSR also related to environmental conditions.

Another new trend in sunflower production in the world is mid and high oleic type sunflower due to its higher oil quality to use as frying oil, thus supplying healthy oil to consumers. While the mid oleic type is popular in US (80% NUSUN (mid oleic), 10% high oleic and 10% conventional) and in Argentina, high oleic type is mostly predominant in European countries. High oleic type sunflower production has reached 75% in France 50% in Spain and 10% in Hungary. Its possible use as a biodiesel source will also increase planted areas and demands for an oleic type in the region.

CONCLUSIONS

Based on data and country report statistics examined, Black Sea countries will continue to be the major sunflower and sunflower oil producers and exporters of the world. WTO memberships of Ukraine and Russia as well as EU-membership of Turkey will facilitate much free trade. Furthermore, oleic type sunflower will also rapidly spread in the BSR in the near future for potential uses as frying oil and energy crops bringing new trends and movement to the region. However, conventional sunflower production will also remain in the region for edible oil demands.

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The future potential of oleic type sunflower in Turkey

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ABSTRACT

Whereas traditional sunflower with high linoleic acid is predominant, the worldwide demand for oleic-type sunflower has been increasing gradually. However, while oleic-type of sunflower has been raised in the USA since 1995 with the mid-oleic NuSun trademark, sunflower produced in Turkey is still almost only of the linoleic acid type and few steps have been taken so far to make the oleic-type more widespread in both production and usage in this country. Aside from the U.S., the most important European sunflower production has totally shifted to oleic-type and now even 75–80% of the share has been reached in France. Actually, Turkey has a big potential for oleic-type of sunflower oil since 600–700 t of worldwide sunflower oil consumption of around 10 million t is consumed by Turkish people. Besides, contrary to the traditional linoleic-type, oleic type sunflower oil totally conforms to EU Biodiesel Standard of EN 14214 and Turkish Biodiesel Standard of TS EN 1421 due to its lower iodine value. Therefore, oleic-type of sunflower may be an alternative for biodiesel production in Turkey while Turkish crude petroleum imports may reach even 20 Billion USD in 2008 with current record petroleum prices. Moreover, at least half of Turkish edible refined sunflower oil is consumed via collective (catering firms, hotels, restaurants, etc) ways and so multi-usage advantage (improved frying and cooking performance) of oleic-type sunflower oil will reduce the dependency of Turkey with lower imports.

Key words: biodiesel – oleic – sunflower – Turkey.

INTRODUCTION

Turkey's varied ecology allows farmers to grow many crops, but most arable land and the greater part of the farm population have been traditionally allocated to producing cereal crops, which are mostly wheat and barley. Other grain crops including rye, oat, corn, and rice, are produced in most parts of Turkey. Industrial crops follow the grains, i.e. cotton, sunflower, sugar beet, tobacco etc. However, it should not be forgotten that pulses such as chickpea and lentils, forage crops such as alfalfa, vetches, sainfoin, are considered as being the main field crops of Turkey.

Sunflower is the most important oilseed of Turkey as sunflower oil has a 70% (600–700.000 t) dominance in Turkish liquid vegetable oil consumption. However, sunflower production of around 800,000 t is not enough to meet the domestic sunflower oil requirements of Turkey. Therefore, Turkey is second in the world ranking in both sunflower and sunflower oil imports. Unlike the emerging worldwide trend, currently all sunflower production is still unfortunately of the conventional linoleic-type in Turkey. Actually, there is a big potential for oleic-type sunflower in Turkey due to a higher edible sunflower oil consumption and bigger biodiesel demand.

The potential planting areas of oleic type sunflower in Turkey

Turkey is among the largest countries in Europe (and is in the world's top 20). Its size is comparable to that of France and the United Kingdom together and Turkey's area is around 20% of the EU- 25 surface. The most important land use for crop production is wheat, which supplies 70% of Turkey's food consumption in terms of calories, with an area of about 10 million ha. With an average wheat harvest of 20 million t per year, Turkey's wheat production amounts to approximately 15% of the EU-25 wheat harvest. The total production area of cereals is about 13 million ha out of a total of 18 million ha of cultivated areas. About 5 million ha of the agricultural area could be considered as fallow land, which is an important form of land use especially in dry regions (Table 1). Crops are the most important products with 55.8% of total value of agricultural production, split between cereals (11.6%), industrial crops such as sugar beet and tobacco (6.4%), vegetables (13.7%), fruits (17.4%) and other crops. Wheat is the most important single crop with 7.9% of total output value in Turkey. Therefore, wheat has long been the basic food in the Turkish diet, generally eaten in the form of bread, whose Turkish per capita consumption ranks among the highest in the world.

Table 1. Land use in Turkey (1,000 ha)[1]

Agricultural Area	41,690
Arable & Permanent Crops	28,523
Arable Land	25,938
Wheat Planted Area	9,400
Barley Planted Area	3,500
Maize Planted Area	800
Fallow Land	5,000
Irrigated Land	5,215
Perm. Crops	2,585
Perm. Pasture	13,167
Non Arable Permanent	48,440
Land Area	76,963
Total Area	77,482

[1]FAO Statistical Database

Based on this situation, Turkey has enough potential both for its domestic needs and also export to Middle East other Middle Asian countries and North Africa due to its geographical location and also its close relationships. Therefore, Turkey allocates or directs farmers to apply for subsidies to produce more oil crops, especially sunflower due to its greater adaptation capability and also low labor needs. At the moment, 70–80% of sunflower production is raised in Trakya, the European part of Turkey. However, Anatolia is also quite suitable for sunflower. If Turkish farmers become convinced that oleic-type of sunflower crop is more lucrative vis-à-vis alternative crops of wheat, sugar beet etc, sunflower production will be also equally widespread in South and East Marmara, Black Sea Region, Middle Anatolia, Cukurova (Adana city and around), GAP Region (near Syrian border) and Aegean Region as second crop in Turkey (Fig. 1).

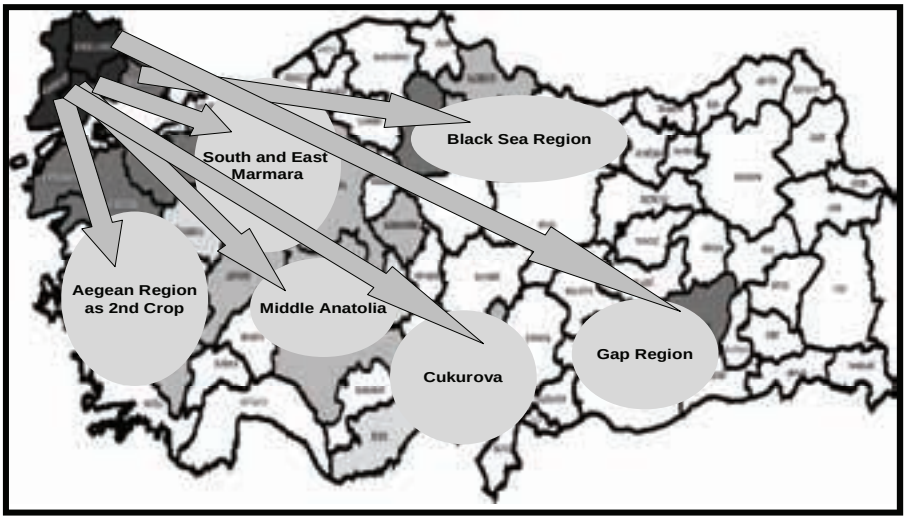

Fig. 1. Oleic type sunflower potential areas by regions in Turkey

The potential use of oleic type sunflower as biodiesel source in Turkey

Sunflower is produced in the world generally for human and non-food purposes (cosmetics, paints, etc.) due to the oil and fatty acid composition of the seed being adapted to these uses. Normally, world-wide sunflower use for edible oil, but non-food consumption has increased in recent years, especially after the planting of higher oleic types in Europe. High oleic sunflower which was first discovered by Soldatov (1976) in Russia, has become popular in recent years in the US, Argentina, and some European countries. However, while mid-oleic ones (60-70% oleic level) are more popular in US and Argentina especially as frying oil in fast food and chips, planting areas of high oleic types (over 80-85%) have gradually been increasing year by year in main sunflower producer countries in Europe such as France (75%), Spain (50%), Hungary (10%), etc. Oleic sunflower production and consumption started rapidly both for healthy frying oil, and also non-food purposes like biodiesel in recent years, but there is not yet enough production for biodiesel due to the high demand for frying oil in Europe.

Due to the especially low iodine value and higher oxidative stability of mid-oleic and high-oleic sunflower oil versus currently dominant linoleic sunflower oil (Vannozzi, 2006; Kaya et al., 2007a, b) oleic-type sunflower oil may also be an alternative biodiesel source in Turkey (Table 2). Oleic sunflower oil conforms to both EU Biodiesel Standard of EN 14214 and Turkish Biodiesel Standard of TS EN 1421. This means that domestically produced oleic sunflower oil could be easily used as a biodiesel source either for domestic consumption or for export to European Union or other destinations.

Table 2. Physical and Chemical Properties of Oil [1]

Oil Type	Iodine Value	Cetane Number	Lower Heating Value (kJ/kg)	Viscosity (mm2/sn)	Cloud Point (ºC)	Pour Point (ºC)	Flashing Point (ºC)
Normal Diesel	115-120	40-55	43-45.000	1,3-4.1	-15 - 5	-35 - 15	120-130
Biodiesel US ASTM standard	93	45	-	1.9-6.0	-	-	>130
EU Biodiesel standard	115	49	-	3.5-5.0	-	-10	100
Canola Oil	94-120	37.6	39.709	3,7	-3.9	-31.7	246
Mid Oleic Sunflower Oil	**94 – 122**		**-**	**4,1**	**-**	**-33**	**250**
High Oleic Sunflower Oil	**88-115**	**49-53**	**-**	**4.8**	**-10**	**-27**	**270**
Linoleic type sunflower Oil	110-143	37.1	39.575	3,7	7.2	-15	274
High oleic Safflower Oil	90-100	49.1	39.516	4,1	-12.2	-20.6	293
Safflower Oil	126-152	41.3	39.519	3,1	18.3	-6.7	260
Sesame Oil	104-120	40.2	39.349	3,5	-3.9	-9.4	260
Cottonseed Oil	90-119	41.8	39.468	3,35	1.7	-15	234
Palm Oil	36-61	42.0	-	-	-	-	-
Soybean Oil	117-143	37.9	39.623	3,3	-4.9	-12.2	254

[1]Albiyobir, 2007

Turkey is one of the biggest crude petroleum importer countries in the world. Import demand is getting higher year by year and Turkey imports about 23 million t crude petroleum each year. Furthermore, with record crude petroleum prices over 100 $/barrel, the invoice of crude petroleum imports in the Turkish budget has reached 12 Billion USD in recent years. When vegetable oil imports are added to petroleum products, both items incur the largest amount of costs to the Turkish Economy (Table 3).

At the present time, Turkish biodiesel production capacities exceed over 1.5 million t and Turkey ranks second with this capacity in Europe after Germany (Albiyobir, 2007). However, the capacity usage ratio never exceeds 20% due to supply shortages. Currently, canola is the main biodiesel oilseed raw material while canola production is just 25–50.000 t in Turkey. Therefore, the largest part of the raw material is provided via imports (245.000 t, 104 million USD in 2007). Based on PETDER report (2007), Turkish annual diesel consumption was around 16 million m^3 in 2006 and in Jan / Sep 07 consumption already reached 11.53 million m^3. From these figures, the current annual Turkish diesel production could be reckoned to be around 15–16 million m^3.

By Turkish Cabinet Decree No. 2006/11202, announced in Official Gazette No. 26370 dated December 8th, 2006, the Government lowered by 2% the biodiesel OTV/Special Consumption Tax (957 YTL/m^3 for Diesel 50) (PETDER Report, 2007) if the biodiesel was manufactured from domestically raised oilseeds.

Table 3. Vegetable seed and oil as well as crude petroleum imports (USD) by years in Turkey[1]

Years	Vegetable Seed + Oil +Meal	Crude Petroleum	Total
	Million USD	Million USD	Million USD
2004	985	6,092	7,077
2005	1,286	8,650	9,936
2006	1,354	10,707	12,061
2007[2]	1,588	12,000*	13,588

[1] TUIK, Turkish Statistical Institute, [2]Forecast

Even with 2% biodiesel directives (unfortunately there are still no directives in Turkey), Turkish biodiesel requirement should be a minimum of 275.000 t. If biodiesel directives are increased to 5.75% like the EU-27 2010 target rate, the requirement should be increased to around 900.000 t. Aside from Turkey, EU requirement with 5.75% directives will reach 18 million t in 2010 and it is totally impossible to produce this quantity in EU arable lands. Please note that current EU biodiesel manufacturing is around 6-6.5 million t and will be increased to 8–9 million t maximum in 2010. In other words, at least half of the EU requirements have to be supplied by imports. At this point, Turkish biodiesel production could play a big role by using its logistics advantages both for Europe and also higher domestic consumption and reduced high import costs (Kleindorfer and Oktem, 2007).

The potential use of oleic type sunflower as edible oil in Turkey

Vegetable oils processed by crude-oil processing industries have an important role in human nourishment as well as human health. Turkish people consume 19.5 kg per capita vegetable oil (in 2005) per year. Turkish annual sunflower oil consumption is around 600–700.000 t and constitutes 70% of domestic liquid vegetable oil consumption (Table 4). Another use of sunflower oil in Turkey is in margarine using for direct consumption, for breakfast and other meals, and in the food industry too (Table 5). The most important objective, with the expansion of oleic-sunflower oil in the market, instead of unconscious and meaningless classifications like only sunflower oil, corn oil, soybean oil, is that building up consumer awareness, with a new classification like frying oil, cooking oil, salad oil or dressing etc, as it will really appear on the Turkish oil market.

Table 4. The using purposes of vegetable oils (t) in Turkey

	2002-2003	2003-2004	2004-2005
Refined sunflower oil	452,000	537,000	579,000
Refined corn oil	108,000	71,000	102,000
Refined Soybean Oil	57,000	35,000	81,000
Others	118,000	95,000	88,000
Liquid total	735,000	738,000	850,000
Margarine total	419,000	447,000	491,000
Total (t)	1,154,000	1,185,000	1,341,000

Table 5. The Using purposes of margarines (1,000 t) in Turkey

The Usage	1997	1998	1999	2000	2001	2002	2003	2004	2005
Breakfast Margarine	167	168	182	171	160	171	160	161	168
Meal Margarine	74	64	77	66	70	65	59	57	65
Industrial Use	163	136	161	162	163	183	200	229	258
Total	404	368	420	399	393	419	419	447	491

Despite there not being any typical research on this theme, it is assumed that at least half of sunflower oil consumption is via collective ways like catering firms, hotels, restaurants etc in Turkey. Therefore, there is a very big potential for this area too, and after introducing oleic sunflower oil, meal and frying quality will improve, because stomach problems frequently appear in Turkey due to the poor quality of the oil used in the restaurants.

Catering area has been one of the most popular sectors in Turkey in recent years and its capacity exceeds over 4.5 Billion $ in 2007. Catering firms which in Turkey have reached 5,000 in number and they serve over 7 million people (Celebi, 2007). However, the sector potential has been estimated as being about 22 million people in the near future. Therefore, oleic sunflower oil will also play an important role in improving served meal quality and rapidly developing this sector.

Furthermore, tourism is another booming sector in Turkey and Turkey has become one of the most preferred destinations in Europe in recent years presenting excellent landscapes, beaches, historical ruins and service in many hotels. Tourists visit Turkey in large numbers and the bed capacity has gradually increased each year, and the five star hotels exceeded over 500 in 2006 (Table 6). Turkish tourism income reached 14 million $ and tourist numbers exceeded over 23 million in 2007 (TUIK, 2007). The tourist sector has a high potential for edible sunflower oil consumption as oleic sunflower oil will contribute to increasing food quality of frying, meals and salad dressing, etc.

Table 6. Tourism potential of Turkey in recent years[1].

Years	No. of Hotels	No. of Rooms	No. of Beds	No. of Tourists	Tourism Income ($)
2001	3,235	284,054	597,866	11,618,969	8,090
2002	3,262	293,299	619,024	13,256,028	8,473
2003	3,370	314,233	663,300	14,029,558	9,676
2004	3,508	336,547	713,714	17,517,610	12,124
2005	3,451	359,128	761,585	21,124,886	13,929
2006	3,344	365,028	783,319	19,819,833	12,554

[1]TUIK

Oleic sunflower oil has multi-usage advantage (perfect frying and cooking performance) versus linoleic sunflower, so it may attract demands from this sector. Moreover, like in the USA, Fritolay/Turkey (Fritolay, 2007) also supports oleic sunflower oil usage in their products due to its longer shelf life and healthier characteristics. Consequently, high oleic sunflower oil has been in the chip products portfolio since November 2007. This will be gradually spread to all Fritolay products.

CONCLUSIONS

To popularize oleic-type of sunflower oil in Turkey vis-à-vis traditional linoleic sunflower oil is absolutely vital to achieve new trends in most major sunflower producer countries. There is no marketing problem as oleic sunflower oil can be easily consumed in both the food (as edible oil etc.) and non-food (as biodiesel etc.) sectors. As oleic sunflower has a price premium compared to linoleic-sunflower, Turkish farmers will also benefit from the cultivation of the oleic type. Oleic sunflower will also reduce the dependency of Turkey by lowering sunflower complex and crude petroleum imports and even increasing sunflower oil and biodiesel exports.

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Oil type sunflower production in Turkey

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ABSTRACT

While sunflower history starts from around 3000 BC in American Indian tribes, its introduction in Turkey took place after the First World War with immigrants from Bulgaria and Romania. At the present time, 70-75% of Turkish sunflower production is carried out in Trakya Region, the European part of Turkey. Most of this production (at least 95%) is oil-bearing type. Now, Turkey is one of the biggest sunflower producer countries in the world. However, Turkish domestic sunflower seed crop of around 700 – 850.000 MT is not able to meet domestic sunflower oil consumption. Therefore, it has been attempted to close the resulting gap with both sunflower seed and sunflower oil imports. At this point, Turkey is the second importer (after EU-27) in the world and the biggest in Black Sea Region for both sunflower seed and oil. Consequently, to reach domestic sunflower oil consumption requirements of 600- 700.000 t and to go on with its sunflower oil exports to mainly Iraq and Syria by using its logistics advantage, as well as to provide enough supplies for sunflower crushers of more than 4 million t, Turkish sunflower would have to be increased to at least 2.0 million t (ie, 2.5 times the current crop).

Key Words: meal – oil – production – seed – sunflower – trade – Turkey.

INTRODUCTION

Turkish sunflower planting areas are mostly found in Trakya Region (70–75%), the European part of Turkey. Other regions comprise South-Marmara Region, Black Sea, Central-Anatolia, Aegean and Mediterranean Region (Fig. 1). Recently, aside from Trakya Region, sunflower planting areas in Adana province and around (60,000 ha) have become widespread. The fact is important because sunflower harvesting in Adana is approximately 1 to 1.5 months earlier than Trakya and provides some supplies for the crushers in Anatolia.

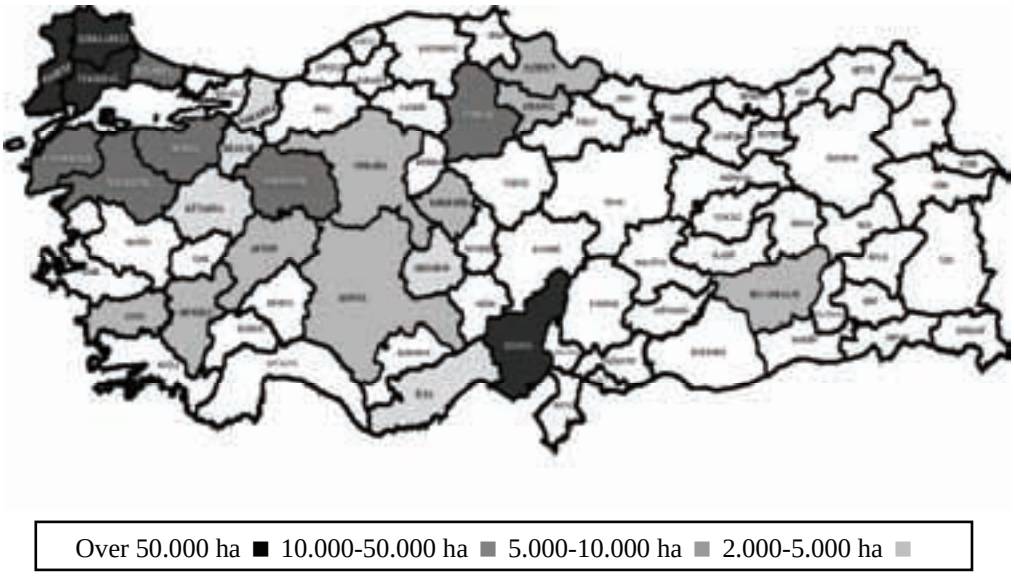

Fig. 1. Normal Sunflower planted areas by provinces in Turkey (2007)

Turkish oil seeds production is around 2.0 – 2.5 million t of which cottonseed has around a 60% share in total production. However, cotton is not of the same importance as sunflower due to its lower oil content of around 20% as well as lower cotton seed oil consumption. Sunflower oil consumption is around 600 – 700.000 t and covers approx 70% of Turkish liquid vegetable oil consumption.

The situation of sunflower acreage, yield and production in Turkey

Sunflower plantings in Turkey were initiated by immigrants from Romania and Bulgaria after the First World War. In the 1950s, sunflower production was around 70.000 t. In the late 1980s, production peaked with 1.25 million t. However, after that year, it has become gradually reduced due to wheat/sunflower parity (the ideal is 1/2.5) at the expense of sunflower (Table 1).

Nevertheless, fortunately, after 2001/02 season when production dipped to 550,000 t, the government finally realized the importance of sunflower as well as other oilseeds and increased their support gradually (especially premium payments per kg), so sunflower experienced an upward trend. The production subsidy program is continuing in order to attract farmers to plant oilseeds particularly sunflower seed and cotton seed. Despite serious delays in these payments, the program is still much appreciated by farmers.

Turkish sunflower crop production has been over 800.000 t in recent years (Table 2). However, in 2007/08 season, production was reduced because the crop suffered the most severe droughts and highest temperatures of the last 70 years in Turkey. Sunflower yields are well above the world yield average of around 1.2 – 1.3 t/ha due to better inputs (hybrid seed, fertilizer, herbicide etc.). Farmers use mostly hybrid cultivars (over 98% percent) in the sunflower production in Turkey and Turkish sunflower hybrid market is about 2,000 t (Kaya, 2004). Due to broomrape problem (appearance of new races F, G, H) especially in Trakya region, sunflower hybrids should incorporate resistance to the new races existing in the region. However, other alternative of broomrape control, the use Imidazolinone (IMI) herbicide-resistant hybrids is being used and these hybrids have covered half of the areas in recent years because they control both broomrape, and the main problem, weeds such as *Xanthium strumarium., Sinapis arvensis,, Chenopodium album., Cirsium arvense., Convolvulus arvensis*, *Avena spp., Datura stramonium, Amaranthus spp*. etc. by applying in post emergence. *Sclerotinia* (both head and stem), *Rhizopus*, *Macrophomina*, *Puccinia* are the main diseases in planting areas but they are not present everywhere and are also not frequently taken into account economically.

Table 1. Sunflower prices and subsidies and sunflower / wheat parity by season in Turkey

Season	Trakya Birlik	Sunflower Subsidies	Sunflower/Wheat Parity	Sunflower/Wheat Parity
	Price (USD/t)	Price (USD/ t)	Including Subsidies	Excluding Subsidies
2000/01	251,4	60,0	2,13	1,72
2001/02	271,1	54,5	2,44	2,03
2002/03	282,4	55,2	2,65	2,21
2003/04	348,5	79,1	1,82	1,48
2004/05	322,3	89,8	1,81	1,42
2005/06	376,6	130,5	1,84	1,49
2006/07	357,7	192,3	2,24	1,60
2007/08	634,5	*	-	2,02

* It is not announced yet

Table 2. Sunflower seed acreage, yield and production by seasons in Turkey[1]

Seasons	Acreage, 1000 ha	Yield, t/ha	Production, 1000 t
2007/08 P	475,000	1,40 - 1,50	650,000 – 700,000
2006/07	500,000	1,70	850,000
2005/06	425,000	1,88	800,000
2004/05	395,000	1,65	650,000
2003/04	420,000	1,43	600,000

[1]Oil Word (2007, 2008), Trakya Birlik Market (2007)

Trakya Birlik and Karadeniz Birlik, two of the leading Agricultural Sales Cooperative Unions, continue to play a very important role in the sustainability of sunflower seed production in Turkey. Trakya Birlik, has 110,000 members constituting 48 cooperatives and is active mainly in the Trakya-Marmara Region, but also has a couple of cooperatives in Middle Anatolia and Aegean Region. On the

other hand, Karadeniz Birlik is mostly located in the Black Sea region but purchases sunflower seed from farmers in other regions such as Middle and Eastern Anatolia too.

These two cooperative unions provide inputs such as seeds, fertilizer and low-cost financing prior to plantings to their members and offer attractive prices during the harvesting season. Trakya Birlik and Karadeniz Birlik purchase more than 50% of Turkish Sunflower crop.

Nowadays, they are also attempting to invest in facilities to produce biodiesel to distribute to their members to help them to reduce input costs.

They have also been effective in encouraging the government to issue production bonuses and import tax protection for commodities that they handle because, due to their high number of farmer members they exert a considerable control on the sunflower domestic trade and production (buying and selling seed, also crushing and refining industry).

Sunflower processing in Turkey

Turkish vegetable oil seed crushing capacities exceed 6 million t and only less than half of this capacity is used. Every year, a new crushing facility is added to the existing 180-odd firms**.** The new high-capacity modern crushers lower the cost of crushing through scale economies, forcing smaller crushers with older technology out of business. Low capacity utilization also remains a problem for the industry due to lack of raw materials. Normally, sunflower crushing is around 1.15 – 1.3 million t in Turkey (Table 3). Turkey's vegetable oil production is mostly used to produce liquid oils and the remainder to make margarine. Turkey imports sunflower seed from the Black Sea region to satisfy the huge crushing demand.

However, in 2007/08 season, sunflower crushers have been experiencing supply difficulties from both domestic and foreign markets due to the high crop losses caused by the historical droughts and high temperatures affecting the Black Sea Region countries like Ukraine, Russia, Bulgaria and Romania, so they were not able to carry out exports to their main destination, Turkey.

Sunflower meal has become increasingly important in recent years due to heavy demands from livestock, dairy and aquaculture industries. Sunflower oil and meal productions in Turkey are around 450–550,000 MT and 600–700,000 MT, respectively. In a tandem with lower crushing rates, the 2007/08 season meal production will also be reduced accordingly (Table 3).

Table 3. Sunflower crushing, oil and meal productions by seasons in Turkey[1,2,3]

Seasons	Crushings (1000 t)	Sunflower Oil Production (1000 t)	Sunflower Meal Production (1000 t)
2007/08 P	975,000 - 1,050,000	380,000 - 410,000	535,000 - 575,000
2006/07	1,325,000	545,000	715,000
2005/06	1,145,000	460,000	620,000
2004/05	1,185,000	480,000	640,000
2003/04	1,275,000	510,00	690,000

[1]Oil Word (2007, 2008) [2]Trakya Birlik Market (2007) [3] BYSD (2007)

Sunflower trade in Turkey

Since the sunflower domestic crop production is far from meeting domestic crushers' demands, Turkey appears as one of the biggest export destinations (the second worldwide after EU-27). Due to their logistics advantage, sunflower imports are mainly carried out from Black Sea countries mostly Bulgaria and Romania (Table 4). Most of Turkish sunflower crushers are located in Trakya Region and so DAF (delivered at frontier) Bulgaria is the most preferred delivery mode. Turkey has around 5–10,000 t of sunflower exports, mainly hybrid seeds and also confectionery. Therefore, in this paper, the details of those exports have been left out.

As sunflower oil production is not available to meet domestic sunflower oil consumption, of around 600 – 700,000 t, sunflower oil imports are required. Sunflower oil imports are mainly carried out from Ukraine and Russia in Turkey (Table 5). Argentina is also preferred, especially for the second half of the season when Black Sea exportable supplies are getting lower. Sunflower imports upon Inward Processing Regime are in a parallel with sunflower oil exports (Table 6).

Table 4. Sunflower seed imports (1000 t / million $) by years in Turkey[1, 2]

Countries	2007	2006	2005	2004	2003
Bulgaria	239,0 / 91,7	157,5 / 44,2	364,9 / 115,7	223,9 / 64,3	151,7 / 39,8
Romania	222,9 / 93,8	96,6 / 28,7	29,3 / 9,6	11,7 / 3,9	45,6 / 11,6
Moldova	48,6 / 15,9	32,7 / 9,4	18,4 / 6,2	22,5 / 7,6	1,7 / 0,5
Ukraine	58,8 / 35,0	7,7 / 2,2	1,1 / 0,4	147,9 / 50,9	177,1 / 47,9
Russia	-	59,9 / 15,5	35,0 / 10,3	55,7 / 19,0	83,3 / 23,4
Uruguay	-	-	-	-	67,3 / 18,3
USA	18,9 / 15,8	15,2 / 12,2	3,7 / 3,1	7,7 / 5,0	7,1 / 3,8
Hungary	1,9 / 1,3	-	31,2 / 10,2	1,2 / 0,4	-
Others	6,1 / 6,7	1,9 / 4,1	3,6 / 5,1	7,5 / 5,3	1,9 / 5,7
Total	596,2 / 260,2	371,5 / 116,3	487,2 / 160,6	478,1 / 156,4	535,7 / 151,0
Avr CIF	436 $/t	313 $/t	330 $/t	327 $/t	282 $/t

[1]TUIK (2007), [2]Trakya Birlik Market (2007)

Table 5. Sunflower oil imports (1000 t / million $) by years in Turkey[1,2]

Countries	2007	2006	2005	2004	2003
Ukraine	55,1/50,2	159,5/94,2	83.4 / 53.8	30.3 / 18.7	67.1 / 38.6
Russia	34,4/26,7	91,9/53,3	53.8 / 35.2	14,3/9,0	10,3/6,0
Argentina	48.2 / 40.1	116.3 / 68.5	4.5 / 3.0	0,3/0,2	10,1/5,9
Bulgaria	4,4 / 3,7	6,9 / 4,0	8,9 / 5,9	11,8 / 7,0	-
Romania	10,7 / 9,1	16,9 / 10,7	37,7 / 26,3	19,4 / 14,2	5,0 / 3,5
Moldova	5,8 / 4,2	-	2,8 / 1,8	-	-
Others	4,5 / 4,0	7,4 / 5,0	10,0 / 6,8	-	-
Total	163,1 / 138,0	398,9 / 235,7	201,1 / 132,8	76,0 / 49,1	92,5 / 54,0
Average CIF	846 $/t	591 $/t	661 $/t	645 $/t	584 $/t

[1]TUIK (2007), [2]Trakya Birlik Market (2007)

Table 6. Sunflower oil exports (1000 t / million $) by years in Turkey[1,2].

Countries	2007	2006	2005	2004	2003
Iraq	54,4 / 63,3	182,6 / 161,9	33,4 / 31,3	7,8 / 6,9	8,4 / 6,7
Syria	12,9 / 16,2	29,3 / 25,8	14,3 / 14,2	1,0 / 0,9	-
Israel	5,3 / 5,6	5,8 / 4,8	2,0 / 1,8	1,8 / 1,5	3,6 / 2,7
Turkish Cyprus	3,8 / 5,0	3,0 / 2,8	2,8 / 2,7	1,5 / 1,4	1,2 / 1,0
South Korea	2,0 / 3,3	1,4 / 1,8	-	-	-
Sudan	2,7 / 2,6	-	-	-	-
Libya	1,7 / 1,9	-	-	-	-
Yemen	1,5 / 1,9	1,9 / 1,6	1,3 / 1,2	1,0 / 0,9	-
Others	9,5 / 12,0	8,9 / 7,5	7,4 / 7,1	5,2 / 4,7	15,3 / 12,3
Total	93,8 / 111,8	232,9 / 206,2	61,2 / 58,3	18,3 / 16,3	28,5 / 22,7
Average CIF	1,193 $/t	885 $/t	953 $/t	891 $/t	795 $/t

[1]Turkish Statistical Institute (2007), [2] Trakya Birlik Market (2007)

Besides, Turkey also exports sunflower oil, particularly refined sunflower oil, and margarine to mainly Iraq and Syria by using its logistics advantage (Table 6). In Turkey, government supports local sunflower oil refiners via the Inward Processing Regime (duty-free, non payment of 22% import-duty on crude sunflower oil imports if they are exported as refined sunflower oil). This creates an added value and increases capacity-usage level of refiners.

Additionally, Turkey is located in a position that for centuries has been a trading crossroads, so it is ideally situated for exporting vegetable oils to many other countries. Turkey has many customers in North Africa, the Middle East, Central Asia and the Black Sea region. Sunflower meal production is also far from being able to meet the huge demands of Turkish livestock sector (Table 7). Sunflower meal imports

mainly originate from neighboring Black Sea countries by using logistics advantage and due to increased availability because of increased crushing capacities.

Table 7. Sunflower meal imports (1000 t / million $) by years in Turkey[1, 2]

Countries	2007	2006	2005	2004	2003
Ukraine	163,8 / 29,9	128,8 / 13,2	58,0 / 7,5	90,6 / 15,2	37,1 / 4,1
Russia	185,2 / 32,5	154,3 / 16,3	97,6 / 12,1	187,8 / 27,9	45,6 / 4,9
Romania	13,4 / 2,0	104,8 / 13,0	85,5 / 11,3	31,9 / 4,7	6,6 / 0,8
Bulgaria	9,9 / 1,3	34,8 / 3,2	23,8 / 2,7	19,3 / 2,4	12,9 / 1,3
Moldova	-	-	3,2 / 0,5	16,0 / 2,7	8,4 / 1,1
Others	1,9 / 0,3	0,3 / 0,1	-	-	2,9 / 0,2
Total	374,2 / 66,0	423,0 / 45,8	268,1 / 34,1	345,6 / 52,9	113,5 / 12,4
Average CIF	176 $/t	108 $/t	127 $/t	153 $/t	109 $/t

[1]Turkish Statistical Institute (2007), [2]Trakya Birlik Market (2007)

Since Turkey is a major importer country in the sunflower trade, domestic prices are mainly driven by import costs. The import duties on sunflower seed and sunflower oil imports are 12% and 22% for all countries except Bosnia and Herzegovina (0%). Turkey also has a bilateral agreement with EU in tariff quotas (sunflower seeds: 1,000 t, 0% duty, sunflower seed oil: 18,400 t, 0% duty).

The interest in producing biodiesel continues increasing year by year in Turkey because of the high price of diesel in the domestic market due to increased world prices and high local taxes (equivalent to sixty percent of the price). As a result, Turkish people use one of the most expensive diesel and benzine fuels in the world. Due to their attractive conditions, especially after the introduction of oleic sunflower into Turkey, oleic types could dominate the planting areas both for providing healthy oil to consumers and for being very suitable for biodiesel production. However, currently, all sunflower oil production is of the linoleic type and the oleic type market is only just starting up.

CONCLUSIONS

At least on a near-term, it is obvious that Turkey will remain as the major importer country of the world to meet domestic sunflower oil consumption requirement of 600-700,000 t and to keep on with its sunflower oil exports, mainly to Iraq and Syria by using its logistics advantage as well as to provide enough supplies for sunflower crushers having more than 4,000,000 t.

To supply enough seed for its factories domestically, sunflower production of Turkey should be increased to a minimum of 2,000,000 t (ie, 2.5 times its current production). This goal is not possible to achieve at least in the near future. Therefore, the government should support its sustainability, and the price parity of sunflower with wheat (as the main rotation crop of sunflower) will be the fundamental factor to be observed.

However, with the current lucrative (after record prices) sunflower agriculture, sunflower production may climb up and exceed 1,000,000 t, or even the record 1.25 million t, achieved in the late 1980s in future seasons. Furthermore, oleic sunflower production will bring a new direction to Turkey and also to Black Sea Region which cultivates half of the world's sunflower production.

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Oilcake as a fuel alternative to wood pellets

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ABSTRACT

Raw sunflower oilcake was obtained from a type of oil mill expeller and was found to contain 6.9-9.5% residual oil and 20.4-21.4 MJ/kg calorific value. Pellet fuels were produced from ground sunflower oil cake using a pelletizer. The capacity of the pelletizer and the characteristics of dried pellets depend on the initial water content of the oil cake. The appropriate values of water contents were about 20 % w.b.

Key words: biomass –oilcake –pellet –production.

INTRODUCTION

Sunflower seeds consist of more than 40% oil; consequently, 60% is classified as oil cake. Although sunflower oil cake can be used as fertilizer, livestock feed, and new materials (Rouilly et al., 2006), it is not easy to use it effectively because of its nutritional properties. As examples of other oil crops, olive oil cake and rape seed oil cake have been used as energy sources (Filiz, 2000; Oktay, 2006; Alkhamis and Kablan, 1999). Results of those studies have demonstrated that oil cake is useful as a potential energy source.

Wood pellets are one way of using biomass as an energy source. They have superior handling characteristics and storability; they are used in various countries. Some burning appliances use wood pellets, but still on a small scale (Sippula et al., 2007).

For this study, we produced a pellet fuel using sunflower oil cakes. It was clear that the capacity of the pelletizer and the characteristics of dried pellets depend on the initial water content of the oil cake. The properties of sunflower oil cake pellets were analyzed following wood pellet standards.

MATERIALS AND METHODS

The variety of oilseed sunflower '63M80' cultivated in the test field of the National Agricultural Research Center was used in the experimental study.

The sunflower seed samples were expressed using a type of oil mill expeller (KEK P0101; Egon Keller GmbH & Co. KG, Germany). Raw sunflower oil cake was obtained as a by-product of the sunflower oil mill. The pressure was adjusted according to the pitch between a press ring and a pressure cone. During the expression, the expulsion rate, surface temperature and the thickness of oilcake was measured. Initially, the oil cake was not uniform in size, thickness, and shape. The sample was ground by rice husk grinder and sprayed with mist to adjust the water content to a value of between 9 and 20% w.b.

The oil cake was pelletized using a ring die type pelletizer equipped with an 11 kW motor. The pelletizer had a 250-mm-diameter and a 40 mm thickness ring die. The obtained pellets were cylindrical with 6 mm diameter. The length was changed optionally; it was set to 30 mm in this test. The ground oilcake was supplied using an auger screw; it was set to 12 rpm for this test.

The product capacity of this pelletizer was calculated from the weight of pellets. The rate of production was calculated from the samples retained on the range of the 4-mm sieve. The pellet temperature was measured using a radiation thermometer. All pellets were stored under ambient conditions for a week to stabilize the water content before testing.

Characteristics of raw oil cake without grinding (ROC) and oil cake pellets (OCP) were measured using approximate standard methods determined in JIS-Z7302, PC WPFS-1, and JHIAN-5651 (JHIA, 2006). After cooling, the ROC was measured for bulk density.

Calorific values were obtained using a standard bomb calorimeter (1013-B; Yoshida Seisakusho Co., Ltd., Japan). Wood pellets were measured as a control pellet.

Cake residual oil was obtained using a fat analyzer (B-815/820; BUCHI, Switzerland).

RESULTS AND DISCUSSION

The results of characteristics of oilcake are presented in Table 1 and the capacities are presented in Table 2. The oil cake characteristics depend on the conditions of compression process. Fastening the expeller tightly, the ROC volume decreased and the thickness decreased. The ROC temperature rose to 82.4°C because of friction heat.

Table 1. Characteristics of oilcake

Expulsion pressure	Thickness (mm)	Surface temperature (°C)	Residual oil (%)
High	4.18	82.4	6.93
Low	8.66	73.0	9.48

Table 2. Capacity of the expeller

Expulsion pressure	Expulsion rate (kg/min)	Gauge index (mm)	Thickness (mm)	Rate of Oil cake (%)
High	0.45	50	4.18	64.5
Low	0.47	55	8.66	67.0

The results of the calorific value are presented in Table 3 along with data of wood pellets. The average calorific value of ROC was 20.99 MJ/kg. When this value is compared to that of white pellets of cedar and pine (c), ROC has a 5% higher calorific value. Although it is difficult to conclude that ROC has a higher calorific value than wood pellets, it shows that sunflower oil cake offers a potential source of energy.

Table 3. Calorific value

		Higher Calorific Value (MJ/kg)
a	ROC	20.99
b	Needle leaf tree	19.30
c	Cedar and Pine (white)	19.91
d	Cedar and Cypress (blend)	19.74
e	Cedar (bark)	19.74

Fig. 1 shows the result of the product. In this test, the initial water content was from 9.0% w.b. to 27.7% w.b. Although the product was from 443.1 g/min to 540.8 g/min at less than 21.0% w.b., it decreased to half at 27.7% w.b. because the flow ability of oil cake decreased with increased water contents.

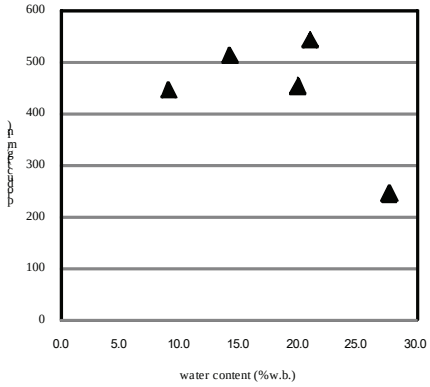

Fig. 1. The relationship between water content and product

Fig. 2 shows that the OCP length of greater than 19.9% w.b. was roughly equal to 30 mm, which is a set length of the pelletizer. The OCP of less than 14.4% w.b was shorter than the set length.

Fig. 3 shows that the shatter indices increase with decreasing initial water content: the indices were 12.5% w.b. at 9.0% w.b. The standard of IWATE requires a shatter index of less than 1%. Therefore, the samples with greater than 19.9%w.b. conformed to the standard.

Fig. 4 presents the relationship between the initial water content and the mechanical strength. The strength increased with increasing initial water content. The strength was found to correlate linearly with initial water content of OCP.

The results showing the product, length, and hardness of pellets showed that the optimum water content was at 19.9–21.0% w.b. in this test.

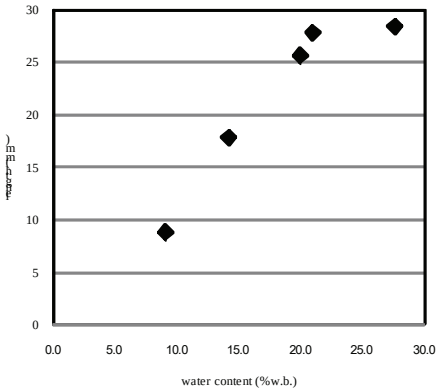

Fig. 2. The relationship between water content and length

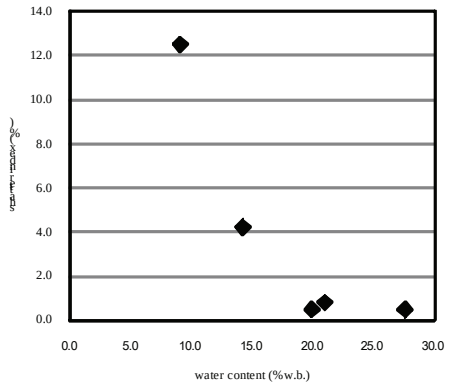

Fig. 3. The relationship between water content and shatter index

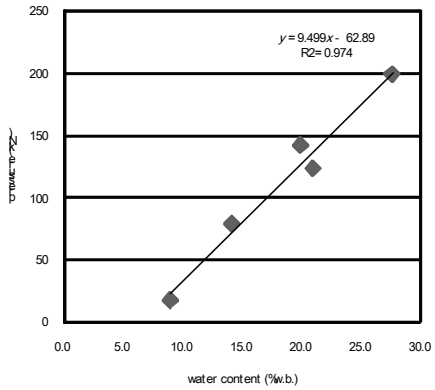

Fig. 4. The Correlation of water content and hardness of pellet

CONCLUSIONS

To use sunflower oilcake as fuel, pellet fuels were produced from sunflower oilcake. The product was stable at least 21%w.b., it decreased to half at 27.7%w.b. because the flow ability of oilcake decreased with increased water contents. The results of the pellet product, length and hardness showed that optimum water content was at 19.9-21.0%w.b. in this test. Oilcake has 20.99MJ/kg calorific values, which are within the standard range of wood pellets.

ACKNOWLEDGEMENTS

This work was supported by KAKENHI (B)-18780195.

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