THIRD INTERNATIONAL SYMPOSIUM ON BROOMRAPE (*Orobanche* spp.) IN SUNFLOWER

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PROCEEDINGS

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Dedicated to the memory of Carlos Alberto Sala and Alexandru Viorel Vrânceanu

Dr. Carlos Alberto Sala (Argentina; 1961-2013) leaded the Biotechnology Department of Nidera for two decades, where he made significant contributions to the development and genetic characterization of new herbicide tolerance technologies for weed and broomrape control in sunflower, particularly CLPlus imidazolinone resistance technology. He was an active member of the Scientific Committee of this Symposium until his final days.

Prof. Alexandru Viorel Vrânceanu (Romania; 1927-2014) is one of the fathers of modern sunflower breeding. He contributed greatly to the commercial introduction of hybrid technology in sunflower and to the development of many improved cultivars. He also developed a set of differential lines to identify sunflower broomrape races and laid the basis for understanding the inheritance of broomrape resistance in sunflower. His crucial contribution to sunflower improvement was recognized by the International Sunflower Association with the V.S. Pustovoit Award.

If I have seen further it is by standing on the shoulders of Giants
Isaac Newton

Foreword

Broomrape (*Orobanche* spp.) is a major constraint for sunflower production in most production areas around the world, particularly in Europe and Asia. The most widespread broomrape species attacking sunflower is *O. cumana* Wallr. The development of resistant cultivars as well as optimized managing strategies is a high priority in sunflower breeding programs all over the world.

The Board of the International Sunflower Association (ISA) proposed in their meeting held in Buenos Aires in February 2012 to celebrate an International Symposium on Broomrape in Sunflower in June 2014 in Spain. This is the third specific symposium on broomrape in sunflower after those held in Turkey in 2008 and Moldova in 2011.

With around 200 participants from 24 countries, and 46 scientific and technical communications, the Symposium represents a unique opportunity to get a complete picture of sunflower broomrape situation around the world, to evaluate the validity of current strategies of control, and to discuss new strategies for the future. The contents of the manuscripts included in these proceedings are the responsibility of the authors and their edition by the Scientific Committee has been reduced to formatting issues and misspellings. 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.

The Symposium has been organized by the Spanish Sunflower Association (Asociación Española del Girasol), which is formed by sunflower researchers and managers from public institutions and private companies involved in the sunflower sector. We are indebted to many individuals, institutions, and companies that have contributed to the success of this event.

Finally, we would like to thank the Scientific Committee for the advice in preparing the scientific sessions and workshops, and all the participants for their stimulating discussions throughout the Symposium. We hope you acquired some valuable information from our speakers, benefited from fruitful discussions with other colleagues, and returned home with new ideas to be developed in the next years. If you also found a friendly environment and enjoyed the social events we prepared, then our efforts in organizing this Symposium will have been worthwhile.

The Organizing Committee

The Spanish Sunflower Association

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SESSION 1

Current situation of sunflower broomrape around the world

Current situation of sunflower broomrape around the world

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ABSTRACT

Orobanche which is a angiosperm parasite one of the most restraining factor in especially Eastern European sunflower-producing countries which has more than half of world sunflower production. There is big challenge between sunflower breeders and broomrape; whenever breeders develop resistant cultivars against this pathogen, broomrape responds by evolving another virulent race overcoming the resistance every 20 years. In the last over 50 years, several research groups have made relevant contributions on sunflower breeding for resistance and dominant genes for resistance to races A, B, C, D, E, and F have been found and incorporated into cultivated sunflower genotypes mostly utilizing classical breeding. The recent studies also indicated that new broomrape populations have been determined also in some countries called G and H races. However, there is still the existing doubt in the description of races and identification of resistance genes in different countries especially recent races so it needs for international cooperation using common differentials and molecular tools. Therefore, sunflower breeders and geneticists have focused on to solve that uncertainty recently and they have achieved significant results in the use of molecular markers for identifying broomrape races. Consequently, in addition to classical breeding methods, marker-assisted selection and some molecular screening tools should be used even more in the future research to obtain broomrape resistance. As well as developing broomrape resistance genes, some research should be performed on also altering the plant anatomy of plant organs, biochemical parameters (mechanical barriers, induced germination, hormones, etc.) aspects of the parasite side such as breeding system and genetics of virulence to understand the dynamics of broomrape populations and race evolution. On the other hand, Clearfield system is also an alternative and efficient control method with using of imidazolinone (IMI) herbicide plus resistant hybrids. The combining both herbicide with genetic resistance could supply more horizontal and durable resistance and successful advances for broomrape control in the future.

Key words: Sunflower – broomrape – races – distribution – resistance – breeding

INTRODUCTION

Sunflower broomrape (*Orobanche cumana* Wallr.) which is the holoparasitic reduces sunflower (*Helianthus annuus* L.) yield until %100 infecting the sunflower roots. In addition to Russia, Ukraine, Romania, Bulgaria, Turkey, and Spain as the main sunflower producers in the world, broomrape is also present in Serbia, Hungary, Moldova, Greece, Tunisia, Israel, Iran, Kazakhstan, China, Mongolia, and Australia, and possibly in a few other countries as well (Dedic et al., 2009; Molinero-Ruiz et al., 2009; Burlov and Burlov, 2011; Pacureanu-Joita et al., 2012; Amri et al., 2012, Ma et al., 2012; Kaya et al., 2012b; Antonova et al., 2012a,b, 2013; Cantamutto, 2012; Miladinović et al., 2012b; Eizenberg et al., 2012, Gisca et al., 2013; Marinkovic et al., 2014). Due to having light and producing many seed in one plant, it is dispersing easily with spreading of sunflower growing area. Therefore, broomrape needs to be controlled by using available strategies, and breeding for resistance proved to be the most reliable way to control the parasite (Škorić, 2012; Fernández- Martínez et al., 2012; Kaya et al., 2013).

One of the best options for controlling broomrape uses resistant and tolerant cultivars in the sunflower production. The rapid changes in the race composition of broomrape requires search for new resistant sources persistently. Therefore, it need to identify sunflower lines and other genetic material firstly for resistance to highly virulent races of broomrape, which is of great importance for current breeding programs, as source of resistance for gene transferring and sunflower hybrid production. After the determination of genes controlling resistance and their action, inbred lines could be used for transferring resistance in inbred lines with good agronomic traits using currently in the breeding programs, or could be used directly for developing commercial hybrids (Škorić et al., 2010; Škorić and Pacureanu- Joita, 2010, Fernández- Martínez et al., 2010, 2011; Škorić, 2012; Cvejic et al., 2012; Kaya et al., 2012a).

RECENT STIUATION OF THE DISTRIBUTION OF NEW BROOMRAPE RACES IN SUNFLOWER AREAS

Broomrape races on sunflower are designated by the letters of Latin alphabet: A, B, C, D, E are old races and F, G and H are the last and the most virulent ones. They were found in the course of the last decade at first in Romania Spain and in Turkey (Fernández-Escobar et al., 2009; Pacureanu-Joita et al., 2012; Fernández-Martínez et al., 2012; Kaya et al., 2012a). To identify the recent races of broomrape; Romanian differentials are using generally by sunflower researchers, resistant to races D, E, F, G; the inbred line P-1380 (differential for E race), the inbred line LC 1093 (the differential for F race), the Spanish line P 96, resistant to race F under control of two recessive genes *or6 or7* and two Russian sunflower lines: VK 623 and VT 62, resistant to new broomrape races respectively in Russia (Antonova et al., 2009, 2010, 2011, 2012a b; 2013; Pacureanu-Joita et al., 2010, 2012; Gisca et al., 2013). The recent development in broomrape races in last four years in sunflower production areas by countries were given below.

Broomrape populations which overcame the resistance gene *Or5* (race F) were identified in Spain in the mid 90's and F race has infested widespread in the main sunflower growing areas of the country for 20 years. On the other hand race G populations were also observed especially in Southern Spain (Andalucía) and Central Spain (Cuenca) which were main sunflower planting areas and exhibited similar aggressiveness with Turkey populations (Molinero-Ruiz et al., 2009, 2014; Fernández-Martínez et al., 2009, 2010; 2012; Velasco et al. 2012; Akhtouch et al 2013).

In Romania, more than 60% of the sunflower cultivated area is infested with broomrape. There are three important areas, as the presence of the broomrape races and infestation degree, situated in Braila, Constanta and Tulcea locations. The three more spread broomrape populations in the largest area cultivated with sunflower, are very different regarding the virulence and dissemination of the parasite The race G was definitely found in Tulcea and Constanta counties in Romania and latest surveys showed possible appearance of even more virulent race (Pacureanu et al., 2009, 2012).

During the last years in the Russian Federation, the biotypes of broomrape have appeared and propagated especially after free exchange of sunflower sowing material between the countries, then overcame rapidly other sunflower production areas (Antonova et al., 2009; 2013). The Rostov region was covered by the broomrape mostly and had the most virulent races affecting highly of all studied sunflower genotypes. While the broomrape populations in Volgograd region were less virulent and mixed with E and F races, the race F existed commonly in Stavropol region. In Krasnodar region, the mix of races C, D, E prevailed but in the northern districts bordering on Rostov region, the biotypes of a parasite affecting differential resistant to F and G races were observed. Thus, high virulent broomrape races overcoming action of known *Or5, Or6, Or7, or6o7* sunflower resistance genes have spread in the South of the Russian Federation (Goncharov, 2009; Gorbachenko et al., 2011; Antonova et al., 2010, 2011, 2012a, b, 2013).

The new broomrape races have developed in last years in Republic of Moldova. The broomrape population were observed more virulent than the race E, but not as aggressive as the F race is in Balti area, in the North part of the Republic of Moldova. A high infestation with virulent F race was found also in the Central and South areas of the Republic of Moldova (Duca et al., 2010, 2013; Glijin, 2012; Gisca et al., 2013).

New F race infestation have spread recently almost all part of in Trakya region which is European part of Turkey and covers more than 50% of sunflower areas in Turkey (Semerci et al., 2010) . Moreover, new races have spread to other sunflower areas recently such as Cukurova, Middle Anatolia and Black Sea regions that were immune areas 4-5 years ago. On the other hand, there was no race determination study in Turkey so it could not be indicated that which new races as G or H existed other than F (Evci et al., 2011; Kaya et al 2009, 2012a,b).

Burlov and Burlov (2011) performed a combined research in artificial conditions also with comparing Turkish and Russian broomrape races with Ukrainian ones. They indicated that there were more virulent G and H races of the parasite in populations of broomrape collected in four regions in Ukraine. They observed that new F and race was not found in the Russian Rostov population and the new virulent races F and G and, possibly, race H as well (especially the Turkish population of the parasite) was observed in Odessa, Donetsk and Turkish populations of broomrape includes.

In Bulgaria, Shindrova and Penchev (2012) observed that E and G races were most widely distributed in Bulgaria but E race population gradually decreased but it was still predominant in the region of northeastern Bulgaria. They mentioned also that race F was rare and random occurrence, race G populations were in only the central north Bulgarian regions nowadays and its infestation could increase gradually in southeastern Bulgaria and in other regions as well in the upcoming years.

Broomrape in sunflower fields in Serbia has observed with varying intensity almost every year. Broomrape population in Serbia, race E was dominant but some broomrapes also have noticed in several regions in Vojvodina (North part of Serbia) on some resistance sunflower hybrids against races A-E

indicating possibly changes in broomrape population. Monitoring of the broomrape population in Serbia has been performing regularly to observe in race composition changes spreading or not of more virulent races from neighboring countries (Dedic et al., 2009; Maširević and Medić-Pap, 2009; Hladni et al., 2009, 2010, 2012; Masirevic 2012a b; Cvejic et al., 2012; Miladinović et al., 2012a; Marinkovic et al., 2014).

Pacureanu et al. (2012) evaluated fourteen populations of broomrape collected from Bulgaria, China, Moldova, Romania, Russia, Serbia, Turkey, Spain and Ukraine in the artificial infestation conditions to determine in differentiation of broomrape races utilizing RAPD markers. They observed that the most aggressive populations were in Moldova, Romania, Russia and Turkey there were two or three different populations of broomrape in each country. They also mentioned that the broomrape populations in Russia are very similar to Romanian ones, except from Rostov's, this being almost similar with the most virulent Turkish population. They also observed that from Chinese population has not attacked to P-1380 line (race E) and Serbian one has not attacked to LC 1093 (race F), as well as Ukrainian population, Bulgarian and Spain populations attacked in lower rate to LC 1093. Their RAPD markers analyze results indicated that the ten studied broomrape populations divided in three groups and the genetic distance was not correlated with the geographic area.

On the other hand, Miladinovic et al. (2012a) also indicated that climatic parameters such as the altitude, latitude, longitude, mean hottest month temperature, mean coolest month temperature, average rainfall of 10 habitats and as well as soil parameters such as soil texture, total N, humus and calcareous content and pH had no effect on *Orobanche* attack intensity. However, Cantamutto et al. (2012) indicated broomrape seed dormancy was this most important parameter even than temperature and day length (latitude dependent) and winter temperature could adjust the germination time of the weed.

However, Molinero-Ruiz et al., (2014) indicated that homogeneous groups of highly virulent broomrape populations were related to geographical origin in Europe. They also found that some molecular groups related to only to one pathogenic group (i.e. race F from Southern Spain), while others include different pathogenic groups from the same geographical origin (i.e. races F and G from Turkey).

BROOMRAPE PARASITE BIOLOGY AND CONTROL METHODS

On the other hand, to understand sunflower resistance mechanism, the studies should be focus on parasite side such as the reproductive biology of parasite, the genetic structure of the populations, inheritance mode of traits related to parasitism, host relationships, germination factors and environmental factors affecting development of parasite, etc... Recent studies on seed production of *Orobanche* revealed that broomrape was self-compatible and tolerated seed production under isolation, both under self-fertilization and controlled hybridization (Ephrath et al., 2010; Plakhine and Joel, 2010; Cantamutto et al., 2012; Dongo et al., 2012; Gevezova et al., 2012; Rodríguez-Ojeda et al., 2012; Spring and Raupp, 2012; Eizenberg et al., 2012, 2013; Duca et al., 2010, 2013; Duca and Glijin, 2013; Seiler, 2011, 2012; Dimitrijević et al., 2013; Habimana et al., 2014). However, the bag type used for isolation clearly played important role on determining seed production and micro-perforated transparent plastic bags were the best for not differing in germination and infectivity capacity (Rodríguez-Ojeda et al., 2010). On the other hand, the inheritance of unpigmented mutant lacking anthocyanin was studied by Rodríguez-Ojeda et al. (2011) and they mentioned that the unpigmented trait was controlled by recessive alleles at a single locus, with the F_1 hybrid being phenotypically distinguishable from both pigmented and unpigmented parents.

Clearfield System with Imidazolinone (IMI) herbicides controls efficiently both major weeds and also broomrape, this system presents practical solutions to farmers so it has increased market share rapidly in recent years especially in Eastern European countries. The resistant IMI genes transferred from wild types to cultivated types, the trait is controlled by one gene *Imr1*, also known as *Ahasl1-1*, exhibiting partial dominance, also affected by a second modifier *Imr2* gene. Therefore, to obtain IMI herbicide resistant hybrids both inbred lines should be IMI resistant. On the other hand, a new IMI-resistant trait called CLHA-plus was developed by mutagenesis and selection controlled by partially dominant allele *Ahasl1-3*. Lines and hybrids carrying the CLPlus mutation have better tolerance to IMI herbicides then it need only one parent of a Clearfield hybrid allowing hybrid development rapidly(Demirci and Kaya, 2009; Fernández- Martínez et al., 2009, 2010, 2012; Sala et al., 2012; Evci et al., 2011, 2012; Kaya et al., 2012, 2013). New sources of resistance to IMI herbicides have been recently identified in populations of wild *H. annuus* and *H. argophylus* (Christov et al., 2010).

Another approach to control broomrape is utilizing from germinations stimulants to decrease broomrape population in the soil when sunflower did not plant. Honiges et al. (2009) indicated that the most widely known such broomrape germination stimulants are strigol, electrol, orobanchol, and GR 24 synthetic stimulant. In addition to dehydrocostus lactone (Joel et al., 2012), Raupp and Spring (2013) also mentioned that germination stimulant from sunflower root exudates as dehydrocostus lactone, a sesquiterpene lactone, dehydrocostus lactone, costunolide, tomentosin, and 8-epixanthatin were purified

and identified spectroscopically and that compounds induced germination of *O. cumana* at nano to micromolar concentrations. On the other hand, Zhang et al. (2013) indicated that soybeans could induce sunflower broomrape germination and could be used potentially as a trap crop for sunflower broomrape. Likewise, Ma et al (2012) observed similar effect on Chinese herb and in another studies (2013) in maize production.

CLASSICAL AND MOLECULAR BREEDING STUDIES ON GENETIC RESISTANCE AND FINDING NEW GENETIC RESISTANT SOURCES AGAINST BROOMRAPE

Since broomrape is an extremely variable parasite and the breakdown of resistance is a so common and frequent occasion, resistance breeding is continuous process and multiple sources of resistance are needed. Sources of resistance to broomrape races found in the early sunflower breeding programs in the Former Soviet Union originated from land races of cultivated sunflower, later from some chemical or radiation mutations but genetic resistance was also introduced into susceptible sunflower from wild species, mainly *H. tuberosus.* Recent studies on evaluation of sunflower germplasm for resistance to broomrape races proved that wild *Helianthus* species constitute the major reservoir of genes conferring resistance to new virulence races (Evci et al., 2009; Pacureanu-Joita et al., 2010; Terzic et al., 2010; Škorić and Pacureanu-Joita, 2010, 2011; Petcu and Pâcureanu, 2011; Škorić, 2012; Fernández-Martínez et al., 2010, 2012; Kaya et al., 2012a, 2014).

Progressions of old broomrape races (A, B, C, D, E) are controlled by five single dominant genes *Or1* to *Or5*, respectively. A new race F that overcomes the gene *Or5* was identified in Spain, Romania, and Turkey. Furthermore, more virulent race G that affects cultivars resistant to race F, was identified (Škorić et al., 2010; Pacureanu-Joita et al., 2011; Fernández-Martínez et al., 2012). Wild *Helianthus* species remain the main source of resistance to new virulent races of pathogen. The resistance to races E, F, G have been found in certain wild species of the genus *Helianthus* and incorporated into cultivated sunflower genotypes by interspecific hybridization. The crosses with the wild sunflower species *H. maximiliani, H. grosseserratus,* and *H. divaricatus* with cultivated sunflower and developed populations contributed greatly to develop sunflower varieties through interspecific hybridization and also recently *H. tuberosus* was used as the donor of *Or5* and *Or6* genes (Škorić et al., 2010).

Sunflower researchers mostly focused on wild species recently to find new resistance genes and develop new resources against broomrape. Velasco et al. (2012) found resistance to race G in a wild *Helianthus debilis* subsp. *tardiflorus* and the F_1 plants from the cross with cultivated sunflower were resistant indicating dominance of resistance gene(s). Seiler and Marek (2011) indicated that while especially perennial wild species exhibited higher resistance, annual species demonstrated lower resistance to Race F. However, although there are some difficulties on crossing perennial species with cultivated sunflower, some novel techniques such embryo culture and chromosome doubling of the F1s, amphiploids, etc. could facilitate the transfer of broomrape-resistant genes from the wild perennial species. Consequently, some amphiploids were obtained from perennial wild species *H. grosseserratus*, *H. maximiliani*, and *H. divaricatus* resistant to Race F releasing of four germplasm populations resistant to Race F named BR1 through BR4. 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 derived from interspecific amphiploids of *H. annuus* and of two wild perennials, H. divaricatus and *H. grosseserratus* controlled by a single dominant gene. However, recent studies indicated that the resistance of the sunflower germplasm derived from *H. grosseserratus* proved to be digenic, the second gene being influenced by environmental factors (Seiler and Jan, 2010; Seiler, 2012; Škorić, 2012; Škorić and Pacureanu-Joita, 2011; Fernández et al. 2012).

Cvejic et al (2012) found that new source of resistance to race G and other new races in population from interspecific hybridization with *H. divaricatus* and *H. tuberosus* and then developed later inbred lines resistant to race G. They also indicated that different sunflower germplasm sources could be used reservoir of genes conferring resistance to new virulent races of broomrape in the future. The latest research conducted in Romania under field and greenhouse conditions showed that sources of resistance to the newest population of *Orobanche* that were found in Romania, Turkey and Spain are present in the lines LC-009 and AO-548 (Rodríguez-Ojeda et al., 2013).

The differences in sources of the resistance have led to development of lines that are resistant to the same race of broomrape but vary in their genetic constitution (Imerovski et al., 2011, 2013). Until today, different authors reported different modes of inheritance of resistance to race F; controlled by single dominant gene, *Or6* or two recessive genes or two partially dominant genes. *Or7*, whose expression was influenced by the environment. Changes in the race composition of broomrape in Romania have been reviewed by Pacureanu-Joita et al. (2008). Preliminary results of resistance to race G indicate that it is controlled by dominant alleles at a single locus (Velasco et al., 2012). Studies by Antonova et al. (2009)

and Goncharov (2009) both discuss the dynamic change of broomrape races in Russia. It is known that *Orobanche* races change frequently in Ukraine and Moldova too, and that, although no public reports have been made yet, there are at least seven races of the pathogen in the two countries.

New races F and G of broomrape have spread in many part of Bulgaria and that races appeared one after another and have complicated the sunflower breeding process. Christov et al. (2009) have achieved outstanding results via interspecific hybridization in identifying broomrape resistant genes in wild *Helianthus* species incorporating them into cultivated sunflower and developing elite sunflower lines. Christov (2013) indicated that some genetic materials originating from wild sunflower species such as *H. tuberosus, H. pauciflorus, H. eggertii, H. x laetiflorus, H. decapetalus, H. hirsutus, H. divaricatus, H. giganteus, H. maximiliani, H. nuttallii* ssp. *rydbergii, H. salicifolius, H. smithii, H. annuus* (wild), *H. argophyllus, H. debilis, H. petiolaris* and *H. praecox*) and also five species from genera *Calendula, Carduus, Grindelia, Inula* and *Tithonia* of family Compositae to obtain resistance to broomrape. He also indicated that some developed inbred lines were demonstrated fully resistance to new races. Encheva et al. (2013) mentioned that also some inbred lines originated from wild *H. salicifolius* showed 100% resistance to *Orobanche* against artificial infection both in field and laboratory conditions developed by interspecific hybridization. Additionally, Encheva and Shindrova (2012) indicated that AAL and DIA accessions of wild *H. annuus* and *H. petiolaris* accessions were completely resistant to broomrape and *H. petiolaris* could have a great potential for broomrape resistance for cultivated sunflower.

To improve the durability of broomrape resistance, different breeding strategies should be applied like gene pyramiding or combining vertical and horizontal resistance mechanisms using more genes from different sources. That mechanism could be obtained by a resistant hybrid from the combination of lines carrying two different resistant mechanisms consisting one of failing of *Orobanche* seed germination and other one supplying necrosis of the parasite structures at early development stage (Fernández-Martínez et al., 2012; Škorić, 2012).

Molecular markers

Due to the influence of environmental factors, inadequate amount of broomrape seeds in the soil, hard process to collect broomrape seeds, its seed germination problems, etc., broomrape tests do not produce reliable results always. In order to attain their breeding goals and identify sources of broomrape resistance certainly, sunflower breeders choose the appropriate inoculation method and molecular marker technique (MAS). Since the most reliable and most easily applicable method of screening breeding materials for broomrape resistance is the use of molecular markers. QTL, RFLP, RAPD, TRAOP, and SSR markers have so far been used for this purpose (Imerovski et al., 2013; Pérez-Vich et al., 2013). Molecular research for the purposes of race characterization and mapping is developing rapidly. For instance, Pacureanu et al. (2009) found that the resistance to the race F could be controlled with either two recessive genes or one dominant gene, in inbred lines depending on the origin of the inbred lines. These results were proved that by Imerovski et al. (2011) at the molecular level, and indicating that the marker is specific to the material on which it has been developed.

The utilizing from RFLP markers for the characterization of broomrape races, a linkage group containing the *Or5* gene were integrated with the GIE Cartisol RFLP map. Besides working on the development of markers for *Or5*, current research is aimed to the mapping of a new gene that provides resistance to broomrape races higher than F (Cvejić et al., 2012). By comparing the molecular profile of resistant and susceptible genotype, a polymorphism was observed on LG3 of SSR map. Accordingly, it can be assumed that the new gene is located on this linkage group (Imerovski et al., 2012). Further tests on the mapping population will determine the exact position of the gene, and enable the development of specific molecular marker that will accelerate the introduction of resistance to new races of broomrape into the commercial sunflower lines.

Pineda-Martos, et al. (2014) studied genetic diversity of 50 broomrape populations in Spain utilizing 15 microsatellite markers and observed the existence of two distant gene pools, one in Cuenca province and other in the Guadalquivir Valley. They indicated that also both inter- and intrapopulation variability were extremely low within each gene pool and but genetic recombination between distant gene pools was an important mechanism for creating new variation and also having an effect on race evolution. In another study, Pineda-Martos, et al. (2014) used 4200 simple sequence repeat (SSR) markers to identify and characterize broomrape and 217 SSR primer pairs were used for validation. From them, 87 SSR primers produced reproducible, high quality amplicons of the expected size that were polymorphic among 18 broomrape populations from different locations. They also indicated that *O. cumana* SSR markers were highly transferable to the closely related species *Orobanche cernua* and utilizing that SSR markers could be possible to classify of *Orobanche* spp. samples into species (*O. cumana* and *O. cernua*), geographical origin and host properly.

CONCLUSIONS AND FUTURE PROSPECTS

Sunflower breeders recently have big challenge recently both developing high yielding cultivars and also adaptable to marginal environments due to sunflower production being pushed into lower-fertility soils. In addition, that abiotic stress conditions, some biotic factors such as broomrape, downy mildew, and other diseases also limit and threaten sunflower production recently in increasingly year-by-year. The limited genetic variability and missing resistant genes for broomrape in cultivated sunflower provided especially from wild species. Recent studies in especially MAS proved that novel molecular techniques could be so valuable in sunflower breeding programs for resistance gene identification, accelerating and facilitating broomrape resistance breeding.

The virulence of broomrape populations varies differently in the world and the most aggressive parasitic races are in the Black Sea having more than 50% of sunflower planting areas with disseminating rapidly. However sunflower breeders and geneticists found resistant genes from some wild *Helianthus* genus and then incorporated them into sunflower inbred lines to develop *Orobanche*-resistant hybrids. However, rapid changes in the race composition of broomrape need urgently to set up an international project on both investigating and screening of all wild sunflower species natural and artificial conditions against new races of broomrape and molecular screening too. There is also an urgent need of collaboration among public institutions and private companies to establish a proper set of differential lines for the new races appeared in Russia, Romania, Ukraine, Turkey, Bulgaria, etc. These efforts will help and lead to launch universal methods be established for both screening for resistance to broomrape in field and greenhouse conditions and molecular methods more accurately.

On the other hand, combining IMI or SU herbicide resistance or both of them together with broomrape resistance will supply choose to sunflower farmers with controlling both broomrape and major broad leaf weeds together. Similarly, some studies focused on broomrape parasite biology and germination stimulants. To speed up the progress of sunflower breeding for resistance to *Orobanche*, there should be a greater level of collaboration between the breeders from public institutions and private companies.

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Current situation of sunflower broomrape in Spain

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ABSTRACT

A review of the situation of *O. cumana* in Spain is presented in this manuscript. The research conducted in the country on characterization of populations of the parasite along the last decades is described in parallel to the evolution of the crop of sunflower in Spain from the 1970's up to the present. A large collection of parasite populations has been studied by their ability to infect the most frequently used sources of resistance against *O. cumana* at each moment. All this research has been developed at the same time than new and effective sources of resistance against broomrape were identified and the resistance incorporated into sunflower of agronomic value. While initial works about characterization of *O. cumana* were focused on populations of virulence lower than F, populations identified as race F have been analysed in the last years. A quite complete understanding of the structure of *O. cumana* infecting sunflower in Spain has been reached using pathogenic and molecular approaches, and new research challenges have arisen in the way.

Key words: Genes of resistance – parasite ecotypes – *Helianthus annuus* L. – *Orobanche cumana* Wallr. – pathotypes – parasite races – virulence

THE SUNFLOWER IN SPAIN

Sunflower (*Helianthus annuus* L.) is grown in Spain since the 16th century, when it was introduced by Spanish explorers. It was first used as an ornamental horticulture plant and later to provide foods. As of the introduction of the plant species, sunflowers were grown in gardens and also in small farms because of their value as an edible crop. Cuenca, in Castilla-La Mancha, has been for centuries the traditional area for growing confectionary sunflower types. Sunflower became a "new" crop in Spain in the 1970's, when it was reintroduced as an oilseed crop. Open pollinated varieties with increased oil content that were initially grown were later replaced by hybrid cultivars. From 166.000 ha grown in 1970, the area devoted to sunflower in Spain reached 10^6 ha in the mid 1980's. Since then, the total acreage of the crop of sunflower is between 800 and 100 10^3 ha every growing season, with occasional variations (Fig. 1).

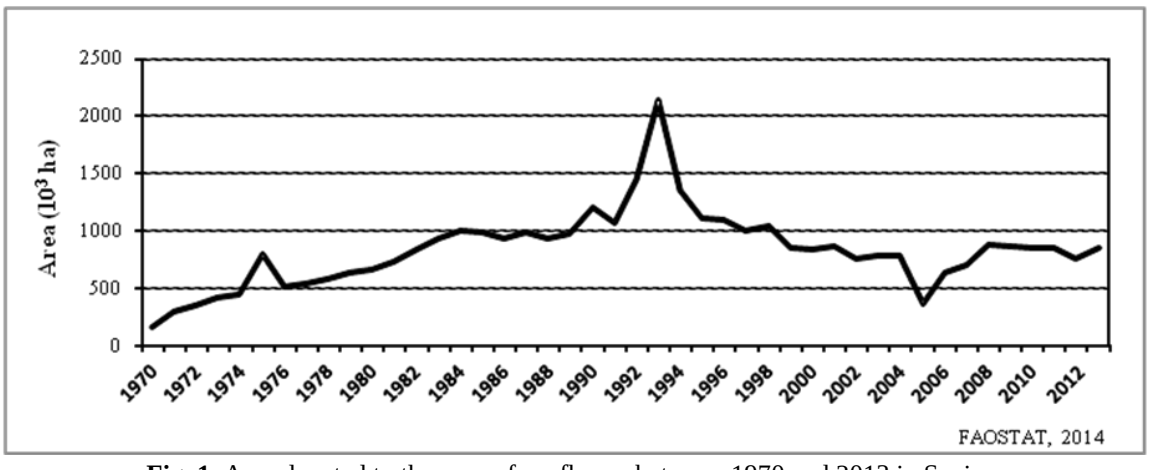

Fig. 1. Area devoted to the crop of sunflower between 1970 and 2013 in Spain.

The current sunflower acreage in Spain is distributed in three main areas: Castilla-La Mancha (in the Central plateau), Andalusia (the Guadalquivir valley, in Southern Spain) and Castilla-León (in Northern Spain). The biggest area where sunflower is grown is located in Andalusia and Castilla-León, where 38 and 35% of the total acreage respectively were cropped in 2013. Also in 2013, sunflower fields in Castilla-La Mancha accounted for 22% of the total acreage (Fig. 2).

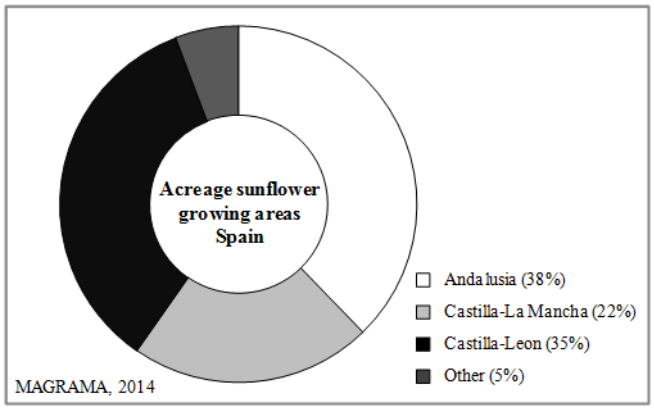

Fig. 2. Percentage distribution of the total acreage cropped to sunflower in the main growing areas of Spain in 2013.

HISTORICAL PERSPECTIVE OF SUNFLOWER BROOMRAPE

Orobanche cumana Wallr. was first reported in Spain in 1958 in Castilla-La Mancha (Díaz-Celayeta, 1974), where susceptible confectionary sunflower had been grown for decades. The introduction of confectionary cultivars from countries where the parasite was not known to occur, as well as of oilseed cultivars from Eastern Europe, favoured the crop expansion in Spain in the 1970's and early 1980's. Most of the oilseed cultivars came from the Soviet Union, where important research on sunflower was conducted as of the first years of the 20th century. V.S. Pustovoit, in a successful breeding program of sunflower, selected open pollinated varieties with increased oil content but also with resistance to broomrape races A and B. This resistance to *O. cumana* was introduced from the perennial *H. tuberosus* L. (in Sackston, 1992). Oilseed cultivars grown at that moment in Spain were, among others, Kruglik A-41, Saratovski 169, Jdanovski 8281 (J8281 from now on), VNIIMK 1646, VNIIMK 8931, Peredovik and Armavirsky 3497, all of which had been developed in the Soviet Union.

Simultaneous to the increase of acreage cropped to sunflower along the 1970's, broomrape attacks became more frequent. Confectionary sunflower was highly susceptible to parasite populations, particularly those cultivars that had been produced abroad Spain and therefore in absence of the parasite pressure that existed in Castilla-La Mancha. Oilseed sunflower was less severely affected than confectionary sunflower, but scattered and slight broomrape attacks occurred in oilseed cultivars grown in Castilla-La Mancha in 1976 (Sackston, 1978). In this same crop area, 30% affected plants and severities of 29 broomrape stems per sunflower plant were reported in 'Peredovik' in 1979 (González-Torres et al., 1982).

The big expansion of the sunflower crop in Spain took place in the 1980's and early 1990's, when open pollinated populations were replaced by highly productive oilseed hybrids. Also at this moment the geographical distribution of races of *O. cumana* in Spain began to be investigated. Single major genes, Or_1 to Or_5, were reported in Romania to control races A–E of *O. cumana*, each of them also conferring resistance to the previously described races (what has been called cumulative resistance) (Vrânceanu et al., 1980). Table 1 summarizes the reaction of resistant germplasms developed in the former USSR and in Romania against parasite populations from Spain, either in field trials or in experiments conducted under controlled conditions, during the 20th century. González-Torres et al. (1982) were the first to study the infection of *O. cumana* in different genotypes of sunflower. They quantified the importance of broomrape attacks on confectionary and oilseed sunflower and identified similar proportions of affected confectionary and oilseed sunflower fields within the total fields of each sunflower type that were prospected. Although confectionary cultivars were more severely affected than oilseed cultivars, the main interest of their work lays in the first report, in terms of incidence and severity, of a quite important infection of broomrape in the oilseed sunflower variety Peredovik. This variety was obtained in the Soviet Union as resistant to races A and B of *O. cumana*, and therefore González-Torres et al. (1982) were also determining that the broomrape ecotype infecting 'Peredovik' in Castilla-La Mancha was a more virulent pathotype than A or B.

Table 1. Reaction of resistant germplasms developed in the former USSR and in Romania against populations of *O. cumana* from Spain, either in field experiments or in experiments conducted under controlled conditions, during the 20th century

Population of *O. cumana*	Geographic origin[1]	Year (collection or field exp.)	Resistant germplasm								References
			AD66	Kruglik A-41	VNIIMK 8931	J8281	Peredovik	Record	S1358	P1380	
BS-78	C	1978	-	-	-	-	S	-	-	-	González-Torres et al., 1982
BF-79	A	1979	-	-	-	-	S	-	-	-	González-Torres et al., 1982
PT-78	C	1978	-	-	-	-	S	-	-	-	González-Torres et al., 1982
El Coronil	A	?	S	S	-	R	-	S	R	R	Melero-Vara et al., 1989
Huéscar	A	?	S	S*[2]	-	R	-	S	S*	R	Melero-Vara et al., 1989
Villarejo	C	?	S	S*	-	R	-	S	S*	R	Melero-Vara et al., 1989
El Coronil	A	1993	S	S	S	-	-	S	R	R	Refoyo and Fernández, 1994
Rozalén	C	1993	S	S	S	-	-	S	S	-	Refoyo and Fernández, 1994

[1] C = Central Spain, A = Andalusia.
[2] S* = broomrape incidence ≤ 33%.

Subsequent works suggested that races of *O. cumana* in Spain did not correlate with races reported in other countries. When three ecotypes of the parasite were inoculated to the set of sunflower genotypes that were used to differentiate races of *O. cumana* in Romania, the control by Or_1 to Or_5 genes did not follow the cumulative pattern observed by Vrânceanu et al. (1980). In particular, the varieties J8281 (Or_2) and Record (Or_3) presented reverse reactions than expected: the three ecotypes from Spain infected 'Record' with values that varied between 50 and 66% infected plants, but were effectively controlled by the resistance into J8281 (Or_2) (Melero-Vara et al., 1989). Another interesting finding of the work by Melero-Vara et al. (1989) was that while the inbred line S1358 (Or_4) was resistant to the ecotype from Seville (in the Guadalquivir valley), the other two ecotypes caused slight infections on the same line (disease incidences of 22 and 33%). In other words: the presence of two different pathotypes of the parasite in Spain, one in the South and another in the Centre of the country, was suggested for the first time. Some years later the difference between *O. cumana* ecotypes from Castilla-La Mancha (Cuenca) and Andalusia (Seville), in terms of pathogenicity, was again pointed up by the reaction of S1358 (Or_4), which presented a resistant reaction to the ecotype from Seville but not to the one from Cuenca (Refoyo and Fernández, 1994).

Unfortunately, the association of pathogenic traits of *O. cumana* and geographic origin of the parasite within Spain could not be confirmed when 36 populations collected in the country from 1989 to 1992 were characterized (Saavedra del Río et al., 1994), because no information about the location where the populations were collected from is available. However, in the work by Saavedra del Río et al. (1994) all except one of the populations were effectively controlled by J8281 (Or_2) and P1380 (Or_5). This meant new evidence that the cumulative pattern of resistance that was effective against races of *O. cumana* in Romania was not applicable to parasite populations from Spain, and that the resistance into J8281 (Or_2) was highly effective for the control of *O. cumana* in the country. Finally, the most important finding of Saavedra del Río et al. (1994) was that one population of *O. cumana*, what happened to be collected in 1992, was associated to moderately resistant reactions of J8281 (Or_2) but also of P1380 (Or_5). This was the first report of a new race of *O. cumana*, or race F, which was not controlled by the Or_5 gene, which conferred the most effective resistance at that moment. It could also be the first sign that the extensive cropping of sunflower in the country was resulting in a selection of highly virulent components within populations of the parasite. Moderately resistant reactions of P1380 (Or_5) observed by Saavedra del Río et al. (1994) might be the precedent to the severe *O. cumana* attacks reported in oilseed hybrids in the following years (Alonso et al., 1996).

EVOLUTION OF PATHOTYPES OF *O. cumana* AND BREEDING FOR RESISTANCE

From the 1990's on, the attacks on sunflower oilseed hybrids by the race F of *O. cumana* in Andalusia became frequent and a real constraint to the Spanish sunflower production. Several researchers reported on the incidence and severities of field infections (Alonso et al., 1996; Domínguez et al., 1996). The acreage cropped to sunflower at that moment in Spain was around one million ha, and the effect of the parasite attacks in susceptible hybrids was quantified in 50% of loss in seed production (Domínguez, 1996).

Early breeding of sunflower for resistance to races of *O. cumana* in Russia and Romania had consisted on the selection of open pollinated varieties of cultivated sunflower. Resistance to the race E of *O. cumana*, also from cultivated sunflower, was incorporated into several germplasm lines released by the USDA (Fargo ND, USA) and the Junta de Andalucía (Córdoba, Spain) in 1997 (Miller and Domínguez, 2000). Similarly, screenings of wild *Helianthus* species and lines derived from interspecific crosses with cultivated sunflower identified resistance to the race E from Spain in most of the perennial species as well as in some annual species and wild derived lines (Ruso et al., 1996). After the report of the race F of *O. cumana*, and as the result of a breeding programme for resistance against it, an outstanding work for identifying and incorporating effective resistance into cultivated germplasm was carried out by Spanish scientists. As a result, resistance to the race F was identified in wild, mostly perennial, species and transferred into cultivated sunflower (Sukno et al., 1998; Fernández-Martínez et al., 2000; 2012). Lines to be used as sources of germplasm for the development of new material were registered (Jan et al., 2002; Fernández-Martínez et al., 2004; Pérez-Vich et al., 2006). Details about Spanish research on breeding sunflower for resistance against *O. cumana* and its progress in time have been excellently reviewed by other authors (Melero-Vara et al., 2000; Fernández-Martínez et al., 2008; 2012).

Simultaneous to the efforts in breeding for resistance against broomrape, important research was devoted to the study of populations of the parasite. Along the 2000's *O. cumana* race F became widely distributed in Andalusia (Molinero-Ruiz et al., 2006; 2009). Although infections of sunflower by *O. cumana* have not been observed in Castilla-León for many years, two importantly infected fields were detected in this area in 2008 (in Valladolid province) and in 2013 (in Soria province). The parasite collected in Valladolid was identified as a race F (Fernández-Escobar et al., 2008), as has been suggested for the population from Soria by recent works of our research group (Molinero-Ruiz, unpublished results). Due to the large area cropped to sunflower in Castilla-León, a potential expansion of the parasite might importantly affect seed production in the area. Therefore, growing sunflower hybrids with resistance to *O. cumana* is advisable as a protective measure against it in this area.

CURRENT STATUS OF *O. cumana* IN SPAIN

An extensive characterization of populations of *O. cumana* from different locations in Spain was carried out from the beginning of the 21st century. The information about populations and reactions of inbred lines currently used as differentials for races of the parasite is presented in Table 2.

Old populations of *O. cumana* were studied by Molinero-Ruiz et al. (2008). In 2005, 38 populations collected along 20 years in Andalusia and Castilla-La Mancha either onto confectionary or oilseed sunflower, were initially assessed by their infectivity on the confectionary susceptible cultivar B117. The results by Molinero-Ruiz et al. (2008) showed that seeds of *O. cumana* can keep their viability and infectivity for at least 17 years when stored at room temperature. Highly interesting were the results when the inbred lines NR5, L86 and P96, incorporating resistance to races E and F (together with B117, confectionary susceptible) were subsequently inoculated with the 12 oldest viable populations of the parasite which had been collected from 1988 to 1999. All the populations infected NR5 with final degrees of attack that ranged from 0.6 to 17.5 broomrape stems per plant, suggesting that, at least as of 1988, highly virulent (not controlled by Or_5) components existed in populations of *O. cumana* from Spain. Concerning the reactions of L86 and P96 to some of the populations, they were not fully resistant. These results pointed again to diverse genotypes composing broomrape populations. Therefore, a complete resistance of sunflower to a given population of *O. cumana* might only occur when the genes of resistance into the crop are able of effectively control all the parasite genotypes in that population. Alternatively, consistent incidences of small number of *O. cumana* stems in some genotypes of sunflower are the sign that the resistance into these genotypes is able of controlling some but not all the genetic components in the parasite population. The main conclusions of the work by Molinero-Ruiz et al. (2008) were the following: a) the race F of *O. cumana* infected both confectionary and oleaginous cultivars in Andalusia as early as 1988, b) one of these race F populations, collected in 1995 in Andalusia, hold, in unknown ratio, highly virulent genotypes, and c) all the populations characterized as race E were genetically

heterogeneous, and were collected on oleaginous cultivars in Andalusia and Castilla-La Mancha between 1993 and 1997.

Table 2. Populations of *O. cumana* and reactions of inbred lines currently used as differentials for races of the parasite

Population of *O. cumana*	Geographic origin	Year of collection	Inbred line			References [1]
			NR5	L86	P96	
SE188	A	1988	S	R	R	II
SE489	A	1989	S	R	R	II
SE193	A	1993	R*[2]	R	R	II
SE194	A	1994	R*	R	R	II
CA195	A	1995	R*	R	R	II
SE195	A	1995	S	R	R	II
SE295	A	1995	S	R*	R*	II
CO196	A	1996	R*	R	R	II
SE296	A	1996	S	R	R	II
F196	C	1996	R	S	R	I
S396	C	1996	R	S	R	I
U296	C	1996	R	S	R*	I
MO197	C	1997	R*	R	R	II, IV
LC198	A	1998	S	R	R	II
LC299	A	1999	S	PR[3]	R	III, IV
MN199	A	1999	S	R	R	II
CU200	C	2000	R	S	R	IV
HE200	A	2000	S	PR	R	III, IV
LR100	A	2000	S	PR	R*	I, III, IV
SE500	A	2000	S	R	R	III, IV
CO101	A	2001	S	PR	R	III, IV
PA101	C	2001	S	S	R*	IV
AA702	A	2002	S	R	R	III, IV
CO1002	A	2002	S	PR	R	III, IV
CR202	A	2002	S	PR	R	I, III, IV
CU1102	C	2002	R	S	R	IV
JC302	A	2002	S	PR	R	III, IV
LG602	A	2002	S	PR	R	III, IV
PG502	A	2002	S	PR	R	III, IV
SP102	A	2002	S	PR	R	III, IV
TP402	A	2002	S	PR	R	III, IV
CN503	A	2003	S	PR	R	III, IV
CP203	A	2003	S	PR	R	III, IV
CT803	A	2003	S	PR	R	III, IV
CT1503	A	2003	S	PR	R	III, IV
EC403	A	2003	S	PR	R	III, IV
LRB1603	A	2003	S	PR	R	III
O1503	A	2003	S	PR	R*	I
P803	A	2003	S	PR	R	I
TOM106	A	2006	S	R	R	I, II

[1] I: Molinero-Ruiz et al., 2006, II: Molinero-Ruiz et al., 2008, III: Molinero-Ruiz et al., 2009, IV: Molinero-Ruiz et al., 2014.

[2] R* = high broomrape incidences and very low number of broomrape stems per sunflower plant; no statistical difference with the resistant control. The reaction is considered as resistant and associated to diversity of genotypes within populations of *O. cumana*.

[2] PR = high broomrape incidences and moderate number of broomrape stems per sunflower plant; statistical difference with the resistant control. The reaction is considered as partially resistant.

The works about sunflower breeding for resistance against race F that have been previously commented, together with the incorporation of this resistance into cultivated sunflower by means of breeding programs of public institutions and also of private companies, lead to the substitution of oilseed hybrids with resistance to race E by hybrids with resistance to race F. Oilseed hybrids resistant to race F of *O. cumana* began to be grown in Spain in the early 2000's. Nowadays hybrids of sunflower with this type of broomrape resistance are grown all over Andalusia, and they are increasingly used in Castilla-León.

Consequently, research about characterization of *O. cumana* in the last years has been focused on populations infecting many of the hybrids of sunflower with resistance to race F. Consistent broomrape attacks of low intensities were observed on these hybrids when they began to be used. These attacks were initially attributed to the occurrence of mixture of races within populations of *O. cumana* race F in fields of Andalusia, but results by our group showed that they were not associated to "new races" of the parasite; partial resistance of the hybrids was instead identified (Pérez-Vich et al., 2004; 2006; Molinero-Ruiz et al., 2006). The level of resistance and the benefits of sunflower hybrids with horizontal resistance against broomrape *versus* susceptible hybrids were later confirmed and quantified under field conditions (Molinero-Ruiz et al., 2009). With low *O. cumana* infestation levels, both the resistant and the susceptible hybrids performed equally well. Infections of resistant sunflower by *O. cumana* depended not only on the level of field infestation, but also on the water availability; drought conditions favoured infections of resistant hybrids. Consequently, and despite low water availability and high intensities of *O. cumana* attack (BI and FDA of partially resistant hybrids only reduced to ca. 50% of the susceptible controls), seed yield of resistant hybrids was still 124% or higher when compared with susceptible controls (Molinero-Ruiz et al., 2009). On the other hand, two groups of subpopulations (races E and F) were studied by their pathogenic traits. No different virulences were detected within any of the groups inoculated onto resistant sunflower genotypes, but race F subpopulations caused statistically different infection values in the genotypes (Molinero-Ruiz et al., 2006). This was the first evidence that populations of *O. cumana* race F from Spain are not homogeneous, and that this heterogeneity is not related to "new races" within parasite populations. This work was also the first report of a not cumulative resistance against *O. cumana*: L86, which was registered as resistant to race F, was extremely susceptible to the less virulent race E. Many *O. cumana* populations infecting sunflower hybrids with partial resistance to race F, together with populations overcoming Or_5, were later characterized. Twenty populations, collected in Andalusia between 1999 and 2006, were compared by their ability to infect, in glasshouse experiments, three inbred lines carrying known sources of resistance against the parasite. All the parasite populations infected NR5, but none of them was able to infect P96, which indicated, as in previous results, that no new *O. cumana* races have developed with a virulence factor more advanced than F (Molinero-Ruiz et al., 2009). Also in this work different levels of aggressiveness of *O. cumana* in NR5 were identified, suggesting again the genetic heterogeneity within these populations (Molinero-Ruiz, et al., 2009).

When the genetic diversity of 50 populations of *O. cumana* collected between 1988 and 2008 in Spain, most of them identified as race <E, E or F, was molecularly analyzed, two distant gene pools were identified, one in Castilla-La Mancha and another one in Andalusia. Both inter- and intra-population variability were extremely low within each pool, which might be due to separate introduction events. Additionally, different races occurred within the same gene pool, suggesting that, in each area, *O. cumana* pathotypes (races) might have evolved from a common genetic background (Pineda-Martos et al., 2013). Also a group of 27 *O. cumana* populations that overcame the Or_5 gene in different fields of Spain, Hungary and Turkey, and therefore characterized as race F, were molecularly characterized. Samples included DNA from broomrape stems infecting NR5 (-N) but also DNA from some stems infecting L86 (-L) and P96 (-P). The results identified four molecular clusters, respectively, grouping populations from Central Spain, Hungary, South Spain and Turkey. Populations from Turkey were molecularly close to those from Andalusia. Moreover, the work revealed the high genetic homogeneity within the analysed race F populations from Spain, because no differences were found between N-, L- and P- DNA samples (Molinero-Ruiz et al., 2014) and confirmed that low infection levels in L86 and P96 (Molinero-Ruiz et al., 2006; 2008) were due to the quantitative resistance of the inbred lines to race F (Pérez-Vich et al., 2004; 2006).The finding of two molecular groups of race F parasite populations, one in Castilla-La Mancha, and another in Andalusia (Molinero-Ruiz et al., 2014), agrees with those, enclosing a variety of races, identified by Pineda-Martos et al. (2013). On the other hand, molecular analyses have not identified race F as a genetically differentiated group of populations, which points to a polyphyletic origin for race F (Molinero-Ruiz et al., 2014), arising independently at different geographical areas, as suggested by Pineda-Martos et al. (2013).

Overall, and from the molecular point of view, populations of *O. cumana* from Spain are clearly

distinguished according to their geographical origin (Castilla-La Mancha and Andalusia). Within each area, a variety of pathotypes may have evolved from a diverse genetic background and probably as a consequence of the great selection pressure exerted for virulence on the parasite by growing resistant sunflower. Evidence of selection of highly virulent components within genetically heterogeneous parasite populations was presented some years ago (Molinero-Ruiz et al., 2008).

Genetic recombination may also have played an important driving force for race evolution in *O. cumana* on account of the relatively high rate of outcrossing (>20%) observed in this species (Rodríguez-Ojeda et al., 2013a) allowing different virulence genes get into contact and subsequent genetic recombination (Pineda-Martos et al., 2013).

Also genetic studies of *O. cumana* have recently been conducted. When analysing the inheritance of avirulence genes, the gene-for-gene interaction in the *O. cumana*-sunflower parasitic system for races E/F and the dominant sunflower gene Or_5 were confirmed (Rodríguez-Ojeda et al., 2013b). A similar approach on genetics of *O. cumana* might be useful in order to gain an accurate knowledge about genetic composition of broomrape populations.

CONCLUSIONS

The geographical distribution of races of *O. cumana* in Spain began to be investigated more than 30 years ago, when open pollinated varieties of sunflower were replaced by highly productive commercial oilseed hybrids. Because the cumulative pattern of resistance that was effective against races of *O. cumana* in Romania and other countries was not applicable to parasite populations from Spain, races of *O. cumana* did not correlate with those races reported in other countries.

As early as 1989, the existence of two different pathotypes of *O. cumana*, one in the South and another in the Centre of Spain, was suggested for the first time. Recent molecular results have identified two distant gene pools, one in each of these two areas, with extremely low inter- and intra-population variability within each pool. Even two molecular groups of *O. cumana* race F have also been determined in the same areas.

The attacks of oilseed hybrids by the race F of *O. cumana* in Andalusia became frequent and a constraint to the Spanish sunflower production in the 1990's. Although genetic resistance against this parasitic race was soon identified and incorporated into cultivated hybrids, slight and consistent infections were frequent in resistant material. Research about performance of resistant hybrids showed evidence that, in most cases, resistance to race F is not complete, but partial and expressed as horizontal resistance in the field.

When populations of *O. cumana* from Spain were pathogenically characterized, their genetic heterogeneity was suggested. Unlike parasite populations with virulence lower than F, where highly virulent components seemed to be present, the heterogeneity within race F populations was not related to the presence of "new races" within them.

Overall, two distant gene pools of *O. cumana* are found in Spain, one in the Centre and another one in the South of the country. Within each area, a variety of pathotypes may have evolved from a diverse genetic background and probably as a consequence of the great selection pressure exerted for virulence on the parasite by growing resistant sunflower. Genetic studies of *O. cumana* might be the most plausible approach to gain knowledge on diverse genotypes composing populations of the parasite from Spain.

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Current situation of sunflower broomrape in France

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ABSTRACT

For several years, in France, sunflower crop have been facing a new bioagressor, sunflower broomrape (*Orobanche cumana* Wallr). This parasitic plant root, specific to sunflower crops, causes serious economic damage around the world, especially in SE Europe, Middle East and SW Asia. Its presence on the French territory was established in 2007. Since 2011, prospecting sunflower plots has allowed us to determine two main infested areas, located in the southwest, corresponding to less than 5 % of the sunflower cultivation area. Other minor spots were identified in Vendée and Poitou-Charentes. Currently, broomrape management is done through prophylaxis, and chemical methods (herbicide-tolerant varieties) combined or not to the use of resistant varieties to broomrape populations. Further work is needed to identify and follow the broomrape populations in time, in order to give out better advice in the choice of varieties. Different projects are in progress, carried by French private and public research, in order to contribute to sunflower broomrape control.

Key words: *Orobanche cumana* – *Helianthus annuus* L.– distribution – control

INTRODUCTION

Sunflower broomrape (*Orobanche cumana* Wallr.) has become a major threat to the sustainability of the sunflower crop in many countries. This holoparasitic angiosperm infects sunflower roots and diverts nutrients and water of the sunflower for its growth and development. The yield losses caused by this parasitic weed can reach 100% for the susceptible cultivars which are heavily infested. The distribution of *O. cumana* is centred on SE Europe, the Middle East, SW Asia, but also in China (Parker, 2013). Different parasitic weed has already been observed on the sunflower crop in France: *Phelipanche ramosa* L. (Pomel) (Nowak and Leflon, 2010) and *Orobanche reticulata* Wallr (Guillaumin, 1975). But none of them is an economic problem for this crop. In contrast to several neighboring countries, broomrape sunflower (*O. cumana*) is a recent problem in France. Since the appearance of this new phenomenon, different stakeholders (private and public research, technical institute, seed companies…) are mobilized to participate in the management of *O. cumana*.

AN EMERGING PROBLEM IN FRANCE

In France, The presence of broomrape sunflower (*O. cumana*) was only established in 2007, in contrast to neighboring countries where it has been observed for several decades. Faced with this new risk, since 2011, the CETIOM (technical institute for the oilseed, hemp and pulse crops) has established a reporting system online (www.cetiom.fr/orobanche_cumana) in order to evaluate the status and the progress of this parasitic weed on the French territory. In addition to this participatory strategy, prospecting in field was conducted in the main sunflower growing areas in 2011 and 2012. We reported that *O. cumana* was present in 8 of the 96 district of this country at the end of 2013, corresponding to 152 plots, and mainly in the south-west (south of Tarn and Garonne, and east of Haute-Garonne) (Fig. 1). The number of infested plots is probably more important, because in low infestation, which is often the case in France, it is very difficult to identify them. In south west of France, CETIOM estimated that less than 5% of sunflower's areas are concerned by *O. cumana*. It is an emerging problem which could increase during the next years. Isolated plots were also observed in Poitou-Charentes and Vendée (Fig. 1). According to the level of infestation, the yield losses are variable (from 16 to 91%). These yield losses seem to depend on the aggressiveness of broomrape, the variety used, the earliness of broomrape's emergence and the deep of soil. In addition to direct losses caused by *O. cumana*, indirect losses were observed with a shattering before harvest. For heavily infested plots, the sunflower crop was completely destroyed by broomrape. It

was observed that more than 50% of plots were weakly infested. The south of Tarn-et-Garonne is particularly exposed.

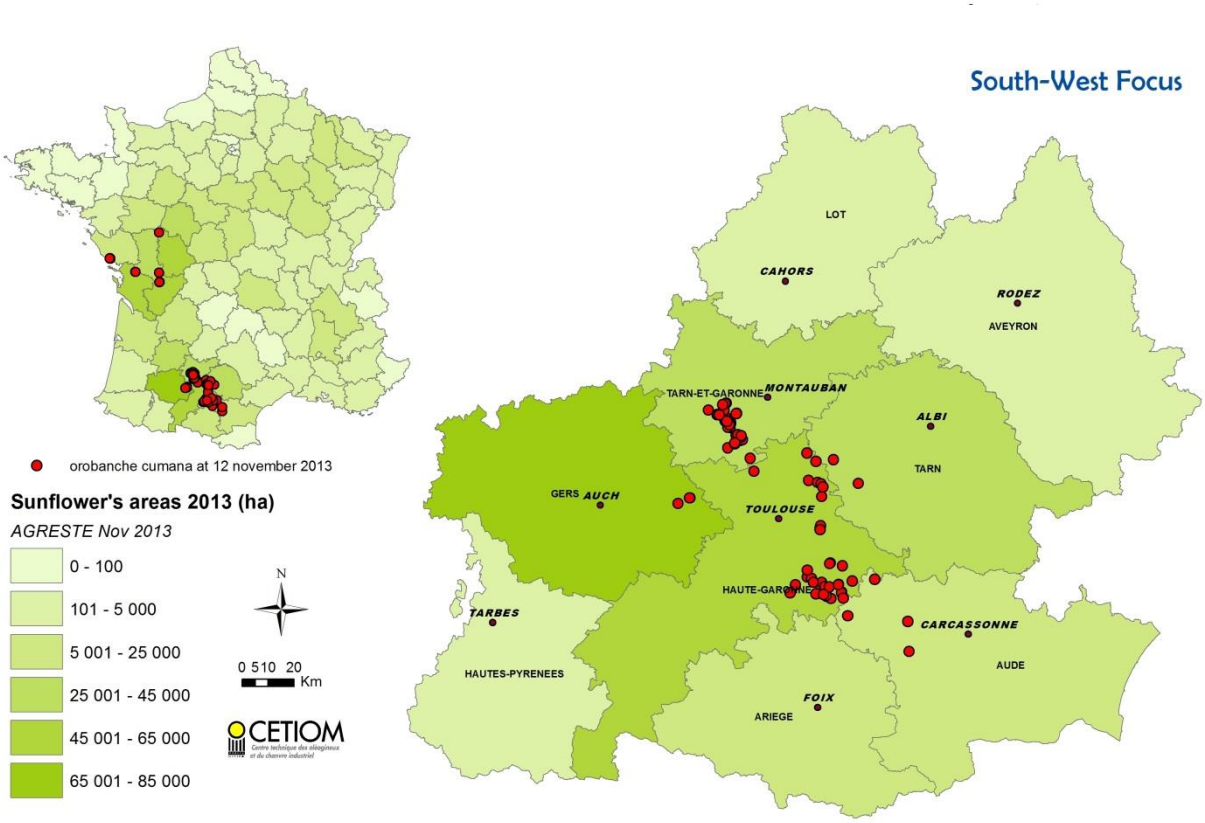

Fig. 1. Mapping of sunflower broomrape on French territory at the end of 2013 (n=152 plots). Each red point corresponds to one city with at least one infested plot.

It is not again clear on the composition of broomrape races which are present in France, but the first results suggest a mixture of races, including race E and more. In south west of France, first data show differences of populations (aggressiveness/virulence) between south of Tarn-et-Garonne and east of Haute-Garonne. Geographical discontinuity infested plots and the potential mixture of races, suggest several events of infestation, but the source of infestation is not established.

Currently the main vector of seed dispersal is probably the human factor through farm equipment. To date, we have only an overview of infested plots. In French crop system, sunflower comes back every 2-3 years, so several years would be necessary before to observe a potential spread of *O. cumana*.

BROOMRAPE MANAGEMENT

In France, the management of sunflower broomrape is done through different ways:

Prophylaxis:
Communications were conducted to alert stakeholders from the agricultural sector to this new problem. This awareness has also allowed people to reference themselves infested plots on the CETIOM website (www.cetiom.fr/orobanche_cumana). We suggest also eradicating the first broomrapes for weakly infested plots, harvesting the healthy plots before the infested plots, burying stubbles after harvest to avoid the spread of seeds by the wind, and thoroughly cleaning the equipment after use. Moreover, we suggest waiting as long as possible the introduction of sunflower in infested plots to limit the multiplication of seeds.

Use of resistant cultivars:
In order to limit the increase of broomrape seed bank and the yield losses, use of resistant cultivars to sunflower broomrape is recommended. First data enable to advice producers on some cultivars to use in case of broomrape. Only a few varieties are available to farmers. Indeed, varieties proposed for the French market are not always characterized for resistance to broomrape populations due to the recent phenomenon. Works should be performed to characterize the French broomrape populations and phenotype varieties with different races which are present on the French territory.

Depending on the situation (broomrape absent in and around from plot, presence of broomrape in the plot, absence of broomrape but presence on the adjacent plot), CETIOM recommends using either solution single (genetic method) or in combination (clearfield® + pulsar variety which is resistant to *O. cumana*). In presence of broomrape in the plot or just around, combination clearfield® + pulsar with variety resistant to *O. cumana* is advised in order to combine efficiency and sustainability of the solution.

Chemical method:
In 2013, CETIOM conducted a field experiment to evaluate herbicide strategies (optimal application timing and dosing) to control sunflower broomrape using imazamox (Pulsar) combined to clearfield® varieties (imidazolinone-tolerant varieties). Two clearfield® varieties showing a contrasting response to French sunflower broomrape populations were used for this trial: LG5658 (susceptible) and LG5663 (partially resistant). Previous experiments had shown that imazamox had efficiency against *O. cumana* much higher than tribenuron-methyl. These results led to recommendations in the chemical control of sunflower broomrape:

For susceptible variety, later application (at 8-10-leaf stage) to 50 g/ha of imazamox was the treatment the most effective, taking into account the number of broomrape shoot emergence and the vigor of sunflower. However, this application would require an additional treatment in pre-emergence to control other weeds. Application to 6-10-leaf stage or subdivision of application in 4 then 6-8-leaf stage is also possible, but it showed a lower efficiency than 8-10-leaf stage. Control was insufficient when imazamox was applied at 4-leaf stage: its efficacy on the level of infestation was only 60% and there was injury to sunflower.

For partially resistant variety, no major difference was observed between one application to 50g/ha and two split applications, at any of the application timing, taking into account the measured traits (level of infestation and vigor of sunflower). However, when treatment (dose of 50 g/ha) was divided into two applications, it was possible to obtain a better control of other weeds.

Otherwise, it was also shown that the foliar absorption of imazamox is effective to control broomrape, and root absorption of herbicide can lead to the same result in rainy years and loam soil.

Other solutions, as thermal method were tested by CETIOM. In a field naturally infested, the impact of thermal weeder (1200 °C) on the germination capacity of *O. cumana* was assessed at different soil depth (0-3 and 3-10 cm) and two processing speeds (0.2 and 1.3km / h). The results showed that if the inhibition of germination was not complete, the treatment showed satisfactory results: the germination rate was up to 7 times lower compared to the untreated control. Despite the fact that this is an effective control strategy, the method is difficult to implement on large areas, and so not used.

RESEARCH PROJECT IN PROGRESS

To date, private and public research began to mobilize in order to struggle against this new scourge in France. Different collaborative projects are in progress:

DIVO Project (funded by FSRSO; partner: RAGT 2N, SOLTIS, CETIOM, Biogemma).The objective of this project is, through a molecular approach, firstly to clarify the phylogenetic relationships between the two species *O. cernua* and *O. cumana* and secondly to assess the genetic diversity within the species *O. cumana*. Confrontation between molecular classification and phenotypic, will make it possible to define an optimzed SNP set for the characterization of *O. cumana* populations.

CTPS Orobanche Project (funded by CTPS; partner: Syngenta, GEVES, CETIOM, LBPV-University of Nantes) aims to (i) develop a molecular tool to detect the broomrape seeds (including *O. cumana* and other parasitic weed species) in the seed lots, (ii) to provide an evaluation method sunflower varieties for *O. cumana*, for the registration of varieties (CTPS).

Promosol Orobanche project (funded by Promosol; partner: INRA, CSIC Córdoba) aims (i) firstly to develop know-how on phenotyping the interaction *Helianthus * O.cumana* at different scales, in order to decipher the genetic background of the different steps involved in the resistance/susceptibility process; (ii) secondly, to build a publicly available set of differential sunflower with the purpose to improve the characterization of races.

HELIOS Project (financially supported by the French FUI - Fonds Unique Interministériel - Single Interministerial Fund, the agglomeration of Saint-Malo, and the Region of Bretagne, and promoted by the Pôle Mer Bretagne; partener : Timac Agro International, Groupe Roullier, MAISADOUR Semences, University of Nantes -Laboratory of Plant Biology and Pathology and CETIOM). This project aims to develop new marine bioactive compounds inhibiting the growth of *Orobanchaceae* (see also poster in this congress).

CONCLUSION

In France, sunflower broomrape is a new biological constraint to the cultivation of sunflower. Genetic and chemical solutions are currently combined to control this parasitic weed. Monitoring sunflower plots should continue to evaluate the potential extension of *O. cumana*. Work is undertaken to better identify the races of *O. cumana* and follow their evolution in the time. Such results should provide better advice in varietal choice which, to date, remains incomplete.

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Current situation of sunflower broomrape in Hungary

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ABSTRACT

Sunflower broomrape (*Orobanche cumana* Wallr) is the most important problem in the sunflower crop area around Black Sea, and dissemination and dispersion of the races of this parasite continue in neighboring territories, including Hungary. Although economic damage was not reported in Hungary, phytophathological and economical risk might increase especially under dry conditions, starting from the South-Central to North-West, simultaneously with the appearance of more virulent populations which cover races D and E in last years. In order to better understanding of evolution of this parasite, Limagrain Hungary has developed a big project for farm monitoring in areas infested with *Orobanche*, using questionnaire and seeds sampling for laboratory test. The study carried out showed, that the most common race in Hungary is race E, but some spots on sunflower growing area in the South are infected with race more dangerous than race E.

Current situation of sunflower broomrape in Serbia

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ABSTRACT

Historical overview of broomrape (*Orobanche cumana* Wallr.) behaviour on sunflower in Serbia is characterized by two epiphytotic occurrences. First, there was epiphytosis of race B in 1950's and then epiphytosis of race E in 1990's. The parasite population in Serbia is stable at the moment as no change in the racial composition has been observed, that is, race E is still prevalent and no new broomrape races have appeared. The areal distribution of broomrape in Serbia has expanded, but the most vulnerable regions are still northern Bačka and Tisa shore in Vojvodina and Timočka Krajina in eastern Serbia. Since the problem of resistance to broomrape race E is fully resolved, as all commercial NS sunflower hybrids have incorporated resistance gene Or_5, the main goal in sunflower breeding program for the market in Serbia in the terms of resistance to broomrape is to find new sources of resistance genes to the new broomrape races which are present in Europe. In a line with this goal, testing of the wild relatives of the sunflower (long-term program) as well as inbred lines in gene bank (short-term program) is carried out.

Key words: Sunflower – broomrape – race - resistance genes

STATUS OF BROOMRAPE POPULATIONS IN SERBIA

Genus *Orobanche* L. is characterized by a great diversity, with 200 different species discovered around the world (Pusch and Gunter, 2009). Twenty-six different species, 56 varieties and 226 different forms of this genus have been discovered in Serbia so far, that makes 308 different taxa (Maširević and Kojić, 2002). Among them, broomrape (*Orobanche cumana* [Wallr.]) causes the economically most significant damage on sunflower. Depending on the intensity of the attack and stage of the development of sunflower at the time of infection, yield decrease could vary from 5 to 100%. Beside *O. cumana*, sunflower could be attacked by other species of this genus such as *O. coerulescens*, *O. minor*, *O. ramosa*, *O. brassicae*, *O. aegyptica* and *O. crenata* (Maširević et al., 2012). *O. aegyptica* and *O. crenata* have not been observed in Serbia, so far.

Sunflower cultivation for oil production has a relatively long tradition in Serbia. Industrial processing of sunflower and oil production started in 1923, while more significant sunflower production and oil refining has begun in 1934 (Čurović and Berić, 2009). This led to significant expansion of sunflower as a field crop, primarily in Vojvodina and eastern Serbia. However, sunflower acreage till the Second World War (WWII) did not exceed 20.000 ha (Tarčinski, 1938). There are no reports on broomrape in sunflower crop in Serbia in this period. The varieties mostly grown at that time were low-oil varieties form the greenies group (with grey hull and white stripes) in Vojvodina and old varieties from the fuchsia group (with dark hull with purple pigment - anthocyanin) in eastern Serbia. Since these varieties are extremely susceptible to all broomrape races, it is most probable that there were no broomrape in Serbia during that period. Evgenije Gibšman started sunflower breeding program after WWII. He used Russian variety Saratovski-169 as a starting material and created first Serbian varieties - NS-4, NS-8 as well as high-oil variety NS-18. These varieties were only resistant to broomrape race A and since no broomrape was observed on them it could be assumed that there broomrape was not present in Serbia at that time.

Appearance of race B

The first report on broomrape in sunflower in Serbia dates from 1951, when broomrape appearance in sunflower crop in Vojvodina was observed (Aćimović, 1977). The first scientific paper on this parasitic weed was published by Bošković in 1962. According to Bošković (1962) broomrape was probably introduced into Serbia *via* seeds of Russian varieties imported from Ukraine. The same author analysed broomrape populations from 31 sites in Vojvodina, and found that there is a certain level of variability among them regarding virulence. Although no regularity was found, he concluded that Bačka region of Vojvodina was more endangered than Banat and Srem. Intensity of the broomrape attack increased during the years and in the end of 1950's almost completely hampered sunflower production. This led to change of varieties that were cultivated and introduction of high-oil Russian varieties, resistant to broomrape and

sunflower moth, such Peredovik, Smena, Jenisej and VNIMK 8931. Although there are no data in the studies on the racial composition of broomrape in Serbia at the time, it was almost certainly the race B, as Russian varieties resistant to this broomrape race solved the problems in sunflower cultivation and brought back this crop to the fields in Vojvodina. Russian high-oil varieties, and the first hybrids that were introduced into production in 1978, resistant to race B of broomrape, enabled the increase in sunflower acreage to 150-200,000 ha which is maintained to the present day.

There are not many papers on broomrape in the period between the end of 1950's and the end of 1990's, since *O. cumana*, except for sporadic occurrences in a completely susceptible confectionery varieties, was not a problem in the sunflower production. The occurrence of broomrape was recorded only in 1976 in north-east part of Vojvodina in three different sites on unidentified sunflower varieties (Aćimović, 1980). The observed broomrape populations differed regarding their virulence.

Appearance of race E

At the beginning of the 1990's Mihaljčević (1996a) observed mass occurrence of O. *cumana* on hybrids resistant to race B in north and partly middle Bačka, as well as along the Tisa shore and Romanian border in Banat. Greenhouse tests confirmed the appearance of new, more virulent race E in Vojvodina. Inter- and intra-population variability tests showed that broomrape populations were homogenous throughout north of Bačka. It was found that dominant gene Or_5, originating from hexaploid wild species *Helianthus tuberosus*, provides complete resistance to this new broomrape race. Thanks to these studies, new sunflower hybrids completely resistant to broomrape race E were created in Institute of Field and Vegetable Crops (IFVC) (Škorić and Jocić, 2005). The introduction of these new hybrids, such as Perun, Bačvanin and Šumadinac, into production in 1996 prevented the new broomrape races to significantly endanger sunflower production in Serbia.

Period since appearance of new broomrape race E in Serbia up today is characterized by studies that verify if there are changes in race composition of broomrape in Serbia. It was found that broomrape population is stable and that there are no changes in race composition, but that broomrape spread into the sunflower production regions where it has not been observed before. In his study, Maširević (2001) found that in 2000, that was extremely dry and favourable for broomrape development, broomrape spread into new regions, and that the intensity of the attack was high, as it in some cases caused yield reduction was up to 50-70%. The same author confirmed that there is no change in race composition of broomrape in Vojvodina, and that it only has expanded to new regions in Vojvodina. Dedić et al. (2009) found that broomrape spread further into some regions in eastern Serbia, and that the race present was race E. Common characteristics of the regions where broomrape was found is that these are arid regions, characterized by soils with pH value over 6.7 (Miladinović et al., 2012).

In contrast to frequent changes in broomrape race composition in Russia, southern Ukraine, Moldova, Romania, Turkey and Spain, chronological survey of appearance of *O. cumana* on sunflower in Serbia during the 20th century and the first decade of the 21st century, supports the widely accepted opinion that slow evolution of physiological races of this parasitic weed is taking place. This slow evolution is a result of a very low percentage of mutations of the virulence genes in the parasite population. In the agro-ecological conditions of Vojvodina, this interpretation could be fully accepted, as there were only two significant changes in broomrape population over the last six decades (in late 1950's and early 1990's). Appearance of new races could be autochthonous or the primary inoculum could be introduced from geographically distant regions *via* infected sunflower seed during uncontrolled import. Thus, the emergence of virulent race B is a consequence of seed import from Ukraine in the period before the 1950's, while the last change in *O. cumana* population could be at least partly explained as a result of processes within the indigenous population. It is known that the site where the first occurrence of virulent race E is observed is known for its small but constant production of confectionery sunflower varieties imported from Hungary ("striped" sunflower for bird feeding and variety Gigant), so it is possible that this new broomrape race was imported with the seeds of these varieties (Mihaljčević, 1996c). Selection pressure on the parasite population during three decades of cultivation of the varieties and then hybrids with the same genetic basis (resistance genes Or_1 and Or_2), inevitable lead to favouring of races with different virulence genes. More stable situation in the population of broomrape in Serbia compared to other countries where the parasite is present could also be explained by the origin broomrape in Serbia. Since broomrape was introduced by the seeds from Ukraine, the broomrape population in Serbia originate from a relatively small number of individuals and is therefore characterized by low genetic variability and a lower potential for change of racial composition. It should be noted that there are no reports on appearance of broomrape races C and D in Serbia.

Future prospects

Constant selection pressure on the parasite population and especially its ability to change due to mutations or recombination of virulence genes, could lead to epiphytotic disease appearance. In order to understand the changes that occur in the parasite population, it is necessary to know that *O. cumana* is self-pollinated parasitic flowering plant, but that percentage of cross fertilization could be up to 21.5 to 28.8% (Rodrígez-Ojeda et al., 2013). Besides that, the emergence of a number of new races in almost all the countries where broomrape is present and free trade of sunflower seeds across the Europe, suggests that the changes in the broomrape population and appearance of new races in Serbia will occur sooner or later. These new broomrape races will probably not be completely new, but the ones already present in the countries that are more vulnerable to the broomrape attack. Based on the experience from the previous two outbreaks of the parasite, it is clear that changes in the population structure of the parasite, that is, the emergence of new races, inevitably leads to the complete change of the range of varieties and hybrids that are cultivated.

STATUS OF THE RESEARCH ON BREEDING FOR RESISTANCE TO BROOMRAPE IN SERBIA

Work on sunflower breeding in Serbia started in 1930's. The main centre for sunflower breeding, since its establishment in 1938, was and is still today the Institute of Field and Vegetable Crops (IFVC) in Novi Sad. The first NS varieties had low oil content and no resistance to broomrape, but they were, since introduction of hybrids in 1965, completely replaced by the hybrids which are high-oil, resistant to pathogens and to broomrape (Jocić et al., 2012).

Breeding for resistance

The achieved levels of broomrape resistance in NS sunflower hybrids, as well as the requirements set forth in the creation of a suitable model of hybrids for conditions of Serbia are complicating the process of creating of hybrids resistant the new broomrape races for at least two reasons. The first reason is that the long standing presence of *Phomopsis helianthi* in Serbia favoured the production of sunflower hybrids tolerant to this parasite. All the varieties, hybrids and the parental lines used as the sources of genes for resistance to the new broomrape races originate mostly from Romania, Spain and Turkey, and thus show a high degree of susceptibility to *Phomopsis helianthi*. The second reason comes from the fact that the broomrape is parasitic flowering plant of arid regions. The material of foreign origin, resistant to the new broomrape races and selected primarily for agro-ecological conditions of southern Spain, eastern Romania and Turkey, expressed in our conditions, in many years of trials, an extremely high susceptibility to white rot on root. All this made the transfer of desirable *Or* genes much more complicated. The lines donors of resistance to the new broomrape races are frequently characterized by extremely poor agronomic characteristics. During the introduction of *Or* genes, it is necessary to maintain the tolerance to *Phomopsis helianthi* in the receiving line at the same level and to avoid the introduction of susceptibility to white rot, while preserving the potential for seed yield and oil content. Ultimately, the main goal of sunflower breeding is not resistance to pathogens but the oil yield, that is, its most important components, seed yield and oil content.

New sources of resistance

Since the problem of resistance to broomrape race E is fully resolved, as all commercial NS sunflower hybrids have incorporated resistance gene Or_5, the main goal in sunflower breeding program for the market in Serbia in the terms of resistance to broomrape is to find new sources of resistance genes to the new broomrape races which are present in Europe. In a line with this goal testing of the wild relatives of the sunflower (long-term program) as well as inbred lines in gene bank (short-term program) has been carried out (Kaya et al., 2012).

IFVC has one of the largest collections of wild sunflower species that contains 447 populations of 21 perennial and 7 annual species of the genus *Helianthus*. Terzić et al. (2010) tested six accessions of wild perennial sunflower species (*H. tuberosus*, *H. grosseserratus*, *H. mollis* and *H. nuttalii*), fourteen F_1 interspecific hybrids between perennial wild species and the cultivated sunflower, as well as 40 accessions of annual wild sunflower species in the greenhouse and in the field for resistance to broomrape. All tested perennial species and 12 out of 14 F_1 interspecific hybrids were found to be resistant to broomrape. In annual species, most of accessions of *H. neglectus* and *H. petiolaris* were resistant to this parasitic weed, while *H. praecox*, *H. petiolaris* and *H. debilis* had some accessions that were resistant to broomrape. Dedić et al. (2011) retested annual wild sunflower accessions in the field and

confirmed the resistance in four accessions of *H. petiolaris*, two accessions *H. praecox* and one accession of *H. neglectus*. Miladinović et al. (2013) tested several populations of *H. annuus* and *H. petiolaris* originating from Argentina, and found that all tested *H. annuus* accessions were susceptible and all tested *H. petiolaris* accessions were resistant to broomrape. Based on these results, crosses with cultivated sunflower were done and backcrosses are currently in progress where each generation is tested for broomrape resistance.

Beside wild species, broomrape resistance testing is done on cultivated sunflower genotypes. Usually, the tests are performed first in Serbia, for resistance to race E, in the greenhouse and field conditions, and then the genotypes with complete resistance to broomrape race E are further tested in Spain, Turkey and Romania. In this way, the lines resistant to broomrape races F and G were detected in NS breeding material (Cvejić et al., 2012; 2014). These lines were obtained from interspecific crosses with *H. tuberosus* and *H. divaricatus*. Preliminary results of studies of the mode of inheritance of resistance to the broomrape races higher than F indicate that this trait is controlled by recessive gene(s). This means that it is necessary to introduce resistance genes into both parental lines in order to obtain resistant hybrids (Cvejić et al., 2014).

Molecular studies on sunflower

In the process of creating resistant lines, it is necessary to have a reliable assessment of breeding material. Molecular markers, when they are close to the resistance genes, can detect the gene of interest and provide a more reliable assessment of the evaluation of the resistance of plants in the field (Miladinović et al., 2014). Due to the fact that the *Or* genes are introduced into cultivated sunflower from various sources, the origin of the genetic material is an important factor when it comes to their position and the mode of inheritance. That is why the work on the development of markers for the detection of resistance to broomrape in IFVC is primarily focused on the identification markers suitable for use in the NS genotypes. Imerovski et al. (2013) screened twenty NS sunflower genotypes resistant to different broomrape races (A-F) with SSR markers, and by contingency analysis found a statistically significant relationship between gene Or_6 and alleles ORS1036_240 and ORS1114_265. Or_4 gene showed a significant association with ORS665_281 and ORS1114_264 while ORS1114_260 allele was detected only in the differential line for Or_2. The observed link between markers from LG3 and three different broomrape resistance genes indicates that there is a possibility that the *Or* genes are closely related, and on the same LG. Current research is focused on mapping genes from new resistance sources from the broomrape races higher than F (Cvejić et al., 2012; Cvejić et al., 2014), where the high polymorphism on LG3 was observed indicating that new resistance genes are probably located on this LG (Imerovski et al., 2012). Molecular analyses have been started on other detected resistance sources with the aim to determine exact position of the resistance genes which should enable the development of specific molecular markers that will speed up the introduction of resistance to new races of broomrape into NS sunflower lines (Imerovski et al., 2014).

Molecular studies on broomrape

Work on the molecular characterization of broomrape plants requires the use of different types of markers for the analysis of the genome of the plant. The aim of the research is to analyse the diversity of the parasite, as well as to identify the markers that could be potentially used to distinguish different broomrape races. For the analysis of the genome of the parasite it is necessary to isolate sufficient quantity of DNA with good quality. Since the collection and transport of fresh plant tissue and seed samples of broomrape can be problematic, the possibility of using the dry stalks broomrape as starting material for DNA extraction was examined. It was found that the adequate quantity and quality of DNA could be obtained from dry stems preserved at room temperature in well-ventilated area, and by using modified protocol of Rogers and Bendich (1985) for DNA isolation (Dimitrijević et al., 2013). The resulting DNA was used to for analysis of inter- and intra- population diversity broomrape in Vojvodina. No clear connection between geographic and genetic distance of tested broomrape populations was found, with rather low inter and intra-population variability (Dimitrijević et al., 2014).

Herbicide-tolerant hybrids

IFVC has registered the first sunflower hybrid tolerant to imidazolinone herbicides (IMI) in Europe (Jocić et al., 2004). In addition, the IFVC is also one of the few institutions that have managed to simultaneously develop hybrids tolerant to tribenuron-methyl (Jocić et al., 2011). The creation of herbicide tolerant hybrids has opened the possibility of chemical control of broomrape. A number of papers has been published on this subject (Malidža et al., 2003; Malidža et al., 2004; Malidža et al., 2012), along with

molecular studies (Dimitrijević, 2013) and based on years of experiments, it was concluded that the by the use of IMI sunflower hybrids and preparations based on imidazolinone, broomrape in sunflower could be fully controlled regardless of racial composition. It was found that imazamox in imidazolinone tolerant hybrids has significantly higher efficacy in controlling broomrape than tribenuron-methyl in tribenuron-methyl-tolerant hybrids. This increased efficiency is primarily reflected in the duration of protection, which is in imidazolinone herbicide till the end of the growing season and in tribenuron-methyl till the end of flowering.

Future prospects

Changes in race composition of specific parasite do not require imminent change of ideotype of the cultivated plants. The emergence of new races of broomrape is not a particular problem for sunflower breeders, in the technical sense, if the genetics of resistance to these races is known. However, the detection and introduction of resistance genes could be in some cases complicated and requires team work of breeders, phytopathologists and molecular biologists.

As previously said, it is unlikely that entirely new broomrape race, not detected in other broomrape affected countries, will occur in Serbia. There are sources of genes for resistance to the new broomrape races in Romania, Spain and Turkey, with different modes of action and mode of inheritance (Škorić et al., 2010). Tests of the resistance of selected breeding material and hybrids in regions where the occurrence of broomrape is constant could elucidate the genetic basis of certain populations of broomrape (virulence genes) as well as of the tested sunflower genotypes (resistance genes). Combined method, that is, testing of the selected genotypes in the greenhouse and in the field conditions in Serbia, and then in Spain, Turkey, Romania and Russia, can help the breeders to get the full picture of what could happen in the future.

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Current situation of sunflower broomrape (*Orobanche cumana* Wallr.) in Romania

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ABSTRACT

Broomrape (*Orobanche cumana* Wallr.) has a long history of parasitism on sunflower, longer than one century, starting with Russia and followed by other countries in Europe and Asia. During this period, the parasite had some times characterized by different virulence degree, number of races and developed sunflower resistant genotypes. In Romania, broomrape on sunflower has been identified in 1940-1941 period, first in south Moldavia, coming from Russia. The most infested area with this parasite is in Dobrogea (near Black See), south Romania (Baragan) and Moldavia (South and Central part). There are differences between these mentioned areas, depending by virulence of the parasite (different races) and by aggressiveness. The most virulent populations of broomrape are in Dobrogea area, being present the races more virulent comparing with F race (G and H races). In the last years these new virulent populations have spread in a small area, in Baragan, were the dominant races are E and F. In Moldavia the predominant race is race E. Using some sunflower differential lines for races E and F there have been studied 12 broomrape populations in the last two years. The highest infestation degree, including the new populations of the parasite is in Tulcea and Constanta areas, being followed by Calarasi-Ialomita and Braila areas. In these areas the old races (E and F) are still present in high percent. The lowest infestation degree is in Iasi (Moldavia) area. In this area there is not present the race F. The same occurs in some locations from Ialomita-Calarasi area. In infested fields with different races of the parasite, it is observed that the sunflower hybrids which were fully resistant in the first years after going into the commercial market, are losing slowly their resistance. Some of them have 20-25% attack degree after 4-5 years from the first cultivation in one infested area.

Key words: Sunflower – broomrape – virulence – aggressiveness – differentials

INTRODUCTION

Broomrape (*Orobanche cumana* Wallr.) was described for the first time in Russia in 1890 (Saţâperov, 1913). This parasite has remained very important for sunflower crop until now, in the main area cultivated, special in Europe. From Russia, the parasite has spread in Ukraine and other close countries in the Black See (Moldova, Romania, Bulgaria, and Turkey).

In Romania, *O. cumana* was identified in sunflower crop in 1940-1941 period, by Savulescu (Vrânceanu, 2000). The highest attack of the parasite is found in southeastern Moldavia, Dobrogea and eastern Muntenia-Baragan. Iliescu (1984, 1988) characterized the parasite *O. cumana* as being very important for sunflower crop in Romania, in 1981-1983 period, after that being not for so high importance and described the specie *Orobanche* (*Phelipanche*) *ramosa*, but with a minor importance. After 1995 year, broomrape started to become year by year very dangerous for sunflower crop in Romania. In present, the parasite continues to spread in new areas, developing new and very virulent races (Păcureanu-Joiţa et al., 2009). The losses in sunflower seed yield produced by the parasite can to be from 20% to 90%, depending by the number of broomrapes on one sunflower plant.

In the last years broomrape has become of a great importance for sunflower crop in Romania, for this reason, all breeders, from public institutions or private seed companies, being focused in controlling this parasite, by using different methods.

Research work made for studying *Orobanche cumana* and relationship between host plant and parasite was very intense in Romania. There have been identified the races of the parasite and sources for resistance in sunflower germplasm. Vrânceanu et al. (1980) have identified the first 5 races (A, B, C, D, E) of the parasite as well as the sources of resistance for each of these, being established a differential set of sunflower inbred lines. In 1996 year it was identified a new race (race F) of the parasite and the source of resistance for this (Păcureanu-Joiţa et al., 1998). In 2006 year, a new population of the parasite, more virulent, it was identified in Tulcea area, near Black See, until now being not identified a differential line for this. This is because, the inheritance of resistance in sunflower, for these new populations of the

parasite, is an horizontal type, different from a sunflower genotype to other one, the parasite developing new races in a short time.

In this paper we are presenting the results obtained in showing the current situation of the parasite spreading in new areas cultivated with broomrape in Romania as well as of the new virulent populations.

MATERIALS AND METHODS

Different sunflower hybrids as well as the differentials for broomrape races have been used in testing for resistance to broomrape, in natural infestation conditions, in 12 locations situated in all infested areas cultivated with sunflower in Romania. The testing, for some broomrape populations, has been done in the artificial infestation, too. In green house, have been used some pots of 5 liters capacity, having inside a mixture of soil, sand and broomrape seeds. In the artificial infestation conditions there have been used broomrape seeds collected from all twelve locations from main infested areas and two sunflower differentials, for race E and race F of the parasite.

RESULTS AND DISCUSSION

In Romania, 60% of sunflower cultivated area is infested with broomrape. There are three big areas, infested with different populations of the parasite, these areas being separated by the virulence of broomrape populations. Fig. 1 shows the three areas, the first one (Tulcea and Constanta) being the most important as broomrape virulence. In this area, infested with the most virulent populations of the parasite, the difference between locations coming from the broomrape aggressiveness. The second area is situated in Braila-Ialomita-Calarasi, with the old six races of broomrape in high percent, but, there are some locations were, in the last two years the new races have been identified in small percent. The third area is situated in southeastern Moldavia and south Muntenia-Baragan. In this area are present in highest percent the first five races of the parasite, in some locations being identified the race F, even one population more virulent, in one location.

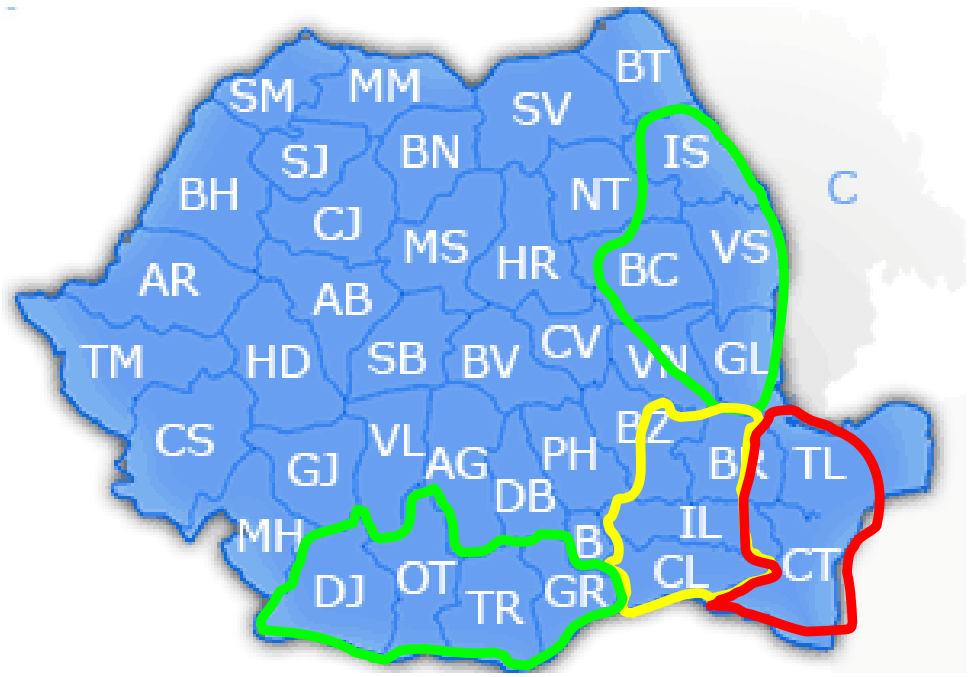

Fig. 1. The most infested areas with the parasite *Orobanche cumana* (broomrape) in Romania.

Table 1 shows areas and locations were tests of resistance for broomrape have been done in infested fields as well as in artificial infestation conditions.

Table 1. The areas infested with broomrape in Romania, were it has been done testing for resistance.

Area	Location	Year 2012	Year 2013
Tulcea 1	Topolog	x	x
Tulcea 2	Lunca	x	x
Constanta 1	Cuza-Voda	x	x
Constanta 2	Gradina	x	x
Constanta 3	Crucea	x	x
Braila 1	Mircea-Voda	x	x
Braila 2	Valea Canepii	x	x
Calarasi 1	Stefan-Voda	x	x
Calarasi 2	Sarulesti	x	x
Alexandria	Troianu	x	x
Iasi	Podu Iloaiei		x
Ialomita	Tandarei		x

Fig. 2 shows the infestation degree of broomrape attack, being observed that in Tulcea-Constanta area showed the highest infestation in both years. In Braila-Ialomita-Calarasi area, in some locations there are present the new races of the parasite, but in small percent. In Muntenia-Baragan area, there is one location (Alexandria-Troianu) with presence of new races of the parasite (very small percent). In Fig. 3, the results obtained by testing in artificial infestation, using the differentials for races E and F are showing that the highest infestation degree on both differentials is in Tulcea 2 and Constanta 1 locations, followed by Constanta 3, Constanta 2, Tulcea 1 and Braila 1. The lowest infestation degree is in Iasi-Podu Iloaiei location. It can be seen that the old races of the parasite are present in high percent in the mixture of races from broomrape populations.

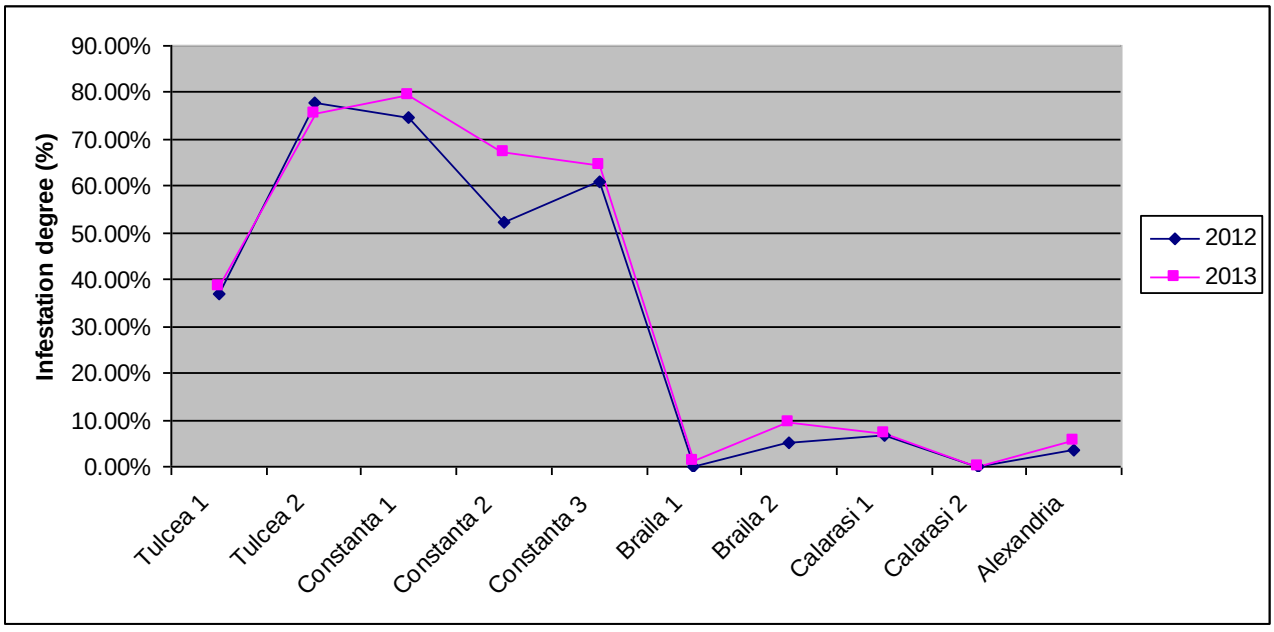

Fig. 2. The infestation degree with the parasite broomrape, in 10 locations in two years, in Romania

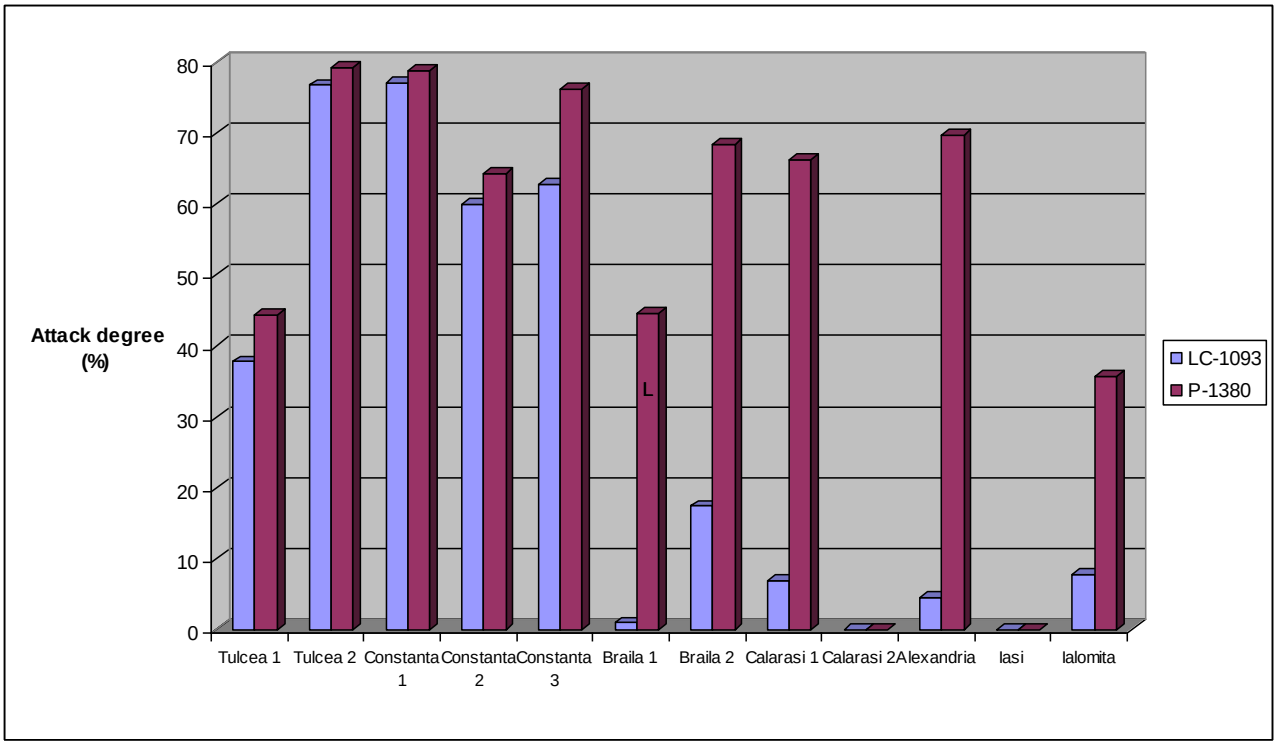

Fig. 3. The broomrape attack degree on the differentials for the races E and F, in 12 locations, in Romania

Testing some hybrids high resistant to the new populations of broomrape, we found that in area were these populations are present in high percent, some of these hybrids have 2-5% attack, some others 22-25% (Fig. 4). In areas were the new races of the parasite came in the last two years, the attack percent is lower. It must to be mentioned that these sunflower hybrids were full resistant when they have been introduced in the commercial market in these areas, but in short time they started to lose their resistance.

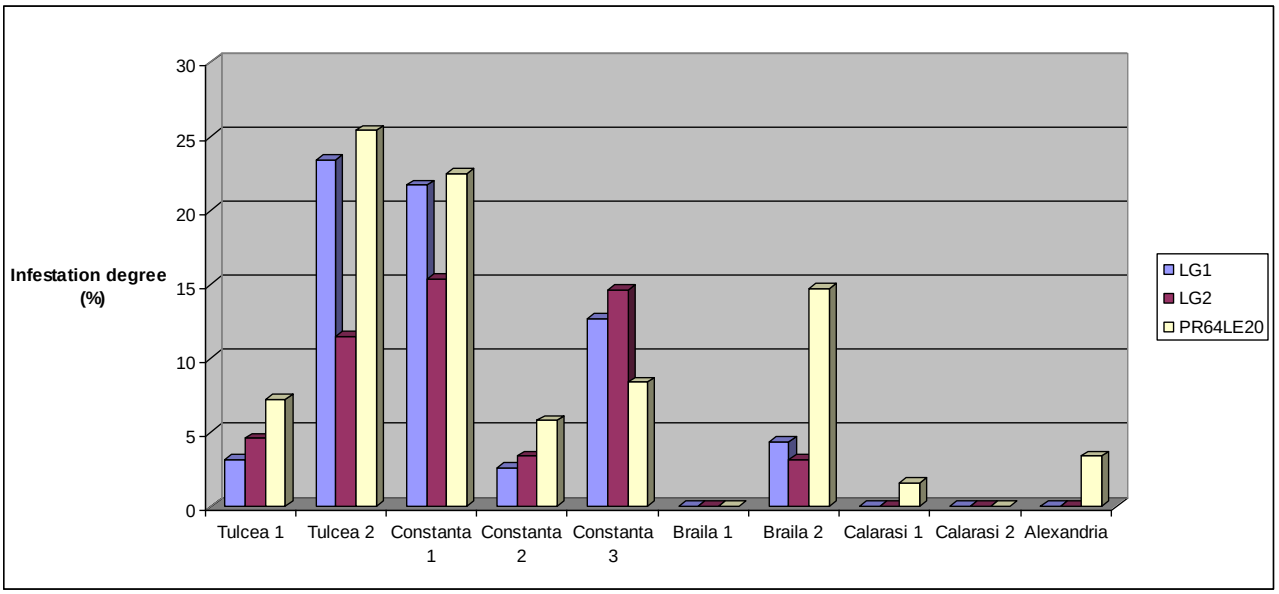

Fig. 4. The broomrape attack degree on some resistant hybrids, in ten locations, in Romania.

CONCLUSIONS

The parasite *Orobanche cumana* Wallr. has become more and more dangerous for sunflower crop in Romania. More than half of area cultivated with sunflower is infested with broomrape. The new populations of this parasite, very virulent, which are spread in areas situated near Black See are changing their virulence in short time, the new sunflower hybrids which are resistant at the beginning of their cultivation in this area, losing quickly their resistance.

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Current situation of sunflower broomrape in the Republic of Moldova

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ABSTRACT

The first document that attests the cultivation of sunflower in the Republic of Moldova (RM) dates back to 1845, while the occurrence of broomrape was first mentioned in 1937. Nowadays, all races of *Orobanche* known in the world are present on the territory of RM and the areas affected by this pest have extended considerably. Starting with 1945 research activities have been carried out in genetics, breeding and improvement of sunflower cultivation technologies, with a special emphasis on resistance to diseases and pests including the broomrape, which can considerably diminish crop productivity. This review summarizes the research progress on sunflower broomrape in the Republic of Moldova providing an overview of the current status, of the potential economic impact, with a perspective for future research strategies to further develop our understanding of the parasite-host interaction.

Key words: broomrape – race – resistance – screening – sunflower– virulence - yield

INTRODUCTION

Helianthus annuus L. is one of the basic oilseed crops in the Republic of Moldova with a wide range of food and industrial uses and with a major economic significance. Sunflower has consistent planting areas and is the third most produced crop after corn and wheat. According to the Food and Agriculture Organization of the United Nations (FAO), in 2012 RM was ranked the 19th biggest producer of sunflower seeds, contributing to 0.8% of the world production with about 296000 tons. However, the production yield of many crops including sunflower, in the local agroecosystems, suffers considerable decrease due to the negative impact of a series of abiotic and biotic factors.

One of the limiting factors for the production of sunflower is the broomrape (*Orobanche cumana* Wallr). This parasitic plant has spread especially in the central and south-eastern regions of the country. Actually, a considerable expansion has been noticed also in the north region with increasingly frequent cases of occurrence during the last 5 years in the districts of Soroca, Drochia, Riscani and Balti. The damage caused by the pathogen is often devastating with reported losses up to 50-90%, leading to a significant reduction in the amount and quality of the oil (Siminel, 1998).

In the last decades, considerable efforts have been invested in order to study the problem and to identify solutions for parasite combating, including:

Investigation of morpho-physiological, biochemical and genetic aspects of the host-parasite interaction *(Moldova State University; University of the Academy of Sciences of Moldova; The Institute of Genetics, Physiology and Plant Protection of the ASM);*

Development of chemical and agro-technical methods to fight the pathogen *(Research Institute of Field Crops "Selectia"; Agrarian State University of Moldova; Moldova State University);*

Evaluation of genetic resistance in artificial and natural conditions *(University of the Academy of Sciences of Moldova; State University of Moldova; Research Institute of Field Crops "Selectia"; AMG – Agroselect, Moldova);*

Obtaining of hybrids resistant to a multitude of *Orobanche* varieties (*Research Institute of Field Crops "Selectia"; AMG – Magroselect; AMG –Agroselect*);

Monitoring of the broomrape impact on the production indices *(Research Institute of Field Crops "Selectia"; University of the Academy of Sciences of Moldova)*.

This paper, is an attempt to review the most prominent studies on distribution, races, host-pathogen interaction, resistance phenomena and impact of sunflower broomrape in Moldova.

HISTORICAL OVERVIEW OF SUNFLOWER CULTIVATION

The first document that attests the cultivation of sunflower on the territory of Moldova dates back to 1845 and the first evidence of oil production from sunflower seeds – to 1867. Since then, the area of sunflower fields have expanded exponentially from 287 ha in 1909 to approximately 11100 ha in 1913 (Lashkov, 1912). In the following years, the dynamics of the planted area as well as the harvested amount, have been constantly rising with occasional drops determined by environmental and cultivation conditions.

Already, starting with the '50s research and cultivation strategies for increasing the overall sunflower production and yield have been planned and implemented (Gordienco, 1959). The use of novel technologies and the substitution of the common varieties with high-yield hybrids during the 1976-1990, correlated with the extension of the cultivated surfaces and have led to a steady increase seed production (Moraru, 1999).

Starting with 1991 the total cultivated surface continued to grow, while the productivity was in a stable decrease (fig. 1). As a result between 1990 and 2000 the surfaces covered with sunflower reached the value of 170 - 200 thousand ha, with an average yield of 1.2 t/ha and a total production of 227 thousand tons. In the 2010s, according to FAO statistics, the planted area increased to about 263 thousand ha, while the average productivity stayed constant (320 thousand tons of total production), which means that by the end of this period the same quantity of seeds as in the '90s was obtained by cultivating an additional surface of 77 thousand hectares (Moraru, 1999; Vronschih et al., 1975; Vronschih et al., 2002).

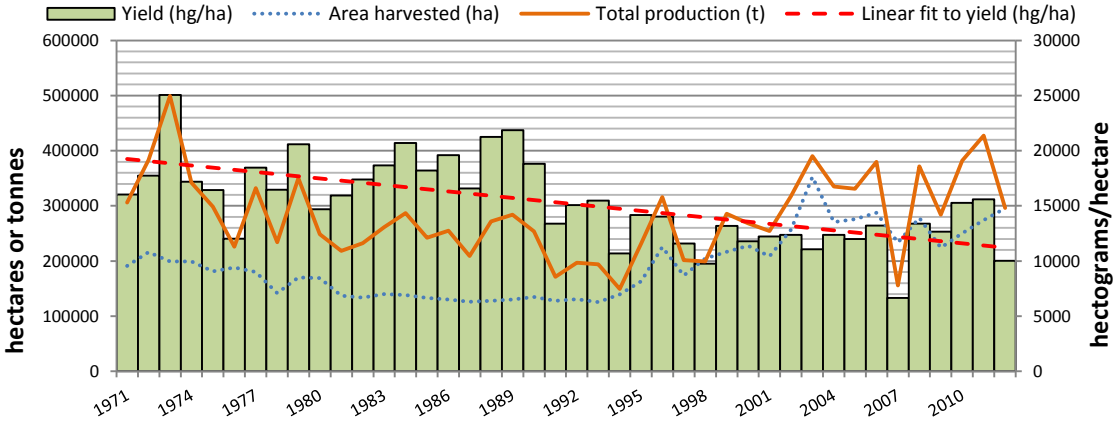

Fig. 1. Total sunflower harvested area, production and yield in Moldova during 1971-2012 (Food and Agriculture Organization of the United Nations).

In these conditions, following correct crop rotation procedures fails and sunflower crops are re-cultured on the same areas after 3 - 4 years. Providing this fact as well as the vulnerability of sunflower to various pathogens, the necessity for obtaining and cultivation of resistant hybrids as well as for strictly respect the cultivation rules becomes evident.

ASSESSMENT OF SUNFLOWER POTENTIAL FOR RESISTANCE TO BROOMRAPE

The knowledge of the mechanisms of resistance to the parasite and of the host-parasite interaction is important to develop strategies for broomrape control.

During the last years, a special attention was focused to triggering chemical signals exuded by the host (Glijin et al., 2011a) and to the host-parasite interaction at various stages of development (Duca et al., 2013a), at different temperatures (28°C and 15°C) (Rotarenco, 2010) and in natural environment (Glijin et al., 2009).

Several studies have been aimed at elucidating the sunflower phenotypic and biochemical modifications (Rotarenco, 2010; Glijin et al., 2011b; Glijin, 2012a; Duca et al., 2012) induced by *O. cumana*. Broomrape infestation in natural environment showed direct consequences on the yield parameters and caused a large reduction in the total achene seed weight per head (Glijin, 2014), the most affected being the weight of 1000-grains (20.1%) and the mass of 1000 kernels (20.7%) (Duca and Glijin, 2014). Also, *O. cumana* infestation had significant effect on protein (Glijin, 2012a) and fat content (Duca et. al., 2012).

Another topic of interest was the evaluation of germplasm in order to select the resistant genotypes for breeding purposes. Sunflower screening (frequency, intensity and attacking rate) of susceptibility to broomrape has been realized in controlled (Rotarenco, 2010; Duca et al., 2008; Duca, 2011a) and natural conditions (Popescu, 2007; Glijin, 2012b). According to the obtained data, all sunflower genotypes (more than 2000) were classified in four groups: resistant (2 - 4% infestation degree), medium-resistant (5 - 20%), susceptible (20 - 40%) and fully susceptible (more than 50%) (Duca et al., 2011b; Duca et al., 2013c; Glijin, 2012b).

Additionally to phenotypic screening, a RAPD (Duca et al., 2013b; Duca et al., 2014) and SCAR analysis (Duca et al., 2009a; Duca et al., 2010a; Duca et al., 2010b; Rotarenco, 2010) were performed. The RAPD profiles showed a genetic polymorphism between susceptible and resistant sunflower genotypes (unpublished data) and presence of the *RTS05* locus (the *Or 5* gene conferring resistance to race E of *O. cumana*) in most of the samples (88.7%) (unpublished data). No correlation was detected between the presence of this genetic marker in the genome and phenotypic resistance to broomrape in most of the analyzed sunflower genotypes (78%) highlighting that the infection was not due to race E, but rather to more aggressive races (Rotarenco, 2010; Duca et al., 2010a,b).

Recently, there have been described some aspects of the multiple defense strategies including the constitutive (Rotarenco, 2010) and inducible defense systems for combating broomrape (Glijin et al., 2011b). In a complementary approach, bioinformatics investigations were performed (Duca et al., 2009b; Duca, 2013).

During the last decades, many efforts have been done in the creation of highly productive hybrids with complex resistance to different pathogens, including sunflower broomrape at the Research Institute of Field Crops "Selectia", AMG – Magroselect; AMG – Agroselect (Buciuceanu, 1988; Buciuceanu et al., 1994a; Vronschih and Lesnic, 2013). Sunflower breeders have used a wide range of cultivated sunflower varieties to search for valuable agronomic and seed quality traits as well as for resistance to insects and diseases (Buciuceanu et al., 1987; Buciuceanu et al., 1994b; Petcovici, 2008; Petcovici and Lungu, 2008; Buciuceanu et al., 2009; Petcovici and Lungu, 2009). Variability of morphological and physiological traits (plant height, flowering period, leaf and achene characteristics, etc.) were used as indicators for sunflower breeding approaches.

CURRENT DISTRIBUTION AND RACIAL STATUS

The first documentary data mentioning the broomrape on Moldovan fields was published in the Annual Report of Agricultural Chamber of Balti in January 31, 1940, based on the evidence of Afanasev and Arhanghelschi (1937). According to their data, a large area of the left side of Nistru River was assigned to the low and moderately infected fields, while the right side of the territory was considered a heavily infected area. Due to the high infection level of resistant sunflower varieties (race A), the authors concluded the presence of a new race (race B) (cited by Sharova, 1977). In order to extend the sunflower cultivated area and to increase the harvest it was recommended to employ on the entire territory of the republic the Jdanovsk 82-81 varietal type and to cultivate this crop on the same ground no more frequently than once in six years (cited by Duca et al., 2011c).

Over many years, researchers at the Moldavian VNIIMK Station investigated broomrape populations from different regions (Edinet, Balti, Chisinau, Slobozia, Causeni, Vulcanesti), establishing a great diversity of physiological races and describing their peculiarities including the attack degree and distribution (Sharova, 1977).

At the beginning of 1970s, another broomrape biotype which began to infect genotypes earlier considered resistant and has rapidly spread over all sunflower growing regions was identified in Moldova (Antonova et al., 2013). This new race that could not be controlled by the genes for resistance to races A and B was called the Moldovan race (Sharova, 1969), or race C (Burlov, 1976).

For the identification of current races belonging to different geographical populations of *O. cumana*, a standard differential set for broomrape races D, E, F and G was used, provided by NARDI Fundulea, Romania. Artificial infection of differentials in pots allowed identification of the races collected from RM

(Balti, Ciadir-Lunga, Stefan-Voda, Soroca, Anenii Noi) and Romania (Tulcea) (Gisca, 2013; Glijin et al., 2014). The authors found a high genetic variability and a differentiation by origin and race of aggression of the analyzed broomrape populations (Gisca, 2013). It was revealed that broomrape from Balti infected the differential for race E, but did not infect the differential for race F. Broomrape from the south part of RM infected differentials that are resistant to race F. This data suggests that *O. cumana* from Stefan-Voda and Ciadir-Lunga belong to race G or more aggressive races, which is in line with other findings (Pacureanu-Joita et al., 2012).

More recently, RAPD analysis and UPGMA clusterization were performed in the University of the Academy of Sciences using *Orobanche cumana* collected from different geographical regions: ten populations from RM, one from Romania (Fundulea) and one from Ukraine (Izmail). A low intra-population and a high inter-population variability were detected. At the morphological level, broomrape seeds from Fundulea (Romania), Taraclia (south part of RM) and Donduseni (north part of RM) significantly differ from other populations.

Based on molecular and phenotypic screening broomrape populations collected from Donduseni, Soroca and Balti belong to race F and the populations from Chisinau, Singera, Rezeni supposedly belong to race ≥ F. Taraclia, Izmail, Fundulea, Cimislia, Stefan Voda and Ciadir-Lunga populations can be attributed to race ≥ G (fig.2) (Glijin et al., 2014).

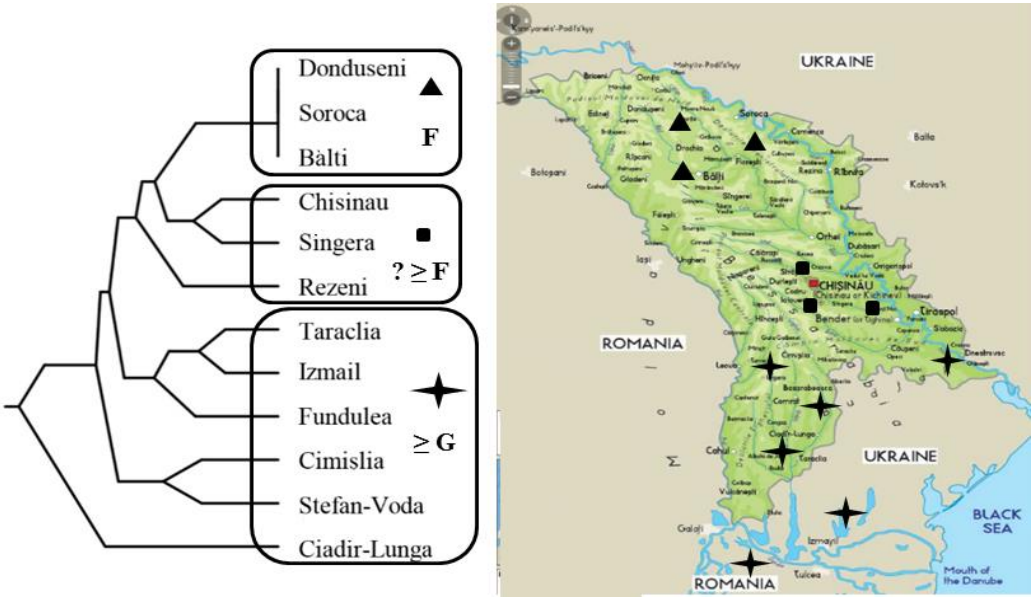

Fig. 2. Dendrogram (UPGMA) derived from RAPD analysis of different geographical populations of broomrape and their racial distribution

The identification of broomrape races should be an ongoing process because, like any obligate parasite, *Orobanche* spp. will develop new races, more virulent, more aggressive and more damaging to sunflower.

ECONOMIC IMPACT

The parasitic plant *Orobanche cumana* is spread across all regions of RM with deriving economic, social and environmental impact. An estimation of the economic cost of sunflower productivity losses is needed for appropriate future prevention and response strategies. Furthermore, avoidance of pests' spreading requires adequate monitoring both by the regulatory authorities and individual farmers or farming enterprises.

Researches carried out for many years *(Moldovan VNIIMK Station, Research Institute of Field Crops "Selectia", Moldova State University, and University of the Academy of Sciences of Moldova)* revealed that the broomrape races are not only widespread, but also very aggressive. There is evidence that this parasitic plant can cause up to 100% yield losses in infected crops.

In the first half of the '60s monitoring of broomrape epidemics was attempted. Thus, in 1963-1964 sunflower crops in the districts of Causeni and Dubasari were affected to an extent of about 70-100% and

in 1972 half of the sunflower crops in Slobozia were affected by broomrape that had reached up to 100% infection rate (Sharova, 1977).

Field experiments demonstrated a major impact of crop rotation on productivity and on the process of appearance of new broomrape races and their rapid spread (Sharova, 1977).

Recently, the influence of natural infection with *O. cumana* on different productivity parameters (Duca and Glijin, 2014) and quality of seeds (Glijin, 2012a; Duca et. al., 2012) was described.

Some estimates of the risk of fabulous losses and economic impact of the parasite have been done in the frames of a project aimed to promote the direct use of Research and Development results by farmers (Glijin et al., 2007).

During the past decades, there have been a limited number of studies attempting to quantify the economic losses caused by the spread of plant pests and to assert the urgent need for appropriate public policies.

CONCLUSIONS AND FUTURE PROSPECTS

The history and current status of sunflower broomrape research in Republic of Moldova, including the morphological, physiological, genetic and molecular aspects of host-pathogen interaction, disease management and economic impact are reviewed in this paper. Nowadays a serious constraint for sunflower productivity is, to a great extent, the failure to respect crop-rotation along with the increase of cultivated surfaces, the low quality of agricultural procedures and protection measures against pests, diseases and weeds.

One of the future challenges is to fortify the collaboration between scientific centers and private companies to exploit new knowledge in order to develop more resistant sunflower varieties or alternative broomrape control strategies. Also, better advances in identification of the current broomrape racial regional distribution and status could be obtained by the international cooperation related to the use of race collections, common differential sets and molecular approaches.

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Current situation of sunflower broomrape in Bulgaria

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ABSTRACT

Plant resistance to diseases and parasites is a very serious problem. It is based on the complex relationship between the host plant and the pathogen. In the process of evolution of plant organisms form a sophisticated protection and restoration systems against various pathogens. Their study is important for improving resistance to diseases and pests and introduction of high quality and complex varieties resistant crops. Parasitic plants of the genera *Orobanche* and *Striga* attack more than 16 million hectares of crops in the Mediterranean and Africa (Rottevelel and Gressel, 1998). There are few cases where researchers have to solve such a complex problem like this with a broomrape. In this regard for many years extensive research was conducted in order to clarify the mechanisms of interaction in the host- parasite system, to develop effective methods for broomrape control, including the development of resistant crop varieties of crops to the parasite. Here the history, perspective, current distribution, racial status and economic impact of broomrape *(Orobanche cumana* Wallr.) on sunflower in Bulgaria is described.

Key words: Broomrape – distribution – races – resistance – sunflower

The Republic of Bulgaria has different types of agro ecological zones. As a result a range of crop species can be grown and the agriculture is a vital part of country's economy. The economic impact of parasitic weeds is tremendous and parasitism by the root parasites often results in significant yield damage.
In Bulgaria 25 species of *Orobanche* are represented. In our country the most important crops attacked by *Orobanche* and *Phelipanche* are sunflower, tobacco and tomato. Sunflower is affected severely by *O. cumana* in Bulgaria. The last year this crop occupies about 800,000 ha (15% arable land in Bulgaria) and it is a major crop for oil production and seeds export.

O. cumana on sunflower was first reported in 1935 (Dobrev, 1945) in the North Eastern part of Bulgaria. It has been suggested by some experts that the parasite was delivered in the country by the import of sunflower seeds from abroad since 1930. In 1945 broomrape got a huge spread and practically in many areas there was not yielding sunflower. The Soviet variety № 643, grown at this time was attacked by *O. cumana* 56.5% and yielded 9.2 kg/ha, and the Bulgarian variety № 3-18 having no resistance to broomrape was attacked 100% and the yield was 0 (Popov et al., 1966). The main reason for this is continuous and strongly increasing the accumulation of *O. cumana* seeds in soil, were sunflower was grown.

Two races of *O. cumana* were described by Knyazkov (1950) - A and B. During the period 1954-1956 in our country the breeder Dr. Petrov selected two varieties Tolbuhin 71 and Tolbuhin 75 that are resistant to broomrape races A and B (Mitov et al., 1972). Later, after 1963 the Russian variety Peredovik (resistant to races A and B) occupies over 90% of the area of sunflower for more than 15 years. Sunflower hybrids NS-26, NS-27 and NS-62 (Yugoslavian) were also introduced in Bulgaria. They were resistant also only to races A and B.

In 1966 it was found that cv. Peredovik which is resistant to races A and B become susceptible and a new broomrape race C appeared (Petrov, 1968). In Dobrudja region the variety Peredovik over two years was attacked at very high level and the yield of highly infested fields was between 10 and 15 kg/ha. Broomrape impact on the yield was significant and requires breeding of new sunflower varieties with resistance to race C of the parasite. Race C was distributed very fast in sunflower fields and in some of them about 80-85% of plants with 60 to 200 broomrapes per 1 m^2 were observed and up to 500 000 seeds from one parasitic plant were counted (Batchvarova, 1978).

In 90's a breeding program for resistance to broomrape races A, B and C in Dobrudja Agricultural Institute started and first Bulgarian hybrids (Super Start, Albena and Dobrich) were introduced into practice. In addition to their resistance to races A, B, and C they were also very high yielding (Encheva and Shindrova, 1994).

After 1990 a massive attack by *O. cumana* on resistant sunflower hybrids and varieties was observed. The reason is due to appearance of new physiological races of the parasite- D and E (Shindrova, 1994).

The breeders overcome the problem with the new races of *O. cumana* by breeding lines N 208 and N 210, resistant to races D and E. From them a new variety Vega was selected (Encheva and Shindrova 1994).

Shindrova and collaborators (Shindrova et al., 1998) published the investigation on the effect of broomrape on some morphological and biochemical indices of sunflower. The results concerning the effect of broomrape infestation was a decrease on plant high from 20 to 50 cm, heads diameter from 9 to 15 cm and the yield per head – from 56 to 105 g.

The effect on fatty acid composition of oil at different rates of attack by broomrape is increasing the linoleic, stearic and palmitic acids and slightly decreasing the oleic acid in kernels (Shindrova et al., 1998).

In 2003 grown resistant cultivars of sunflower again were infested with *Orobanche cumana* and sunflower fields are moderately and severely attacked. Two new races of the parasite - E and F were identified and with highest distribution in all sunflower fields until 2004 was race E (Shindrova, 2006).

Besides the appearance of new races the areas of sunflower fields (from 250 000 ha in 1990 up to 650 000 ha in 2011) and distribution of broomrape were increased. The race G was also identified in 2006-2007 in the sunflower fields. In the beginning it occurred in limited areas but later was isolated from 42.9 % of the collected samples (Shindrova and Penchev, 2012).

In 2007 in the North- Eastern part of Bulgaria, near the boundary with Romania one broomrape sample in one sunflower field was identified for the first and fortunately for the last time as race H of *Orobanche cumana*. The differential set of sunflower genotypes was used for race identification of *Orobanche cumana* (D, E, F, G and H) (Shindrova and Penchev, 2012).

Nowadays the most spread races of the broomrape in sunflower fields in Bulgaria are E (35%), F (16%) and G (80%).

Conducted RAPD analysis of different backgrounds and races of *O. cumana* from Bulgaria and abroad showed the presence of polymorphic DNA regions in this species. By comparing the profiles of race F from Bulgaria, Romania and Spain by RAPD analyses with primer Ubc 215 a polymorphic fragment with size 950 bp was present in the race F from Bulgaria and Romania but is lacking in race F from Spain (Atanassova et al., 2005, Batchvarova, 2004). The additional molecular analysis of *O. cumana* using ISSR markers on population originating from the Balkan region and Russia that have been performed, showed low levels of intraspecies variability. Similar results were obtained through studying the variability of the internal transcribed spacers region of the nuclear ribosomal DNA (ITS) and ribulose-bisphosphate carboxylase pseudogene (RbcL). While small differences between the sequences of *O. cumana* populations were present, a complete homology among the different origins of *O. cumana* have been observed.

To create a resistant to broomrape sunflower forms in Bulgaria the following methods are used: interspecific, intergeneric hybridization and mutagenesis.

Christov and contributors in DAI, Gen. Toshevo obtained by interspecific hybridization sunflower lines with complete resistance to broomrape and mildew by using wild diploid species of *Helianthus* (Christov et al., 1998,Valkova, 2009, Georgiev et al., 2012) . In creating sunflower forms, resistant to broomrape race G, 16 wild *Helianthus* species were used. Complete resistance to the parasite was established with new lines as 7019 R, 7203 R, C23/1, C 41, C 46, C 48 etc. with 5 wild species: *H. pauciflorus, H. tuberosus, H. divaricatus, H. hirsutus and H. bolanderi* (Christov, 2012). Christov (2013) also succeeded to obtain resistant sunflower forms to broomrape by intergeneric hybridization with *Inula helenium, Tithonia rotundifolia, Tithonia speioza, Aster speciosa* and *Verbesina helianthoides*. The same author, by the method of physical mutagenesis obtained sunflower forms resistant to broomrape, which are included in the breeding program of the Institute (Christov, 1996; Christov and Nikolova, 1996).

Venkov and Shindrova (1998) also used the method of chemical mutagenesis, and after treatment with nitrosomethylurea (NMU) obtained sunflower mutants with increased resistance to broomrape, reaching 44.2%. The story of the sunflower shows that the best way to limit losses from parasite is introduction of resistant sunflower varieties in the fields.

Ultrasonic treatment of immature zigotic embryos of sunflower also allows developing a resistance to broomrape. Two mutant restorer lines were obtained with complete resistance to *O. cumana* and stable inheritance in the next generations (Encheva et al.).

In Bulgaria as a biological method to control broomrape by *Phytomyza orobanchia* have been tested by Trentchev (1981). The application of the process has a little effect due to the high propagation coefficient of broomrape seeds.

Bedi (1994), Iliev and colaborators (1998) and Muller-Stoever (Muller-Stoever et al., 2009) obtained very good results to control broomrape in Bulgaria, by treatment of soil in sunflower fields with *Fusarium oxysporum* (Schlecht.) Snyd. et Haus. f. sp. *orthoceras* (App. and Wr.) Bilai.

For sunflower, by using Clearfield technology it is possible to make a variety naturally resistant to a weed-killer herbicide (Pulsar 40- imazamox) that controls most weeds affecting this species, but also its main parasite (broomrape) at its younger stages. This technology has been used successfully for four years in Eastern Europe and also in Bulgaria. A precursor in this technology, LG today holds second position for Clearfield sunflower in Hungary, Romania and Bulgaria. LG introduce its new Clearfield hybrids as Rimisol (resistant to *Plasmopara helianthi* and tolerant to *Phomopsis helianthi* and *Sclerotinia sclerotiorum*) and LG 5697 CLP.

During the last five years the breeders in DAI registered several new hybrids: Yana, Marica, San Luka, Veleka, Vokil, Valin and Alpin. They are high yielding and with complete resistance to mildew and all races of *O. cumana*.

CONCLUSIONS

The problem of *O. cumana* on sunflower is one which has been very largely brought under control in the past thanks to the development of highly resistant varieties. Resistance continues to offer the greatest hope to solve *Orobanche* problem in sunflower fields.

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The situation of broomrape infestation, control methods in sunflower production areas in Turkey

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ABSTRACT

Broomrape (*Orobanche cumana* Wallr.) which is a dangerous parasite is the most limiting factor for sunflower production in Turkey and also some other countries such as Spain, Bulgaria, Russia, etc. This serious parasite developed itself historically against sunflower resistant genotypes and new aggressive races were appeared recently other than 5 known races (A, B, C, D and E). In the last 20 years, first new F race infestation was observed in Trakya region which is European part of Turkey and covers more than 50% of sunflower areas in Turkey. Since last ten years, new races have infested almost all part of Trakya region. Furthermore, new races spread to other important sunflower production areas recently such as Cukurova, Middle Anatolia and Black Sea regions which were immune areas 4-5 years ago. However, there was no any race determination study in Turkey so it could not be concluded that which new races such as G or H existed other than F. Since resistant hybrids which were in the markets exhibited different degree of susceptibility (0-20% frequencies) based on region as well as different rates based on years too. Therefore, it could not be said as resistant about these hybrids, they could be classified only as tolerant ones. On the other hand, seed companies develop new tolerant hybrids every years mostly combining or adding new traits to broomrape tolerance such as downy mildew resistance as well as IMI herbicide resistance together because Clearfield system is one of the best and efficient option to control both broomrape and major broadleaf weeds. On the hand, Sulfonyl Urea herbicide resistance is not preferable trait due to the less control of both broomrape and common weeds in Turkey.

Key words: Sunflower – broomrape – races – resistance – control

Current situation of sunflower broomrape in Ukraine

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ABSTRACT

Ukraine is one of the largest producers of sunflower in the world and largest world exporter of sunflower oil. Estimated 70% of total sunflower surface in Ukraine is affected by drought and also this area is infested of different races of broomrape (*Orobanche cumana* Wallr). Large increase in the area of sunflower cultivation during the last years (from 1.6 million ha in 1990 to 5.8 million ha in 2014) and short rotation has provoked the appearance and quick spread of new virulent races of the parasite. So, sunflower broomrape is the most important problem in the sunflower crop in the South and South-East part of Ukraine (Black See area). The hot and dry conditions and strongly saturated sunflower in the rotation has facilitated the spread of races E, F, and G. So, for better understanding the evolution of broomrape, Limagrain Ukraine began its own project for farm monitoring in areas infested with *Orobanche* and also pay attention on new potential risked areas. For this monitoring, the company Limagrain Ukraine is using the next tools: questionnaire - interviews by sales managers in each broomrape affected region, feedback from farmers, inspection of the fields, and seeds sampling for laboratory test. The study showed that the most spread races in Ukraine are race E (around 70% of sunflower surface) and race G (around 29% of sunflower surface). Also, some spots on sunflower growing areas in Donetsk, Odessa, Zaporozhe and Lugansk regions are infested with more dangerous race.

The history of interconnected evolution of *Orobanche cumana* Wallr. and sunflower in the Russian Federation and Kazakhstan

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ABSTRACT

Currently, *O. cumana* in the Russian Federation overcomes the influence of dominant genes of resistance in sunflower *Or4, Or5, Or6, Or7,* already known in European countries, and the combined impact of the two recessive genes *or6or7*. The most virulent biotypes of parasite G and H are found in many regions of sunflower cultivation: the Rostov, Voronezh, Volgograd, Saratov, Orenburg, Stavropol and Krasnodar regions. The situation is especially unfavorable in the Rostov region where the race G became predominant in many populations of *O. cumana*. In the Krasnodar region broomrape began to spread in recent years (after a long absence) mainly in the northern regions (and adjacent), bordering with the Rostov region. Here the populations of *O. cumana* are a mixture of races of different virulence. The races D and E still often dominate. However, there are already the parasite populations, where the race G is predominant and somewhere biotype H is already present. In a sample of seeds from Kazakhstan is dominating the low virulent race C, among which there is a small admixture of biotype G. This combination of low virulent race with a small amount of highly virulent specimens of biotype G indicates the natural origin of the latter, regardless of the influence of the breeding process of sunflower. Some deviations from the basic model of development of *O. cumana* in ontogenesis that contribute to increase and acceleration of seed preproduction of parasite's specimens are described.

Key words: Broomrape – *Orobanche cumana* – populations – virulence – races – sunflower

The history of *Orobanche cumana* Wallr. parasitism on sunflower in Russia is about 200 years old. In the late 17th century, sunflower in Russia could only be found in the homestead gardens. Its sowing as a field crop began in the first half of the 19th century, first in Saratov and Voronezh provinces. And here it encountered *O. cumana* that parasitized on sea and Austrian wormwoods (*Artemisia maritima incana* Schm. and *A. austriaca* Jacq.) (Beilin, 1947). Sunflower proved to be a more appropriate host for broomrape than wormwood. From here began its spreading to the new areas of sunflower sowing (Sukachyov, 1900). The first report on mass infestation of sunflower with broomrape in Russia appeared in Voronezh in 1866 (A. Oldamov, Voronezh provincial gazette, 62).

By the late 19th and early 20th centuries, the spreading zone of *O. cumana* expanded so much that this parasite has become a serious threat to sunflower crops (Karzin, 1898; Shreiner, 1894, 1904; Maltsev, 1913; Plachek, 1913). Already then Russian breeder A.I Stebut wrote: "The sunflower crops were even abandoned in some areas, as there was no sure way of broomrape control" (Stebut, 1913). By 1920, the efforts of breeders at the Saratov experimental station helped to create varieties resistant to broomrape in this area. However, when these varieties were sown for trials on the fields of the Don experimental station in the Rostov region, they were highly affected by the broomrape prevalent in that region. Hence appeared the terminology: "good" broomrape is in the Saratov region and "evil" broomrape is in the Rostov region. They were called races A and B according to the alphabet. They differed greatly in areas of spreading. Race A was more common in the Saratov and Voronezh regions and race B – in the Rostov and Krasnodar regions, Moldova and in the south of Ukraine (Zhdanov, 1930; Scherbina, 1931; Pustovoit, 1937).

Later, it was discovered that race B is heterogeneous in its composition, so it was called a complex of races B. The immunity to race A consisted in formation of thickening of root tissues around the

broomrape seedling that penetrated into it and died there. Sunflower immunity to the complex of races B did not manifest itself outwardly.

In the mid-20th century *O. cumana* became a mass phenomenon in all areas of Minor and Central Asia, Ukraine, Moldova, on the Caucasus, the Volga region, and in some areas of the Western and Eastern Siberia (Beilin, 1968).

Over the course of history of sunflower cultivation in Soviet Russia (the Soviet Union) there were three periods when severe infestation of crops with broomrape has put the culture under threat of extinction. The interconnected evolution of parasite and host led to the emerging of new races of parasite that were able to overcome the immunity of resistant varieties and hybrids. The last epiphytotic situation developed in the USSR in the early 70's of the 20th century, when the broomrape biotype that first appeared in Moldova and was named the Moldovan race (or race C) began affecting previously resistant varieties and spread rapidly in all regions of sunflower cultivation, especially in the North Caucasus. Successful breeding of new sunflower varieties resistant to the Moldovan race during the next dozen of years helped to solve the problem. The immunity of resistant assortment to the Moldovan broomrape biotype was expressed in lignification of vascular walls of root's xylem, isolating them from the haustorial cells of parasite.

The isolation from water-mineral substances led to the destruction of haustorial cells (Antonova, 1978). Since the late 1970s, the widespread cultivation of resistant varieties with this type of immunity in the Soviet Union caused germination of broomrape seeds and gradually led to the elimination of their main resources in soil (Pustovoit et al., 1983).

Approximately until the late 90's there were no problems with broomrape on sunflower in Russia. It was difficult even to find broomrape somewhere to collect the seeds for testing the resistance of breeding material. However, during the last 20 years the country experiences the increased intensification of cultivation of sunflower as a high-yielding crop: the expansion of planting acreage and non-observance of scientifically based crop rotation. Along with attraction to the country in the 90's of the sunflower hybrids of foreign breeding as a seed grain, which were susceptible to the local broomrape, it contributed to replenishment of resources of parasite seed in soil and accelerated the race formation. Currently, *O. cumana* that has spread in the southern regions of the Russian Federation affects all domestic and foreign assortment of sunflower.

The question of necessity of the race identification of *O. cumana* on sunflower in Russia and establishing of unified international nomenclature of the races of this parasite is essential.

Since 2006, the workers of the laboratory of immunity and electrophoresis of VNIIMK are systematically carrying out the identification of racial structure of *O. cumana* populations in the southern regions of the Russian Federation: Rostov, Volgograd, Stavropol, and Krasnodar regions. For this purpose the known differentiators of resistance to the races D, E, F created in Romania (LC 1002, LC 1003, LC 1093) are used as well as the line P 96 resistant to the race F in Spain and the line IR7 of VNIIMK breeding possessing the immunity to the races A-G. Sunflower variety VNIIMK 8883, that have never been bred for resistance to broomrape, is used as a control variant.

The accumulated data shows that at the beginning of research most populations of parasite had the complete range of biotypes: D, E, F, and G with a predominance of less virulent D and E. But by now, the highly virulent race G with an admixture of the even more virulent biotype H is beginning to dominate in many populations. Thus, currently *O. cumana* in Russia overcomes the influence of dominant genes of resistance in sunflower *OR4, Or5, Or6, Or7,* already known in European countries, and the combined impact of the two recessive genes *or6or7*. The problem of finding new resistance genes is especially acute.

The most frustrating soil condition in terms of infestation with seeds of the highly virulent biotypes of broomrape is observed in the most areas of the Rostov region (Table 1). Everywhere the land users here are returning the sunflower to the previous field after 1-3 years, therefore occurs rapid equalization of structure of broomrape populations toward the predominance of the most virulent biotype. Besides the prevalence of the race G in many populations, the most virulent today biotype H is frequently

encountered here. The situation in the Rostov region reached its pinnacle, in some places the broomrape infestation makes it impossible to receive yield. It became unprofitable economically to cultivate sunflower and land users are already trying to find the substitute for this crop.

Table 1. The degree of infestation* of resistance differentiators of sunflower with broomrape populations of the Rostov and Volgograd regions of the Russian Federation, 2013

Area	VNIIMK 8883 susceptible control	Resistance differentiator of sunflower:					The predominant races in population	The presence of other races in population
		LC 1002	LC 1003	LC 1093	P 96	VT 62		
		D *Or4*	E *Or5***	F *Or6*	F *or6or7*	G *Or7*		
The Rostov region								
Bokovsky	58	52	67	57	26	0	G	F
Morozovsky	98	73	55	22	16	11	F	D, E, G, H
Zernogradsky	80	61	39	44	50	5	G	D, F, H
Kagalnitsky	44	38	40	53	17	0	G	E, F, G
Milyutinsky	68	51	41	43	13	8	G	D, E, H
The Volgograd region								
Gorodishchensky	24	20	5	7	2	11	E	D, G, H
Elansky	78	72	46	25	6	0	F	D, E, G,
Novoanninsky	115	28	57	12	2	0	D	F, G
Surovikinsky	84	10	0	0	1	3	D	F, H

*The degree of infestation – the number of broomrape tubercles per one affected sunflower plant
**Text in italics represents the resistance genes to the specified race in sunflower differentiator

The situation is slightly better in the Volgograd region, where in some populations are often dominated by the less virulent races D and E (Table 1).

The situation is much better in the Krasnodar region. Here broomrape began to spread in recent years, mainly in the northern (and adjacent) areas, bordering with the Rostov region. It should be noted that according to the local laws, in the Krasnodar region sunflower cannot be return to its previous place earlier than after 5 years. The data in Table 2 show that currently in the Krasnodar region the broomrape populations are a mixture of races of different virulence. This testifies to the recent renewal of seeds resources of parasite in soil. Still, the races D or E quite often predominate. At the same time in all studied populations there is an admixture of one or several highly virulent biotypes that overcome the influence of resistance genes *Or4, Or5, Or6, Or7, or6or7* in sunflower. In some areas there are populations of parasite in which the race G is predominant and in some places the biotype H is already present.

Table 2. The degree of infestation* of resistance differentiators of sunflower with broomrape populations of the Krasnodar and Stavropol regions of the Russian Federation, 2013

Area	VNIIMK 8883 susceptible control	Resistance differentiator of sunflower:					The predominant races in population	The presence of other races in population
		LC 1002	LC 1003	LC 1093	P 96	VT 62		
		D *Or4*	E *Or5***	F *Or6*	F *or6or7*	G *Or7*		
The Krasnodar region								
Yeysky	94	78	33	5	0	0	E	D, F, G
Brykhovetsky	41	33	26	6	3 (88)***	4 (33)***	F	D, E, G
Krylovsky	59	56	40	41	20	8	G	E, H
Kushchyovsky	109	62	17	9	9	0	D, E	F, G
Pavlovsky	63	52	39	32	17	5	G	D, E, F, H
The Stavropol region								
Grachevsky	79	42	23	16	3	12	D	E F, G, H
Ipatovsky	92	52	25	31	6	0	D	E, F, G,
Petrovsky	111	86	30	15	2	0	E	D, F, G,
Trunovsky	26	19	25	20	20	4	G	F, H

*The degree of infestation – the number of broomrape tubercles per one affected sunflower plant
**Text in italics represents the resistance genes to the specified race in sunflower differentiator
***The percentage of affected plants is shown in brackets

The data shown in Table 2 indicate that in the Krasnodar region takes place a rapid spread of the highly virulent broomrape biotypes, resistance to which is absent in domestic assortment of sunflower. At the same time, land users, along with the intensification of crop cultivation, tend to grow foreign hybrids resistant to the races E, F, G. Since the seeds of all broomrape biotypes germinate in the presence of roots of resistant hybrids but their seedlings cannot grow in their roots, and because of it the non-virulent races are gradually eliminated from the population. Therefore, in the short term the further equalization of virulent structure of parasite populations toward the predominance of biotypes G and H should be expected. There is the acute problem of finding genes of resistance to these two races specifically.

The situation in the Stavropol region is developing in similar way. The biotypes F, G, H are already detected against the background of the prevalence of the low virulent races D and E. The race G dominates already in some populations (Table 2).

The data in Table 3 and Fig. 1 show how widely the race G spread in the Russian Federation. It already prevails in some populations of the Voronezh, Saratov and Orenburg regions.

At the same time, the sample of broomrape seeds, collected in 2012 in the Shemonaikhinsky district of Kazakhstan near Ust-Kamenogorsk, almost entirely consists of low virulent specimens, not able to overcome resistance gene of sunflower *Or 4*, i.e. of the race C. However, this sample of seeds already has a small percentage of specimens that overcome the influence of dominant resistance gene of sunflower *Or 6* (Table 3). It should be noted that the degree of infestation, equal to one, was observed in 100% of plants of used differentiator line having gene *Or 6*. This eliminates the randomness connected with the detection of a single nodule and indicates the presence of a small number of specimens in parasite population that are able to overcome the influence of gene *Or 6*.

Table 3. The degree of infestation* of resistance differentiators of sunflower with broomrape populations of the Voronezh, Saratov, Orenburg regions of the Russian Federation and the Shemonaikhinsky district of Kazakhstan, 2013

Area, city	VNIIMK 8883 susceptible control	Resistance differentiator of sunflower:					The predominant races in population	The presence of other races in population
		LC 1002	LC 1003	LC 1093	P 96	VT 62		
		D *Or4*	E *Or5***	F *Or6*	F *or6or7*	G *Or7*		
The Voronezh region								
Pavlovsk	73	36	60	43	12	5	G	E, F, H
The Saratov region								
Sovetsky district	58	21	22	42	3	3	G	E, F, H
The Orenburg region								
Alexandrovsky district	34	18	20	21	0	0	G	D
Kazakhstan								
Shemonaikhinsky district, Ust-Kamenogorsk	117	0	0	1***	0	0	**C**	G

*The degree of infestation – the number of broomrape tubercles per one affected sunflower plant
**Text in italics represents the resistance genes to the specified race in sunflower differentiator
***All 100% of plants of differentiator line are affected with degree 1

The presence of a small amount of biotype G among the predominant in a population of race C, non-virulent to the modern assortment of sunflower, may indicate that such instances of highly virulent broomrape have a genuine, natural origin, not related to the "pressure" of the breeding process of sunflower.

The genuine, natural occurrence of specimens with high virulence in low virulent populations of *O. cumana* on sunflower was previously observed in Spain (Molinero-Ruiz et al., 2008). The authors

concluded that the cause of it is not the creation of resistant sunflower hybrids and they are of natural origin. And it proves the necessity of continuous sunflower breeding for immunity to broomrape, refuting the opponents' opinion that breeding will not have time to produce resistant material if it is the reason of generation of highly virulent forms of the parasite. It is obvious that the cause of more rapid spreading of such forms is non-observance of crop rotation, intensification of sunflower cultivation as an economically highly profitable crop.

Fig. 1. The map of spreading of the highly virulent biotypes G and H of broomrape (*O. cumana*), parasitizing on sunflower on the territory of the Russian Federation and Kazakhstan: C, G, and H – names of biotypes (races) of the parasite.

It should be noted that during the Soviet period of the last century there was not broomrape on sunflower in Kazakhstan. The sunflower there was cultivated in three regions: East Kazakhstan (75 % of the total area planted with this crop in Kazakhstan), Semey (the former Semipalatinsk) and Pavlodar (Fig. 1), occupying a relatively small area of 100-200 thousand ha. Other areas of Kazakhstan are very droughty and are unsuitable for sunflower cultivation. Today, the Semey region is combined with the East Kazakhstan. At that time, the experimental breeding station of VNIIMK was in the East Kazakhstan region. Its scientific recommendations on crop cultivation with observance of eight-field crop rotation were strictly followed.

Currently, the planting acreage of sunflower in these regions of Kazakhstan increased considerably and is about 700-800 thousand ha. There also takes place the enhanced intensification of cultivation of this highly profitable crop. The broomrape appeared as a consequence of it. Therefore, it should be expected that in the next few years at least an already existing biotype G will reproduce and spread here.

The analysis of the virulent structure of *O. cumana* populations from different regions of the Russian Federation shows that everywhere the process of its changing develops in a similar manner: there is a

rapid equalization of virulence toward the predominance of the most virulent biotype. Today, it's the race G. We can assume that in the East Kazakhstan region under the conditions of immoderate cultivation of sunflower the situation will evolve in a similar manner, and in a few years this race will also become predominant there.

Our observations of highly virulent parasite populations in the Rostov region showed that here *O. cumana* has a high potential of reproductive function, wide possibilities of adaptation to the conditions of permanent destruction. We would like to draw attention to the fact that at the present stage in the ontogenesis of *O. cumana* there are frequent deviations from the model of development when 1 shoot, or rarely 2 shoots from one tubercle are dominating. The changes have occurred in the development of a tubercle. This structure has become multipolar, capable of setting multiple meristematic zones and development of adventive shoots from them. There is a tendency to the accelerated development of adventive shoots. The apexes of rudimentary roots of tubercle acquired the ability to grow directly into the stem, skipping the stages of penetration into the root adjacent to the parent tuber and the formation of a new tubercle followed by setting in it an apex of additional shoot (Antonova et al., 2012). This hastens a lot the formation of additional seeds from one specimen of parasite and is one of the proofs of its evolution towards increase of the potential of the reproductive function.

Currently, the surprising phenomenon is the ability of *O. cumana* to form flowers, their self-pollination and ripening of seeds in them on certain adventive shoots, hidden in soil due to their underdevelopment and inability to reach the surface (Antonova et al., 2012). The accelerated formation of seeds and increase of seed reproduction by any means are the characteristic features of highly virulent biotypes of *O. cumana* on sunflower in the Rostov region.

In our opinion, there is an acute problem of finding new genes of resistance to *O. cumana* in sunflower. Moreover, it is also necessary to study cause and effect relationship of immunity to highly virulent broomrape in wild species of sunflower, which have been identified by several authors (Ruso et al., 1996; Sukno et. al., 1998; Fernández-Martínez et al.,2000; Terzič et al., 2010; Christov, 2012; Antonova et al., 2011) as unaffected. This huge stratum of routine research work should be done in order to understand what exactly is brought from wild species into cultivated sunflower controlled by resistance genes to *O. cumana* and what, causing the immunity of roots, it loses in the process of breeding of productive and other agronomic characters. It is necessary to conduct a thorough comparative study of physiological-and-biochemical and anatomical-and-morphological characteristics of roots of different genotypes of wild and cultivated sunflower in order to identify acceptable for breeding factors of incompatibilities with the modern most virulent biotypes of *O. cumana*.

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Distribution and race composition of sunflower broomrape (*Orobanche cumana* Wallr.) in Northern China

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ABSTRACT

In order to identify the distribution and race composition of sunflower broomrape (*Orobanche cumana* Wallr.) seeds were collected from the main sunflower production regions in northern China during 2013. A total of 62 samples collected from different districts, including Inner Mongolia, Xinjiang autonomous region, Gansu Province, Jilin Province, Heilongjiang Province and Hebei Province were evaluated against a set of differential lines. A wide distribution of *O. cumana* races from race A to race G or higher was observed. Among all samples, only the samples from Wuyuan district in Inner Mongolia had a high percentage of the *O. cumana* biotype race G or higher.

Key words: Sunflower – broomrape – race composition – distribution – China

INTRODUCTION

Sunflower broomrape (*Orobanche cumana* Wallr.) is a parasitic angiosperm, totally devoid of chlorophyll, that infects the roots of sunflower (*Helianthus annuus* L.), drawing water and nutrients from them. This parasitic plant is regarded as one of the most important constraints for sunflower production in areas of eastern and southern Europe, the Middle East, Russia, Ukraine and China (Parker, 1994). There are eight known races of *O. cumana* parasitizing sunflower: A through H. In the early 1980s, Vrânceanu et al. (1980) identified five pathogenic races, A through E, with a set of sunflower differentials carrying the dominant resistance genes Or_1 through Or_5, each providing resistance to a new race and also to the previous ones. In the 1990s, broomrape populations overcoming all the known resistance genes Or_1 through Or_5 were identified in most of the sunflower cultivation areas by a new biotype population named race F. Shortly after resistance to race F had been incorporated into sunflower cultivars, new broomrape population overcoming race F resistance genes, named race G, was discovered in Spain, Romania, Turkey, Ukraine, and Russia. Sunflower germplasm resistant to the race G broomrape population has been identified in Romania (Pacureanu-Joita et al., 2009) and Turkey (Kaya et al., 2009), and a single dominant resistance gene transferred from *H. debilis* into cultivated background was reported (Velasco et al., 2012). Broomrape populations overcoming race G resistance genes are considered as race H. The last three races were the most virulent during the past 10 years.

Orobanche cumana is known to have been present in China since 1979. After that it rapidly spread over the main sunflower production regions including Inner Mongolia and Xinjiang autonomous regions, Gansu, Jilin, Heilongjiang, Hebei and Shanxi Provinces. The first report of *O. cumana* was from Jilin as race A in 1996 by the Jilin Institute of Sunflower using Spanish differentials. However, recent studies have shown a widespread increase in new virulent broomrape races in China. The purpose of this study was to assess the distribution of virulence and race type of *O. cumana* in northern China.

MATERIALS AND METHODS

Broomrape seeds were collected from the main sunflower production regions during 2013. A total of 62 samples were collected from isolated fields, including 19 samples from three districts in Inner Mongolia autonomous region, 14 samples from five districts in Xinjiang, 6 samples from two districts in Gansu, 12 samples from two districts in Jilin, 1 sample from Heilongjiang, and 10 samples from five districts in Hebei. The collected seeds were used to infect a set of differential lines resistant to broomrape races A to F (Kruglik A-41, J8281, Record, S1358, NR5 and P96) in order to identify the race structure. Each line was also resistant to the previous races. The variety B117 was used as susceptible control. Differentials 1532, 1589, 1506 and 1507 were also used to check the resistance to races C, D, and E. Small pots were prepared and filled with a mixture of sand, vermiculite and soil in a 1:1:3 ratio. The broomrape seeds

were applied at the rate of 10 mg in 50 g of soil mixture. Sunflower plants were planted into the small pots after germination and grown in the incubator until the V4 stage. Then, the seedlings were transferred to regular 6-inch diameter flower pots grown under greenhouse conditions. The observations and race composition were made using standard methods described by Velasco et al. (personal communication).

RESULTS

Results of broomrape samples against the differentials are summarized in Table 1. Among all samples, only one collected from the Wuyuan district in Inner Mongolia appeared to affect the differential P96 (resistant to race F in Spain) with an infection of 13 shoots per plant, indicating the presence of race G or higher virulence. There were some single cases of infection from the samples collected from the Urateqianqi and Uratezhongqi districts in Inner Mongolia, demonstrating a potential high virulence level development. The resistance of the Spanish lines NR5 and 1507 (resistant to race E), were overcome by the samples from Urateqianqi and Uratezhongqi districts by a low degree of infection equal to 3, 5 (NR5) and 16, 7 (1507), respectively, which proved the existence of race F.

In the studies of the Xinjiang autonomous region, samples from five districts showed no effect on P96. Samples from Qitai and Fukang districts slightly infected 1507. However, the differential NR5 was infected by broomrape seeds collected from these two districts by the degree of infection equal to 6 and 12, respectively. This indicates the presence of broomrape race F in these two districts. Differential 1506 showed either none or single stalk infection at Changji, while Altay and Tacheng districts had higher infection rates of 11, 18 and 18, respectively, for differential S1358, suggesting the existence of race E.

In Gansu , the broomrape population was able to overcome both NR5 and 1507, resistant to race E, with a degree of infection equal to 4 and 2, respectively, while the control B117 infection was 16 in the Wuwei district. This indicates the presence of broomrape biotype race F. The broomrape seeds collected from the Jinchang district were less virulent, with degrees of infection equal to 10, 3, 4 and 1, respectively, for control B117, and differentials Kruglik A-41, J8281 and Record, suggesting a mixture of races A, C and D, with a predominance of race A in this region.

In Jilin Province, the broomrape population from the Baicheng district was able to overcome the resistance of S1358. The degree of infection was equal to 5, while that of the control variant B117 was equal to 7. This result indicates the presence of broomrape biotype race E. The degrees of infection for Kruglik A-41, J8281 and Record were 11, 0 and 11, respectively, suggesting a mixture of the races D and E in the Baicheng district. Among nine samples from the Baicheng district, only one sample infected 1507 and was considered to be an outlier. In the Yaonan district, almost the same degree of infection was observed in the control B117 and Kruglik A-41, 24 and 26, respectively, while J8281, Record, and S1358 were 10, 11, and 9, respectively. These results indicated a mixture of races B and E.

The situation in Heilongjiang was similar to the Yaonan district. Samples collected from the Daqing district showed almost the same degree of infection in the control B117, Kruglik A-41, and J8281, equal to 13, 10 and 11, respectively, while Record and S1358 were 3 and 4, respectively. It is likely that a mixture of races C and E exists in this district.

In Hebei, all five populations overcame S1358, but none of them showed virulence toward 1506 (race D). Samples collected from the Xuanhua district showed the same degree of infection among the control B117, Kruglik A-41, and J8281, with 11, 15 and 11, respectively, while those for Record and S1358 were 2 and 1, respectively. The results indicated that a mixture of races C, D and E exist in this district with predominance of race C. In the Yangyuan district, broomrape seeds infected the control B117 (susceptible) and Kruglik A-41, J8281, Record, and S1358 with the of infection equal to 22, 16, 8, 2 and 6, respectively, suggesting a mixture of races A, B, C and E, with a predominance of race B. Samples collected from the Wei district showed the same degree of infection among the control B117, Kruglik A-41, and J8281 equal to 15, 10 and 15, while Record and S1358 were 7 and 4, respectively. This result indicated a mixture of races C, D and E are present, with a predominance of race C. Samples collected from Huaian district showed the same degree of infection among the control B117 and Kruglik A-41, equal to 30 and 28, while J8281, Record and S1358 were 12, 6 and 11, respectively. This fact indicated the presence of a mixture of races B and E. Samples collected from the Wanquan district showed the same degree of infection among the control B117 and Kruglik A-41 with 16 and 17 infections, while the degree for J8281, Record, and S1358 was 0, 5 and 8, respectively. Thus, a mixture of races B and E is likely present.

Table 1. Degree of infection* of differentiating sunflower genotypes with broomrape (*O. cumana*) collected from the districts of Inner Mongolia and Xinjiang autonomous regions, Gansu, Jilin, Heilongjiang and Hebei.

Seed collection location	Differential line										
	B117 (susceptible)	Kruglik A-41 A**	J8281 B	1532 C	1589 C	Record C	S1358 D	1506 D	NR5 E	1507 E	P96 F
					Inner Mongolia						
Urateqianqi	79	39	26	62	59	21	16	4	3	16	1
Uratezhongqi	54	43	9	39	27	19	21	2	5	7	1
Wuyuan	58	23	10	38	68	42	24	3	5	6	13
					Xinjiang						
Qitai	53	27	15	28	30	16	24	1	6	2	0
Fukang	64	30	18	45	21	19	31	6	12	3	0
Changji	35	31	11	6	16	7	11	0	0	0	0
Altay	30	20	6	24	10	2	18	0	0	0	0
Tacheng	29	40	10	5	21	16	18	1	0	0	0
					Gansu						
Wuwei	16	0	2	4	8	10	2	1	4	2	0
Jinchang	10	3	4	2	2	1	0	0	0	0	0
					Jilin						
Baicheng	7	11	0	9	0	11	5	0	0	2	0
Yaonan	24	26	10	15	17	11	9	0	0	0	0
					Heilongjiang						
Daqing	13	10	21	8	10	3	4	0	0	0	0
					Hebei						
Xuanhua	11	15	11	7	2	2	1	0	0	0	0
Yangyuan	22	16	8	5	0	2	6	0	0	0	0
Wei	15	10	15	3	0	7	4	0	0	0	0
Huaian	30	28	12	2	0	6	11	0	0	0	0
Wanquan	16	17	0	13	0	5	8	0	0	0	0

*Average number of broomrape tubercles and shoots per infected plant.
**Letter designation of the broomrape race to which the differential line is resistant.

DISCUSSION

The northern provinces in China are considered to be the traditional sunflower production regions. Sunflower is usually used in the rotation cycle to prevent diseases, improve soil fertility and reduce alkalinity. Sunflower broomrape has been controlled mainly by crop rotation and herbicides. In the past

few decades, there have been a few records of outbreaks in Xinjiang , Jilin, and Shanxi due to years of continually planting the same crop in the same field for the high profitability of oil and confectionery sunflower production. Ten years ago, confectionery hybrid sunflower planting seeds were introduced to the Chinese market. The high yield and the high profitability caused the breaking of crop rotations in almost every sunflower planting region, especially in the Inner Mongolia. As a result, highly virulent broomrape biotypes appeared and rapidly spread over many districts of this region. The broomrape biotypes are considered less virulent in the Xinjiang and Gansu than in Inner Mongolia. The situation in Eastern China is relatively safe, but the migration of highly virulent broomrape biotypes from the neighboring Inner Mongolia to the adjacent districts (Fig. 1) needs to be closely monitored.

Since the first report of broomrape in China, the Chinese Ministry of Agriculture had monitored the situation and tried to establish the path of transmission of sunflower broomrape. During the random inspection of sunflower planting seeds in the market, broomrape seeds have been found in imported sunflower planting seeds from Europe and Eastern Europe and some local varieties. It is believed that the broomrape seeds were carried by the imported sunflower planting seeds from the broomrape infected regions. This may explain the higher virulence that appeared in the Inner Mongolia region which imported the most advanced sunflower varieties every year. Although there are strict regulations of inspection and requirements of a phytosanitary certificate for the imported planting seed, the lack of enforcement makes it almost impossible to prevent the new virulent broomrape races from being seeded in China.

Until now the resistant varieties have not been widely used to prevent sunflower broomrape in China, which explains the mixture of older and newer races in the same field from almost every sample collected. Sooner or later the resistant varieties will be introduced to the market and will start to increase the environmental pressure for selection and force the race to evolve to a higher virulence. Some urgent actions are required to prevent increasing degrees of virulent Races of broomrape.

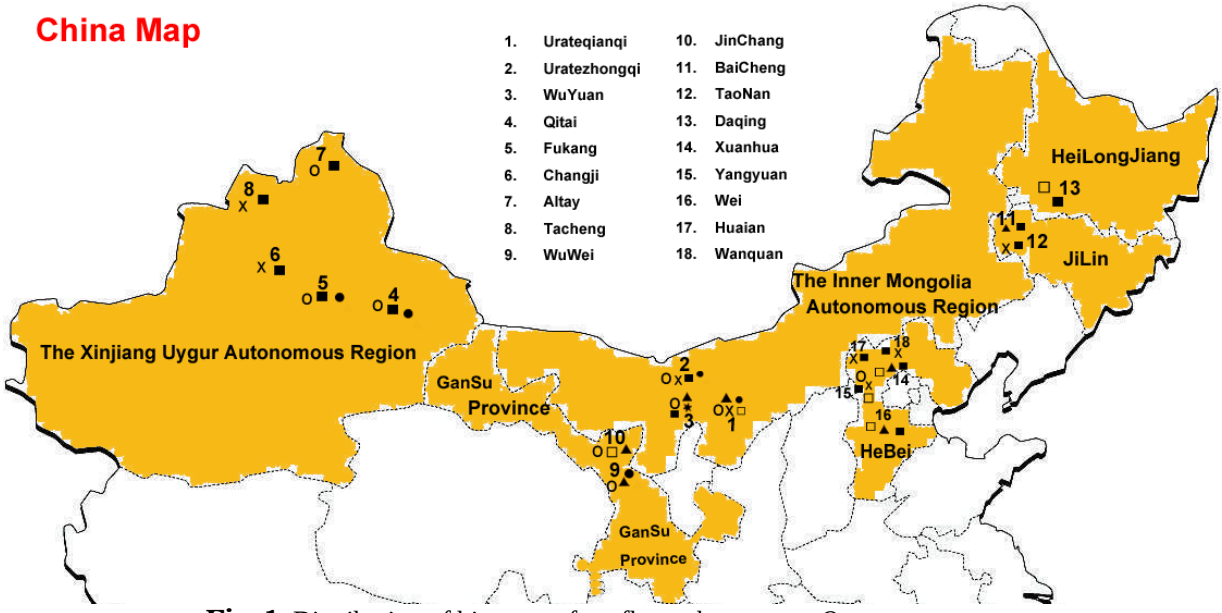

Fig. 1. Distribution of biotypes of sunflower broomrape *O. cumana*
(❍-A, ✕-B, □-C, ▲-D, ■-E, ●-F, ✶-G and higher virulence)

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The race identification of Sunflower broomrape in China

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ABSTRACT

Sunflower broomrape is a kind of parasite weed and influenced the sunflower production in China severely. The most effective way to control this weed is to generate the resistant sunflower varieties. However, for the frequently race differentiation, identification race types is the basis for the resistant breeding. In our study, we identified the race composition of sunflower broomrape in China under lab condition by using a set of differential lines provided by Dr. Dragan Škorić. The preliminary data indicated that race A, D, E and G are the main types of sunflower broomrape in China. Race A is mainly distributed in Mizhi, Shilou, Hunyuan (Shanxi province), Xuanhua (Hebei province) and Tuzuoqi (Middle part of Innermongolia). Race D is found in Xinyuan,Tekesi and Beitun (Xinjiang region). Race E is found in Shihezi (Xinjiang region). Race G is only distributed in Xixiaozhao (West part of Innermongolia). Based on these results, we concluded that Xinjiang and Innermongolia region distributed rather high level race types such as D and G, indicating the high variation frequency happened in these regions.

Key words: Sunflower – broomrape – race identification

SESSION 2

Knowing the parasite: Biology and genetics of Orobanche

Knowing the parasite: Biology and genetics of *Orobanche*

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ABSTRACT

Due to their forms and colors, parasitic plants are most often considered to be botanical curiosities. However, in some cases, these are proved to be also deadly pests with the capacity to exploit other plants. Among the obligate root parasitic weeds, the holoparasites which are devoid of chlorophyll and thus unable to carry out photosynthesis totally rely on their hosts for their water, mineral, and carbohydrate supplies. Members of the genus *Orobanche* and *Phelipanche*, belonging to the *Orobanchaceae* family (the broomrape family), are thus the final result of this evolutionary transition from autotrophism to heterotrophism. The underlying process of this trophic exploitation, governed by a fine-tuned molecular dialogue between both partners, is an extraordinary example of adaptive plant biology operated by these parasitic organisms in the course of evolution. This transition is associated with remarkable morphological and physiological adaptations, such as the requirement for the seeds to germinate to perceive molecules produced by host roots, the development of a novel organ, the *haustorium*, which invades host tissues and establishes a physiological continuum between the parasite and the host, the establishment of a sink strength required for translocation of host resources, the loss of photosynthesis, and a reduced leaf and root architecture.

Key words: Conditioning – germination – *haustorium* – *Orobanche* – sink strength – tubercle

Phylogenetic relationships

The scientific name *Orobanche* derives from the Ancient Greek words ὄροβος (orobos, "bitter vetch") and ἄγχω (ankhō, "to strangle") with reference to the impact of the parasite on its host. From a systematic point of view, the *Orobanchaceae* family, formerly related to the *Scrophulariaceae* family, is a monophyletic group composed of 2060 species distributed in 90 heterotrophic genus, except for a single autotrophic lineage, the genus *Lindenbergia* (Westwood et al., 2010). The parasitic way of life would have a unique origin and would arise from Laurasie in the northern Tethys Ocean (probably in East Asia) during the Tertiary period (Wolfe et al., 2005). Phylogenetic studies based on the association of several molecular markers propose a distribution in six major clades in which the transition from hemiparasitism to holoparasitism would have occurred three times in an independent manner (McNeal et al., 2013). The tribe *Orobancheae sensu stricto* is made of 170 herbaceous holoparasitic species from temperate to subtropical regions, mainly in the northern hemisphere. There is some confusion regarding the classification of the different species from this tribe due to the drastic reduction, induced by the parasitic way of life, of the number of phenotypic traits classically used in taxonomic identification. These species were traditionally grouped in four main sections (*Trionychon* Wallr., *Myzorrhiza* (Philippi), *Gymnocaulis* Nutt., and *Osproleon* Wallr. (= *Orobanche sensu stricto*)). Using plastid and nuclear (ITS) sequences, it has been possible to group the four sections in two phylogenetically distinct genus: the *Orobanche* genus (19 chromosomes) coupling the *Osproleon* section and the *Diphelypaea* genus, and the *Phelipanche* genus (12 chromosomes) including the sections *Gymnocaulis*, *Myzorrhiza*, and *Trionychon* (Joel, 2009). This dichotomy is also supported by studies based on LTR retrotransposon repertoire and repetitive DNA sequences (Piednoël et al., 2012, 2013). However, a degree of ambiguity exists in regard to the name of the *Phelipanche* genus because species were named for a long time *Orobanche* (i.e. *P. ramosa*, *P. aegyptiaca*, *P. mutelii* …). Nevertheless, the improvement of molecular techniques allowed for example the distinction between *O. cernua* and *O. cumana* which have long been considered as a single species (Delavault and Thalouarn, 2002), even though the debate is still open (Schneeweiss et al., 2004). Throughout the text the generic name *Orobanche* will be used when referring to broomrape species.

Among the different broomrape members, only about twenty species should be considered as harmful parasitic weeds in important crops, including *O. cumana* Wallr. on sunflower, *O. crenata* Forsk. and *O. foetida* Poir. on legumes, *P. ramosa* L. Pomel on oilseed rape, and *P. aegyptiaca* Pers. on tomato. They

represent a major economical constraint as approximately 16 million hectares of arable land are threatened around the Mediterranean see and in West Asia.

An original life cycle

Common feature of all holoparasites, the life cycle of *Orobanche* is atypical compare with classical Angiosperms due to its high degree of trophic specialization and its synchronization with that of its host (Gibot-Leclerc et al., 2012) (Fig. 1). This cycle is divided into two distinct phases: the first one which is « independent », from the seed imbibition to radicle development, and occurs thanks to the seed storage reserves. This independency is only a matter of energy since a chemical signal produced by the host roots in the rhizosphere is required for seed germination. The « dependent » second phase starts with the *haustorium* formation and the connection of the parasite to the vascular system of the host. Survival and development of the parasite are then highly dependent on this attachment step (Joel et al., 2007). It is therefore the most susceptible step in the *Orobanche* life cycle. However, the quantity of seeds in the soil is so high that, despite a strong rate of unsuccessful germinations (no attachment to the host), the perennity of the parasitic species is not impacted. Broomrapes are mainly autogamous plants (Musselman et al., 1982) with a strategy consisting in producing up to half a million extremely small seeds (200 à 300 µm) in order to maintain a high level of adaptability to environmental conditions (Irving and Cameron, 2009). Then, most of the life cycle occurs beneath the soil surface and only the inflorescence will end up emerging.

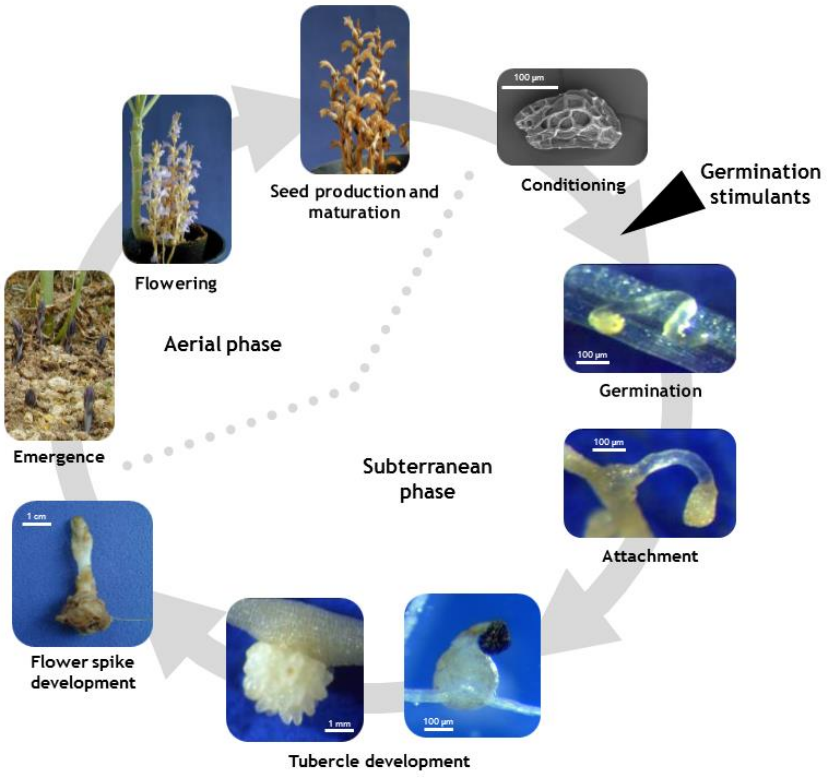

Fig. 1. Life cycle of *Orobanche* (example of *Phelipanche ramosa*).

The seeds

Orobanche seed coat is characterized by a honeycomb pattern resulting from the dehydration of a maternal cell layer (only the cell walls remain visible). These patterns are species specific and could then be used for species identification. Ovoid seeds are composed of lipids, with oleic and linoleic acids as main storage material in *P. aegyptiaca* (Bar-Nun and Mayer, 2002). The endosperm is composed of 3 to 4 cell layers and contains several oil bodies and starch grains. It surrounds a reduced embryo that is composed of a spherical body without a plumule, a radicle or cotyledons. Usually considered as an undifferentiated body, recent works indicated that two distinct regions may be present in the embryo of *P. aegyptiaca* (Joel et al., 2011). It is suggested that the two perisperm cells located between the embryo and the micropyle may contain the putative receptors of germination stimulants (Lechat et al., 2012). The *Orobanche* tiny seeds may easily spread to other fields and can persist in soil for decades (Prider et al., 2013), leading to an accelerated increase of contamination in the infested areas in which susceptible crops might be affected. Broomrape seed dispersal is facilitated by wind, water, humans and their agricultural tools, and animal. They are also suspected of being dispersed by forage and contaminated crop seed lots (Dongo et al., 2011). This last mode of dissemination and the resulting contamination of new areas are of special relevance for seed companies and other institutions supplying crop seeds, as well as for seed transfer regulation.

The conditioning period

After maturation and desiccation, *Orobanche* seeds are more or less buried into the soil where they enter dormancy, a waiting period for environmental conditions suitable for their germination. The dormancy discussed here seems to be specific to broomrape species because some recent works suggest that *P. ramosa* seeds do not have classical physical dormancy (Lechat et al., 2012). In the contrary, these seeds require a conditioning period that would correspond to a moist environment and suitable temperatures and which is specific to each broomrape species. It is suggested that this period correspond to physiological processes resulting in the set-up of the machinery needed for germination stimulant (GS) perception. Seed hydration and major metabolic pathways are initiated during seed conditioning. However, some broomrape species may not require this conditioning phase (Plakhine et al., 2009) but it is still a matter of debate. In *P. ramosa*, it has been shown that seeds require a minimum of 4 days of conditioning to allow optimal germination in response to germination stimulants (Fig. 2). The conditioning period starts with seed imbibition which takes around 1 h. This rapid imbibition is obtained by water entering in the seed through the micropyle which opens after 30 min (Joel et al., 2011). Then several physiological process occur indicating a rapid metabolic reactivation. First, an optimal Adenylate Energy Charge (AEC = 0.9) is reached as of first day of conditioning. A strong decrease in ABA seed content occurred during the same period of conditioning in *P. ramosa* (Lechat et al., 2012) along with a high release in the medium in the case of *O. minor* (Chae et al., 2004). A characteristic pattern of respiration, protein synthesis, and utilization of reducing sugars (Bar-Nun and Mayer, 1993, 2002) occur as well as a strong alternative oxidase activity. Uematsu et al. (2007) also demonstrated an accumulation of cAMP associated to a gibberellin synthesis in *O. minor* seeds while a decrease of cAMP quantity was observed in *P. ramosa* (Personal communication, MM. Lechat). All these events are potentially involved in the set-up of the mechanisms needed for stimulant perception or signal translocation and thus for broomrape seed germination, without however knowing the underlying mechanism. However, recent works indicate that an epigenetic mechanism, a DNA demethylation, may occur during the conditioning phase and would explain the non-responsiveness of seeds to germination stimulants during this period (see below).

It is noteworthy that the overall phenomenon of conditioning is reversible. Indeed, in the absence of a host in the immediate vicinity, seeds enter a secondary dormancy (Kebreab and Murdoch, 1999). This mechanism enables the seeds to face unfavorable environmental conditions or a time lag with the development cycle of the host plant. Seeds will have to go through a new cycle of desiccation and conditioning to be again perceptive to the GS produced by another host plant (Matusova et al., 2004). This phenomenon appears to be a key to longevity of broomrape seed (Joel et al., 2007).

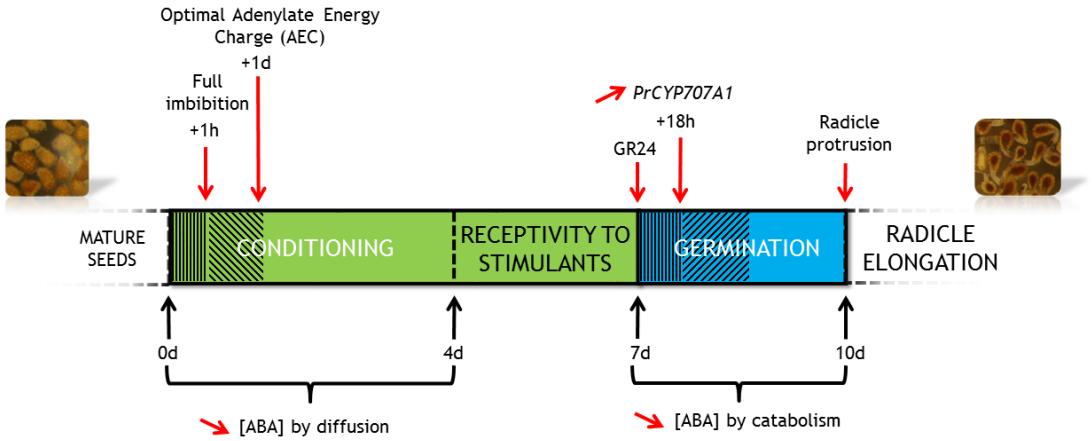

Fig. 2. Molecular mechanisms in conditioning and germination of *P. ramosa* seeds (Lechat et al., 2012). ABA, abscisic acid; *PrCYP707A1*, an ABA 8'-hydrxylase encoding gene.

Germination: the stimulants and their mode of action

Unlike most Angiosperms, seeds of broomrape species are unable to germinate without the stimulation by a chemical stimulus, the germination stimulant, produced and exuded in the rhizosphere by surrounding host roots. Most GS identified thus far belong to the strigolactone (SL) family (Yoneyama et al., 2010). The first SL, strigol, was discovered in 1966 by Cook et al. It was exuded by cotton roots and induced at a very low concentration (1.10^{-11} M) the germination of *Striga* seeds. Since then, more than 15 structural variants of strigol have been discovered and all derive from the carotenoid biosynthesis pathway (Matusova et al., 2005). Interestingly, SL also act as host recognition signals for symbiotic arbuscular mycorrhizal fungi (Besserer et al., 2006) and are considered to be a novel class of plant hormones involved in controlling shoot branching inhibition (Gomez-Roldan et al., 2008). Several studies have also investigated the SL signaling pathway in plants as well as the relationships between SLs and other phytohormones during the control of plant architecture. SLs interact with auxin and cytokinins (CK) in bud outgrowth control, during adventitious root initiation or in nutrient-stress responses. In addition, cross-talk can occur between SLs, auxin and ethylene in the control of root hair elongation.

Although most of the germination stimulants identified so far belong to the SL family and correspond to butenolides signaling molecules, other molecules have also been identified such as sesquiterpene lactones, polyphenols, and isothiocyanates involved in the germination of *O. cumana* (Raupp and Spring, 2013), *O. foetida* (Evidente et al., 2010), and *P. ramosa* (Auger et al., 2012), respectively. In contrast, although the key role of SLs as germination stimulants has been known for several decades, almost nothing is known about the early molecular events governing the germination of root parasitic plants in response to SLs, or about how SLs interact with parasite phytohormones during this process. For a long time, identification of SL receptors has remained elusive and their subcellular location was under debate despite several structure-activity studies. However, recent works have allowed significant progress in understanding the components of this key step in the *Orobanche* biology. First using a transcriptomic approach, Lechat et al. (2012) highlighted the major role of *PrCYP707A1*, an abscisic acid (ABA) catabolic gene, in the germination of *P. ramosa* seeds in response to the SL analogue GR24 (Fig. 2). *PrCYP707A1* is expressed at low levels during conditioning during which an initial decline in ABA levels was recorded. GR24 application after conditioning triggered a strong up-regulation of *PrCYP707A1* during the first 18 h, followed by an 8-fold decrease in ABA levels detectable 3 days after treatment. Concomitant treatments of conditioned seeds with GR24 and exogenous ABA, or Abz-E2B, a specific inhibitor of CYP707A, caused inhibition of germination. These results demonstrated that germination occurs after a dormancy release of the seeds by ABA catabolism mediated by the GR24-dependent activation of *PrCYP707A* gene. Responses of *P. ramosa*, *O. cumana*, *O. minor*, and *S. hermonthica* seeds to different GS - the synthetic strigolactone GR24, the sunflower sesquiterpene lactone dehydrocostus lactone (DCL) and the 2-phenylethyl isothiocyanate (ITC) present in the rhizosphere of oilseed rape -

were also analyzed (Delavault et al., 2013). The seeds displayed differential response patterns according to the species, the stimulants and the applied concentration. Thus, the four species germinated in response to GR24, only the three broomrape species responded to DCL, and only *P. ramosa* germinated in response to ITC. Whatever the GS and the species, when germination was triggered, a *CYP707A* up-regulation was observed. These results revealed the ubiquitous key role of *CYP707A* in parasitic plant seed germination triggered by GS.

In situ hybridization experiments on GR24-treated seeds revealed a specific *PrCYP707A1* mRNA accumulation in the perisperm cells located between the embryo and the micropyle, suggesting that it could be the location of SL receptors in *P. ramosa* (Lechat et al., 2012). The mechanisms involved in perception and signal transduction of germination stimulants upstream of *PrCYP707A1* still remain unknown. However, the group of David Nelson working on karrikins, molecules present in smoke and known to trigger the germination of non-parasitic plant seeds, strongly suggests that the perception system in *Orobanchaceae* employs a mechanism involving KAI2, a α/β-fold hydrolase, as the receptor of SL, and a F-box protein MAX2 as the mediator of the response (Conn et al., 2013). It has been shown also that *P. ramosa* seeds require a conditioning period of at least 4 days to be receptive to the GS and that, during this 4 day period, *PrCYP707A1* is not expressed. Interestingly, a global DNA demethylation process was shown to occur progressively during the conditioning period. DNA methylation is known as an epigenetic modification that affects gene expression in plants, with a high level of cytosine methylation strongly correlated with gene silencing. In the case of *PrCYP707A*, its repression during the minimal 4 day conditioning period seems to be associated to a high degree of DNA methylation in its promoter sequence (Lechat et al., unpublished results).

The *haustorium*, a key organ

A common feature of all parasitic plants is their capacity to attach to a host plant thanks to a specialized endophytic organ called *haustorium* (from the Latin word *haurire* meaning "to draw up"). Once they are stimulated, *Orobanche* seeds produce a germ-tube considered as a radicle and called *procaulôme*. The germ-tube grows by cell elongation toward the host roots probably guided by a positive chemotropism related to an increasing concentration gradient of stimulating molecules produced by the host (Bouwmeester et al., 2003). Once the seedlings reach a host root, radicle elongation ends while processes of radial expansion are initiated and apical cells differentiate into papillae secreting a mucilaginous substance that promotes adhesion to the host. The radicle apex starts then to swell and develops a structure called *appressorium* allowing anchoring to the host root surface and also penetration into the root cortex *via* intrusive cells. Progression of *appressorium* cells into the root tissues occurs through mechanical pressure facilitated by the secretion of enzymes with pectine-methylesterase and polygalacturonase activities (Véronési et al., 2007). These enzyme would adapt the chemical composition and the physical properties of host cell walls to the *haustorium* development, without the involvement of degrading processes that would generate strong defense responses by the host. Endodermis is the last physical barrier that the *appressorium* should pass through to reach the central cylinder containing the host vascular system. This last step would be facilitated by secretion of enzyme with a cutinase activity. After penetration through host endodermis, parasite cells initiate the most important organ of the interaction, the *haustorium sensu stricto*, which "serves as the structural and physiological bridge that allows the parasites to withdraw water and nutrients from the conductive systems of living host plants". Apparition of the invasive *haustorium* in plants was the major event that permits the evolutionary transition toward a parasitic lifestyle. *Orobanchaceae* have two kinds of *haustoria*, lateral *haustoria*, which develop as lateral extensions of parasitic roots, and terminal *haustoria* which develop at the apex of the radicle. The terminal *haustorium* is a characteristic feature of the holoparasitic *Orobanche* clade of *Orobanchaceae*. As mentioned earlier, seed germination of obligate root parasitic plant is triggered by chemical compounds produced by the host. In the case of the *haustorium* formation, these are also chemical signals derived from host which initiate its development in *Orobanchaceae*. These signals are called xenognosins or Haustorium-Inducing Factors (HIFs). However among the *Orobanchaceae*, there are notable exceptions, the *Phelipanche* and *Orobanche* species for which no host factors for *haustorium* development have been to date discovered.

The nature of the conductive system between the *haustorium* and the host vascular system diverges depending on the parasite species. Indeed, obligate hemiparasites such as *Striga* species are xylem-feeders because they produce direct connections with the host xylem *via* a specific organ called *osculum*, but little

or no phloem connections. By contrast, *Orobanche* are "phloem-feeders" because there are clear indications for both phloem and xylem continuums as it has been demonstrated by using specific xylem and phloem-mobile dyes.

Post-attachment development of the parasite

Phloem connections provide *Orobanche* with the ability to extract nitrogen and carbohydrate compounds from their host. Once the connections are established, the *haustorium* growths, distends host root tissues, and rapidly becomes a small tubercle. As it grows, the tubercle produces short adventitious roots with a rudimentary cap but without root hair zone making them unable to uptake water and mineral compounds from the soil. Nevertheless, each of these roots is a growing organ connected to host phloem that contributes to increase the sink strength of the tubercle. This tuberous cell mass, with a not yet defined morphology, constitutes then a storage organ accumulating temporarily mainly hexoses, polyols (mannitol, inositol…), amino acids and starch (Delavault et al., 2002 ; Abbes et al., 2009; Draie et al., 2011). The tubercle exhibits one or more shoot apical meristems that, when the parasite will perceive appropriate host derived signals (nutrients, hormones…), will produce achlorophyllous scaly stems. After the stems emerge from the soil, they develop one or more flower spikes supporting several flowers. The rate of stem branching and the number of flowers per spike depend on the species. Every flower, after (self)-fertilization, gives a capsule containing thousands of seeds. It is noteworthy that the life cycle of *Orobanche* is synchronized with that of the host plant. Every step (bud burst of apical meristem, flowering and seed production) is then realized simultaneously with those of the host (Gibot-Leclerc et al., 2012).

Source-sink relationship, sink strength, and host-parasite transfers

All plant organs require a constant supply in photoassimilates and reduced nitrogen compounds. Autotrophic plants should then proceed to a partitioning of nutrients in order to supply organs unable to produce such compounds. This is referred to as source-sink relationships with, for example, the relation between mature source leaves, which are highly photosynthetic with then an excess of nutritive compounds, and sink organs such as young leaves, buds or roots. The term sink strength can be defined as the competitive ability of an organ to attract assimilates and water coming from conducting tissues. Thus, holoparasites act like supernumerary sink organs which are highly dependent on carbohydrate and nitrogen compounds remobilized from the different source organs of host (Fig. 3). Because *Orobanche* are achlorophyllous holoparasites lacking functional roots, their carbon and nitrogen nutrition relies totally on an acquisition from the host. In plant, nitrogen assimilation is a process highly dependent on carbon and energy. Because of a lack of reducing power and carbon skeletons (usually provides by photosynthesis) and a low nitrate reductase activity in broomrape species, the amino acid uptake from the host seems to be the most efficient nitrogen acquisition in terms of energy (Irving and Cameron, 2009). Moreover, because their transpiration rate is very low, it is almost impossible for *Orobanche* to obtain nutrients by diverting the flow of xylem content of the host, as it is the case in the hemiparasite species (Hibberd et al., 1999). Thus, *Orobanche* derives all of its nutrients needed for its development from the host phloem ("phloem-feeder"). This is done by reducing its osmotic potential thanks to an accumulating high levels of osmotically active compounds such as cations, sugars, amino acids, and polyols, and also to the establishment of a decreasing concentration gradient between the *haustorium* tissues close to the connections and the other organs.

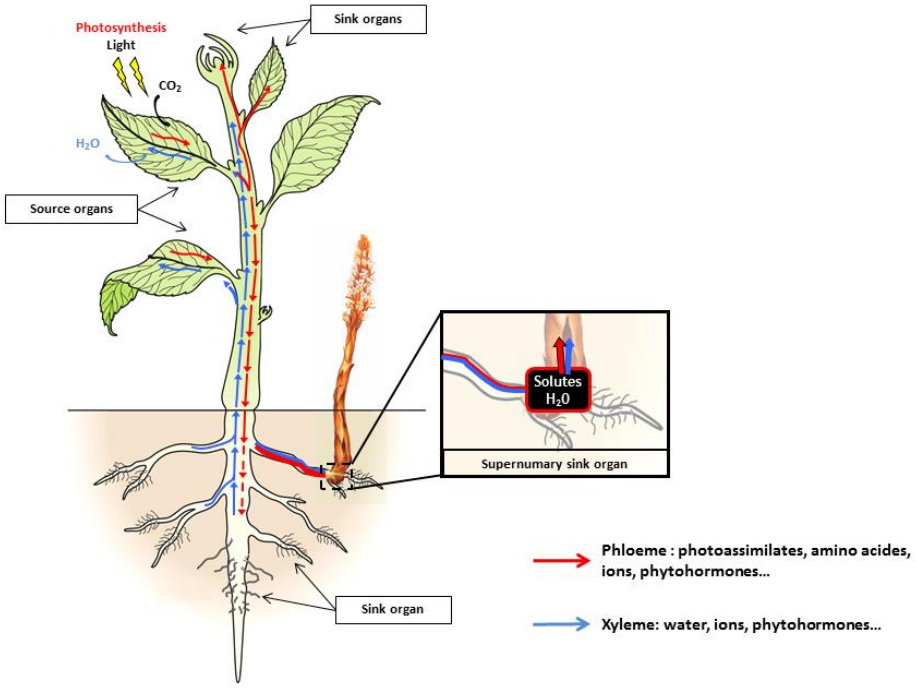

Fig. 3. Source-sink relationship in a host plant-*Orobanche* interaction.

Given the central importance of the *haustorium* function in resource acquisition, it is surprising that the molecular mechanisms involved in the physiology of this organ remain poorly characterized. Concerning carbon, Draie et al. (2011) demonstrated the major role of PrSAI1, a *P. ramosa* vacuolar soluble acid invertase, in the sink strength of the flowering shoot. PrSAI1 would act in the phloem unloading of sucrose from the host and then in the subsequent accumulation of hexoses. Péron et al. (2012) highlighted the role of *PrSus1*, a *P. ramosa* sucrose synthase encoding gene, in the utilization of host-derived sucrose in meristematic areas and in cellulose biosynthesis in differentiating vascular elements. Importance of accumulating mannitol has been long demonstrated in *Orobanche* because it is a useful solute that functions as an osmoticum involved in reducing the osmotic potential, a storage form for reduced carbon, an osmoprotectant, and a scavenger of reactive oxygen species (Delavault et al., 2002). Regarding nitrogen, few studies have been conducted to examine the actors of the sink strength as well as of the nitrogen metabolism in *Orobanche*. Glutamine Synthetase 2 (GS2) and nitrate reductase have been shown to be missing or reduced in activity in several *Orobanche* species. The fact that herbicides, targeting specifically the amino acid biosynthesis, have a major impact on broomrapes tends to prove that these plants have their own machinery for amino acid metabolism (Eizenberg et al., 2012). In *O. foetida*, tubercles accumulates preferentially soluble amino acids, especially aspartate and asparagine suggesting an important role for a glutamine-dependent asparagine synthetase in the N metabolism of the parasite (Abbes et al., 2009). To illustrate the tremendous sink strength developed by the parasite, an example is *O. cernua* that gained 99 % of its carbon and 95 % of its nitrogen from tobacco phloem (Hibberd et al., 1999).

Conclusion and perspectives

The trophic exploitation developed by *Orobanche* impacts the host plant physiology at several levels. From a global point of view, the infection strongly reduces the host plant biomass and its fertility. At the field level, this results in a yield loss in economically important crops that could be total depending on the broomrape species and the host genotype. It is worth noting that the subterranean phase of the parasite development is most detrimental for the crop. Thus, when the infection becomes visible with the parasite emergence form the soil, it is already too late for the crop because the parasite has cause irreversible damage that will reduce crop yield. To date, in spite of intense efforts, means to selectively control the various broomrape species are still scarce and inefficient in terms of sustainability. Given the alarming

impact of *Orobanche* species on world agriculture, deciphering the physiological and molecular events governing the parasite development and the establishment of the interaction with its host is then a necessary step toward the development of targeted control methods. Indeed, every developmental or metabolic particularity of the parasite might be considered as a point of vulnerability that could be potentially exploited. Unfortunately, compare to other plant – pathogen interactions, our knowledge is far too scarce. Progress in understanding the unique biology of broomrapes has been hampered by the lack of genomic resources in *Orobanche* and the lack of protocols allowing gene modification (knock-out or expression modulation) in these parasites. However, most of these barriers have been very recently alleviated with the availability of transcriptomic data for *P. aegyptiaca* (Parasitic Plant genome Project) and *P. ramosa* (P. Delavault, University of Nantes), the possibility of manipulating *P. aegyptiaca* gene expression *via* hairpin RNAi through the genetic transformation of the host plant and the systemic movement of the silencing signal from host to parasite (Aly et al., 2009), and the development of a robust system for *Agrobacterium rhizogenes*-mediated transformation and subsequent regeneration of the *P. aegyptiaca* holoparasitic plant (Fernández-Aparicio et al., 2011). In any cases, the road ahead is still long - "development of effective and low-cost control measures for *Orobanche* remains the "holy grail" for plant pathologists, agronomists, and biotechnologists" (Nickrent, Southern Illinois University).

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Seed structure characteristics of *Orobanche cumana* populations

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ABSTRACT

Sunflower broomrape *Orobanche cumana* WALLR. is a rapidly growing threat to the oilcrop production in many countries. Fast adaptation to new environments and increasing host resistance suggests that phenotypically distinctive populations of the weed may have evolved. The differentiation of such populations on the base of seed micromorphological characters was attempted. Morphometric measurements allowed distinction of *O. cumana* from several other *Orobanche* and *Phelipanche* species and micromorphological details of the testa showed typical traits for *O. cumana*, *O. crenata*, *P. ramosa*, and *P. aegyptiaca*. However, populations of sunflower broomrape from five different European countries could not be separated from each other on the base of size or form of their seeds nor was their ultrastructure of the testa different when analyzed by scanning electron microscopy. This accounted as well for samples of different races (race E and higher).

Key words: Broomrape – *Helianthus annuus* – *Orobanche cumana* – *Phelipanche* – seed micromorphology – sunflower

INTRODUCTION

Seed morphology has been recognized as a suitable feature for the differentiation of parasitic weeds of *Orobanche* and *Phelipanche* species (Abu Sbaih and Jury, 1994; Plaza et al., 2004; Domina and Colombo, 2005). While size and shape of the entire seeds revealed to be highly variable, the ultrastructure of the testa was shown to provide characteristics that are distinctive for the separation of the two taxa, which were formerly placed in two sections (sect. *Trionychon* and sect. *Orobanche*) of the genus *Orobanche sensu lato*. For sunflower broomrape, *O. cumana*, transmission and scanning electron microscopic investigations had unravelled a complex composition of layers forming the typical reticulate ornamentation of the testa (Thomas et al., 1999). The usually oblongoid to ellipsoid seeds have a surface composed of an outer layer of large, thick-walled cells with pitted periclinal inner walls which become visible when the thin outer wall layer collapses due to the loss of water. Attempts to use seed ultrastructure and morphology for population studies in this agro-economically important taxon are rare and so far restricted to collections from the Iberian Peninsula (Plaza et al., 2004). At the same time, phenotypic characters would be extremely helpful for differentiating ecotypes of this weed. The current study, therefore, aims to compare sunflower broomrape seeds from distant origins and of putatively different races.

MATERIALS AND METHODS

Sample preparation for SEM

Dry seeds of different origin (see Table 1) were carefully transferred with a brush on aluminium stubs covered with conductive carbon adhesive tabs. The samples were sputter coated with gold/palladium (SCD 040, Balzers Union, Switzerland) and the morphology and micromorphological characters were examined with a scanning electron microscope (DSM 940, Zeiss, Germany) at 5 kV.

Measurements and statistics

The seed form was classified in 3 categories: 1. narrow elongated to pear shaped elongated, 2. roundish to ovate; 3 pear shaped. Size measurements (length and width) of 50 seeds per sample were recorded on scanning electron micrographs (Adobe Photoshop CS2) and statistically treated (mean value; standard deviation; minimum and maximum value). Analysis of variance (ANOVA) was conducted on the ratio of seed length / width with InfoStat (Version 2013, InfoStat Group, University of Córdoba, Argentina). Effects were considered significant if $P < 0.05$ in the Tukey test.

Plant material

Seed samples of the following origin were used:

Table 1. Seed samples used for scanning electron microscopy.

Taxon	Collecting date	Origin	Host / Race
P. aegyptiaca	2009	Israel (Maro Hawa)[1]	on *Solanum lycopersicum*
P. ramosa	2008	Germany (Neupotz)	on *Nicotiana tabacum*
O. crenata	2007	Israel (Merahusia)[1]	on *Daucus carota*
O. cumana	2003	Spain (Córdoba)[2]	EC403
O. cumana	2005	Spain (Córdoba)[2]	not determined
O. cumana	2008	Spain (Córdoba)[2]	TOM 2008
O. cumana	2012	Serbia (Novi Sad)[3]	on sunflower genotype NS Slatki
O. cumana	2012	Serbia (Lipar)[3]	race E or higher
O. cumana	2012	Serbia (Supliak)[3]	on non resistant host
O. cumana	2012	Serbia (Sombor)[3]	race E or higher
O. cumana	2012	Romania (Alexandria)[4]	not determined
O. cumana	2012	Romania (Cuza-Voda)[4]	not determined
O. cumana	2012	Romania (Iazu)[4]	not determined
O. cumana	2011	Moldova (Chisinau)[5]	race G
O. cumana	2011	Moldova (Chisinau)[5]	race G
O. cumana	2011	Moldova (Balti)[5]	race F
O. cumana	2012	Russia (Krasnodar, Gulkevitchy)[6]	race F or higher
O. cumana	2012	Russia (Rostov, Morozovskiy)[6]	race H or higher
O. cumana	2012	Russia (Stavropol, Krasnogvardeiskiy) [6]	race H or higher

Samples were generously provided by: [1] J. Hershenhorn, Hebrew University Rehovot, Israel; [2] D. Rubiales and M. Molinero-Ruiz, Inst. for Sustainable Agriculture, Córdoba, Spain; [3] D. Miladinovic and B. Dedic, Inst. of Field and Vegetable Crops, Novi Sad, Serbia; [4] M. Pacureanu, Nat. Agric. Res. Inst., Fundulea, Romania; [5] A. Glijin, Academy of Science of Moldova, Chisinau, Moldova; [6] T. Antonova, Academy of Agricultural Sciences, Krasnodar, Russia.

RESULTS

Comparison of seed form and testa micromorphology

The seed forms were variable but could be classified in 3 categories: 1. narrow elongated to pear shaped elongated for *O. cumana* (Fig. 1a) or 2. roundish to ovate as for *O. crenata* and *P. aegyptiaca* (Fig 1b, c) or 3. pear shaped for *P ramosa* (Fig. 1d). Seeds of *O. cumana* showed a testa consisting of ca. 30-35 particularly large, mostly elongated cells. (Fig. 1a) The length of the cells often reached 5 to 6 times of the width.

On dry seeds the anticlinal cell walls of the testa were visible, because the thin outer layer attached to the inner pitted, thick periclinal cell wall is giving the testa a reticulate appearance (Fig. 1). *O. cumana*, *O. crenata*, *P. ramosa*, and *P aegyptiaca* could be distinguished by micromorphological traits. While both *Orobanche* species shared the pitted structure of the inner periclinal wall and lacked the fibrous layer of the *Phelipanche* species (compare Fig. 2), *O. cumana* showed a more granular surface and the anticlinal walls were not crenated, but irregularly thickened (Fig. 2a) in contrast to *O. crenata* (Fig. 2b). Seed samples of different *O. cumana* populations did not show significant differences in the their micromorphology (data not shown). The surface of both *Phelipanche* species was covered with fibrous material and both had crenated anticlinal cell walls Fig. 2c, d). In contrast to *P. ramosa* for *P. aegyptiaca* the pitted structure of the periclinal cell walls under the fibrous layer was visible (Fig. 2d).

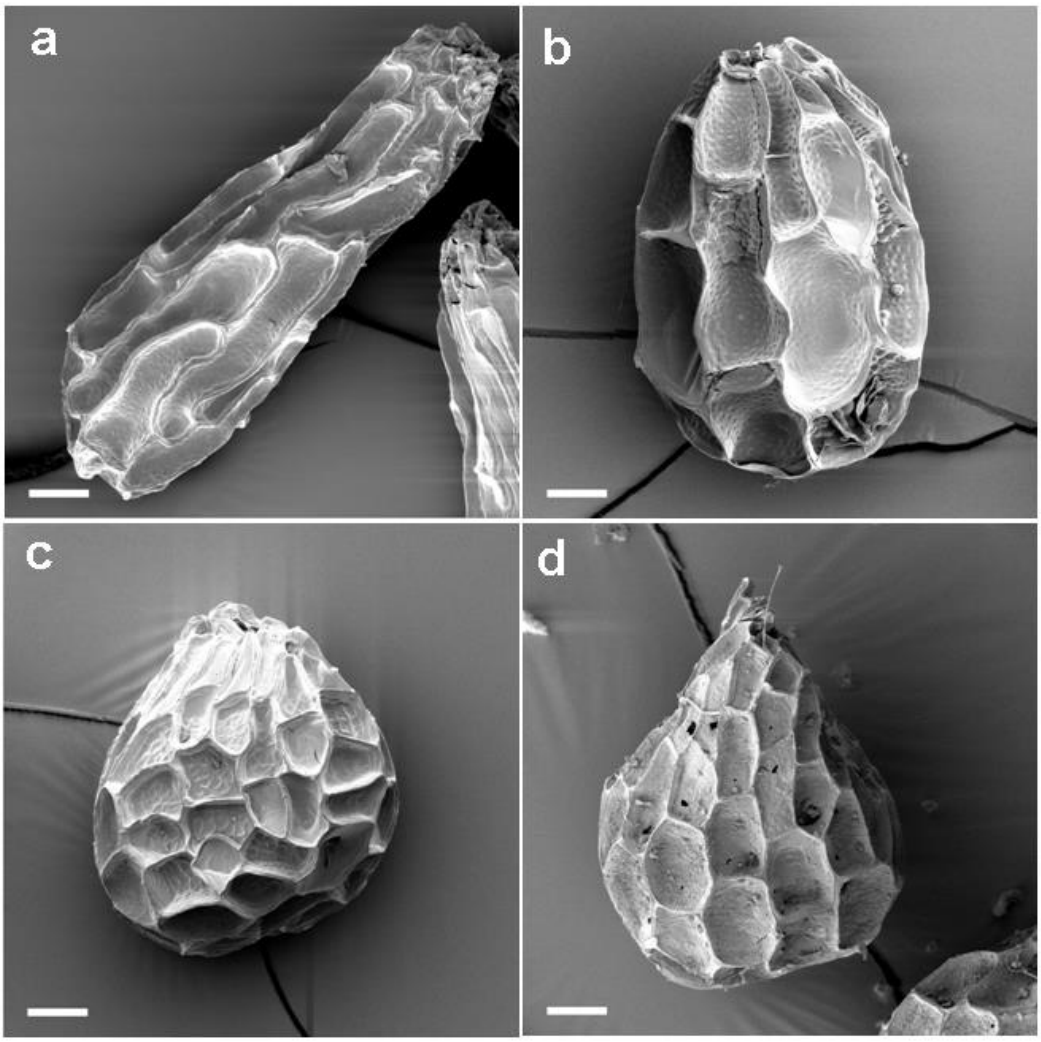

Fig. 1. Scanning electron micrographs showing the typical forms of dry seeds of the four broomrape taxa. a: Narrow elongated seed form of *O. cumana*; b: Roundish ovate form of *O. crenata*; c: Round seed form of *P. aegyptiaca*; d: Pear shaped seed of *P. ramosa*. Scale bar, 50 µm.

Morphometric data

The form of *O. cumana* seeds was quite variable ranging from roundish-ovate to pear shaped and ellipsoid elongate. These different types occurred even within the seed of a single fruit. Nevertheless, the majority of seeds was elongate and mostly more than twice as long as wide. The mean length of 16 populations was 367.3 (SD ± 26.85) µm and the width was 164.3 (SD ± 14.26) µm (Table 2). The absolute seed sizes varied in between the different populations by up to ca. 30 %, but this did not affect the ratio between length and width which was relatively constant at 2.27 (SD ± 0.1) and clearly differentiated *O. cumana* from *O. crenata* (1.63), *P. aegyptiaca* (1.43) and *P. ramosa* (1.40).

The size variation in between the populations of *O. cumana*, although statistically significant (Tukey Test $P < 0.05$) for one sample each from Spain, Romania, and Moldova, was neither correlated to the geographic origin nor to the pathogenicity (in the cases where host preference and resistance of the sunflower genotypes were known).

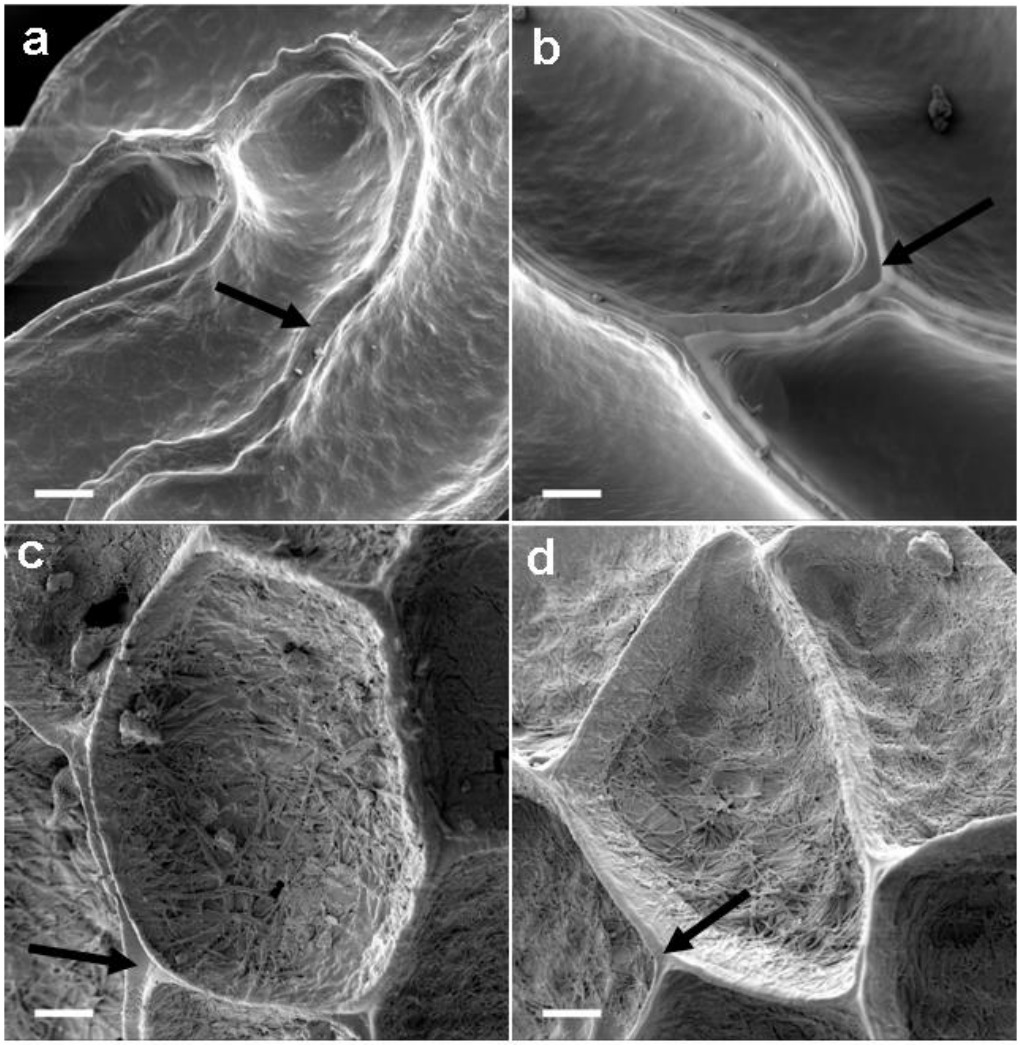

Fig. 2. Scanning electron micrographs showing micromorphology of the testa. a: Detail of the granular surface of the periclinal wall and the irregular bead (arrow) of the anticlinal wall in *O. cumana*; b: Surface and crenated anticlinal cell wall (arrow) in *O. crenata*; c: Detail of fibrous material on the surface of a periclinal wall and crenated anticlinal cell walls (arrow) in *P. ramosa*; d: Similar fibrous material, but additionally a pitted pattern of the surface and also furrowed anticlinal cell walls (arrow) in *P aegyptiaca*; scale bar, 10 µm.

Table 2. Seed size of *Orobanche* and *Phelipanche* samples.

Taxon	Origin	Seed length [µm]	Seed width [µm]	Length/width
P. aegyptiaca	Israel (Maro Hawa)	(220-) 295±41 (-379)	(158-) 207±26 (-259)	1.43±0.23
P. ramosa	Germany (Neupotz)	(211-) 285±32 (-356)	(165-) 205±19 (-240)	1.40±0.20
O. crenata	Israel (Merahusia)	(257-) 346±44 (-447)	(153-) 216±33 (-283)	1.63±0.24
O. cumana	Spain (Córdoba) 2003	(281-) 365±42 (-472)	(113-) 160±19 (-195)	2.31±0.37 ^{ABC}
O. cumana	Spain (Córdoba) 2005	(250-) 339±42 (-420)	(97-) 150±20 (-202)	2.30±0.37 ^{ABC}
O. cumana	Spain (Córdoba) 2008	(264-) 360±51 (-476)	(89-) 150±26 (-202)	2.45±0.42 ^{C}
O. cumana	Serbia (Novi Sad)	(286-) 414±59 (-517)	(136-) 178±21 (-229)	2.37±0.44 ^{BC}
O. cumana	Serbia (Lipar)	(279-) 371±71 (-623)	(116-) 167±44 (-341)	2.27±0.34 ^{ABC}
O. cumana	Serbia (Supliak)	(256-) 351±36 (-428)	(130-) 167±36 (-213)	2.12±0.29 ^{AB}
O. cumana	Serbia (Sombor)	(271-) 382±44 (-425)	(124-) 166±19 (-209)	2.33±0.40 ^{ABC}
O. cumana	Romania (Alexandria)	(280-) 354±40 (-435)	(113-) 150±21 (-211)	2.40±0.38 ^{C}
O. cumana	Romania (Cuza-Voda)	(248-) 332±45 (-417)	(110-) 151±19 (-201)	2.23±0.37 ^{ABC}
O. cumana	Romania (Iazu)	(312-) 381±40 (-452)	(128-) 175±26 (-250)	2.22±0.35 ^{ABC}
O. cumana	Moldova (Chisinau)	(257-) 359±47 (-448)	(116-) 166±25 (-216)	2.21±0.41 ^{ABC}
O. cumana	Moldova (Chisinau)	(212-) 323±52 (-489)	(100-) 140±18 (-175)	2.34±0.48 ^{ABC}
O. cumana	Moldova (Balti)	(274-) 352±39 (-431)	(133-) 170±21 (-206)	2.09±0.31 ^{A}
O. cumana	Russia (Krasnodar, Gulkevitchy)	(297-) 381±39 (-457)	(121-) 165±21 (-213)	2.36±0.42 ^{ABC}
O. cumana	Russia (Rostov, Morozovskiy)	(273-) 415±48 (-550)	(145-) 199±24 (-267)	2.11±0.29 ^{AB}
O. cumana	Russia (Stavro-pol, Krasnogvar-deiskiy)	(312-) 398±39 (-478)	(134-) 174±25 (-248)	2.33±0.38 ^{ABC}

Values represent the mean ± standard deviation of n=50 seeds in µm, numbers in brackets indicate minimum and maximum values. The same letter indicates that differences between lenght/width ratios of *O. cumana*-seed populations are not statistically significant (Tukey test, $P < 0.05$).

DISCUSSION

The study on the seed morphology of *O. cumana* showed clear features suitable to differentiate sunflower broomrape from *O. crenata* and the two species investigated of the sister genus *Phelipanche*. The investigation of the micromorphology of testa cells confirmed the characteristically smooth, pitted inner cell wall of *Orobanche* in comparison to the fibrous texture of *Phelipanche* as previously reported by several groups (Abu Sbaih and Jury, 1994; Thomas et al., 1999; Plaza et al., 2004; Domina and Colombo, 2005; Joel et al., 2012). The irregularly thickened anticlinal walls of the testa were a newly found and unique feature for *O. cumana*, while this wall appeared crenated in the three other taxa studied. The furrowed type of wall is also visible on SEM pictures of several other *Orobanche* species shown in the literature (e.g. Plaza et al., 2004; Domina and Colombo, 2005) and may be worth further attention in future systematic studies of the genus. Amongst the 16 samples of *O. cumana*, no cellular characters were found suitable for the differentiation of geographically defined populations or race (in samples where they could be defined). A similar situation was given for the comparison of morphometric data obtained from seed measurements. The size values found for *P. aegyptiaca*, *P. ramosa* and *O. crenata* were well in the range reported for other samples of these taxa (Abu Sbaih and Jury, 1994; Plaza et al., 2004; Domina and Colombo, 2005). This also accounts for *O. cumana*, for which Plaza et al. (2004) reported size measurements of 360 x 160 to 500 x 250 µm from populations in Spain. The Spanish samples in our study averaged 360 x 155 µm, but also contained single larger seeds up to 476 µm in length and 202 µm in width. Such absolute size differences might be attributed to external factors such as the nutritional situation, climate or unintentional selection of harvested material. In addition, the maturity of seeds at the date of harvest could have an influence. We therefore preferred the comparison of the size ratio between length and width, as we found that this parameter is less affected by the mentioned factors. Taken this into account, *O. cumana* clearly differed from the other species studied by showing a length/width-ratio of well above 2, whereas the other taxa reached values around 1.5. Nevertheless, morphometric measurements did not provide characteristics significant enough for the differentiation of samples from distant origins or different pathotypes. This is in line with several recent reports on the molecular diversity of sunflower broomrape, in which SSR markers (Pineda-Martos et al., 2013) and RAPD amplicons (Molinero-Ruiz et al., 2014) were screened, but also failed to detect race correlation.

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Effect of roots of different sunflower hybrids and bio agent based on *Trichoderma asperellum* on broomrape germination

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ABSTRACT

Broomrape (*Orobanche cumana*) is one of the major sunflower parasites which could cause losses to sunflower production on a worldwide scale. The most efficient way in control of this parasitic plant is growing of resistant or tolerant sunflower hybrids. During breeding process it is very important to test susceptibility of sunflower genotypes to broomrape. The aim of this paper was to evaluate influence of roots of different sunflower hybrids and nutritive media on broomrape seed germination. Hybrids used in the test were: susceptible to broomrape (NS-H-111), high-oleic (HO-B-2) and resistant to broomrape (NORH-34). Evaluation of seed germination was performed on water agar medium with giberellic acid and by adding of sunflower roots. The effect of the biological agent Trifender (based on *Trichoderma asperellum*, Bioved, Hungary) on broomrape seed germination was tested on water agar medium with giberellic acid and by the presence of roots of susceptible hybrid NS-H-111. Seeds of *O. cumana* were collected in the sunflower fields from five localities in Vojvodina during 2009. Surface sterilized seeds were put on nutritive media and incubated at 25°C in the dark. Seed germination and germ length were affected by seed sample, medium (roots of different sunflower hybrids) and interaction between these two factors. The highest number of germinated seeds was observed on media with roots of susceptible NS-H-111 and HO-B-2. Germination and average germ length on medium with bio-agent Trifender plus roots of NS-H-111 was significantly lower.

Key words: Broomrape seed – germination – sunflower hybrids – *Trichoderma asperellum*

INTRODUCTION

The broomrape seed is very small and 1000-seed weight is about 0.001 g. Despite how tiny the seeds are, they can survive in the soil up to the twenty years (Škorić et al., 2012). Seeds of parasitic plants germinate only in the presence of stimulant molecules which are secreted by the root of a suitable host plant (Musselman, 1987; Parker and Riches, 1993). Seed germination is a response to chemical signals from the host plant. *O. cumana* is one of the broomrape species which has intermediate behavior regarding the induction of germination by root exudates of different plant species, although sunflower induced the highest level of germination (Fernández-Aparicio et al., 2009). Moreover, in the absence of the host crop, parasitic seedlings have no chance to survive. Plant resistance to parasitic plants, both non-host and host-specific seems to be a complex multifactorial process (Serghini et al., 2001). This interplay is dependent on the host (species, varieties and populations), the parasite (species and races or populations) and biotic and abiotic factors (Cubero et al., 1994).

In addition to scientific programs which include development and creation of sunflower genotypes resistant to broomrape, intensive research have been conducted to find other ways of the control of this parasite such as chemical and non-chemical control measures. Use of biological control agents has gained its importance in the recent years. Bio-agents could act as germination inhibitors or could cause germ decline and decline of above ground parts of parasitic plants before seed ripening. Biological control in broomrape is associated with fungi from genus *Fusarium* mostly *Fusarium oxysporum* Schlecht. f.sp. *orthoceras* (Appel & Wollenw.) as a broomrape pathogen (Amsellem et al., 2001; Shabana et al., 2003; Muller-Stover et al., 2004; Sauerborn et al., 2007). Thomas et al. (1998) reported that *F. oxysporum* f. sp. *orthoceras* reduced broomrape seed germination and increased sunflower biomass with soil application in controlled conditions. Species such as *F. oxysporum, F. equiseti* and *F. compactum* have been found out as inhibitors of *Orobanche crenata* and *O. ramosa* seed germination (Abouzeid and El-Tarabily, 2010). For many years, *Trichoderma* species are well known as potential bio-agents in control of soil borne plant pathogenic microorganisms (Papavizas, 1985). Their antagonistic effect was registered against some important sunflower pathogens such as *Sclerotinia sclerotiorum* and *Macrophomina phaseolina* (Mansour et al., 2008; Singh et al., 2008).

The aim of this paper was to identify the influence of different sunflower genotypes and bioagent Trifender to germination of broomrape seed from different localities.

MATERIALS AND METHODS

Seeds of *O. cumana* were collected during 2009 in the sunflower fields in Vojvodina at five different localities: Bačka Topola (2/09), Vršac (20/09), Svetozar Miletić (31/09), Vatin (38/09) and Zrenjanin (53/09). Seed samples were kept in the fridge on +4°C to break dormancy and to stimulate germination. The agar germination method is a modified procedure of Hess et al. (1992) and Serghini et al. (2001). Broomrape seed germination was evaluated on 2% water agar medium (WA) with giberellic acid (GA_3, Sigma-Aldrich, USA) which was added in the quantity of 25 mg/L and by presence of 5-8 days old and 5-6 cm long roots of the different sunflower hybrids. Following hybrids were included in the test: susceptible to broomrape (NS-H-111), high-oleic (HO-B-2) and hybrid resistant to broomrape (NORH-34). The effect of the biological agent Trifender (Bioved Hungary, T) on broomrape seed germination was tested on water agar medium with giberellic acid and by presence of roots of susceptible hybrid NS-H-111. Trifender is a biological pesticide from *Trichoderma asperellum* acting as plant growth promoter and biological fertilizer with beneficial side effect to control of soil borne pathogens. Trifender was added in the quantity of 1g/L of WA medium. The check was water agar with GA_3.

Broomrape seeds were surface sterilized in 1% NaOCl for 5 min, rinsed twice in sterile water and incubated on nutritive media at 25°C in the dark. For each seed sample and medium 25 seeds in 4 replicates were used. Germination rate, germ length and distance of germinated broomrape seed from sunflower root were evaluated on 7, 14, 21 and 28 days using binocular microscope. Data were subjected to ArcSin transformation and analyzed by ANOVA and Duncan test using software Statistica 10.

RESULTS

Broomrape seed germination

According to ANOVA there was significant difference in broomrape seed germination after 7 and 28 days (Table 1). Seed germination was affected by seed sample, medium (roots of different sunflower hybrids) and interaction between these two factors. After 7 days seed germination rate ranged from 0-61%. After 28 days the maximum germination rate was 68% and it was achieved by the broomrape seed sample 53/09. Three weeks after the first evaluation, in the majority of tested samples germination rate did not increased significantly, except in the samples 2/09 and 31/09 on the medium with roots of NS-H-111 and 2/09 on the medium with bio-agent Trifender. Differences in germination between media were not observed only for the sample 2/09. In all other tested samples the germination was significantly lower on medium with NORH-34 than on medium with NS-H-111. The samples, 20/09, 38/09 and 53/09 had also significantly lower germination on medium with bio-agent Trifender. On media with susceptible hybrid HOB-2 significantly higher germination was observed for sample 38/09 in comparison to medium with Trifender and NORH-34, as well as for 53/09 in comparison to medium with Trifender.

The broomrape seed sample 2/09 had the best average germination rate (analyzing all media). Good germination rate had also samples 31/09 and 53/09. The poorest germination rate had samples 38/09 and 20/09 originated from Eastern part of Vojvodina. The broomrape seed samples 2/09 and 31/09 originated from Northen West part of Vojvodina.

In general the highest number of germinated seeds was observed on media with roots of susceptible hybrids NS-H-111 and HO-B-2. Germination rate was much lower on media with NORH-34 and on media with bio-agent Trifender. However, the germination rate on GA_3 medium without presence of sunflower roots was very low. On this medium germination was observed for samples 2/09 and 31/09.

Table 1. Germination of broomrape seed on different nutritive media

Broomrape sample	medium	Number of germinated seeds (%)	
		after7 days	after 28 days
2/09	GA+NS-H-111	40.0 ^{abc}	54.0^{abcd}
	GA+HO-B-2	60.0 ^{a}	62.0 ^{abc}
	GA+NORH-34	36.0 ^{abcd}	44.0 ^{abcde}
	GA+T+NS-H-111	14.0 ^{defg}	39.0 ^{bcde}
	GA	0.0^{j}	1.0^{ij}
20/09	GA+NS-H-111	37.0 ^{abc}	41.0 ^{bcde}
	GA+HO-B-2	10.0 ^{fghi}	13.0^{ghi}
	GA+NORH-34	10.0 ^{ghi}	13.0 ^{ghi}
	GA+T+NS-H-111	10.0 ^{efgh}	13.0^{fgh}
	GA	0.0 ^{j}	0.0^{j}
31/09	GA+NS-H-111	51.0 ^{ab}	65.0 ^{ab}
	GA+HO-B-2	32.0 ^{bcd}	35.0 ^{cdef}
	GA+NORH-34	18.0 ^{cdef}	22.0^{efgh}
	GA+T+NS-H-111	31.0 ^{bcde}	41.0 ^{abcde}
	GA	0.0 ^{j}	1.0 ^{ij}
38/09	GA+NS-H-111	33.0 ^{bcde}	35.0 ^{defg}
	GA+HO-B-2	40.0 ^{abc}	40.0 ^{bcde}
	GA+NORH-34	6.0^{ghij}	8.0^{hij}
	GA+T+NS-H-111	2.0 ^{hij}	4.0^{hij}
	GA	0.0^{j}	0.0^{j}
53/09	GA+NS-H-111	61.0 ^{a}	68.0 ^{a}
	GA+HO-B-2	41.0 ^{abc}	47.0 ^{abcde}
	GA+NORH-34	25.0^{cde}	34.0^{defg}
	GA+T+NS-H-111	7.0 ^{fghij}	15.0^{fgh}
	GA	0.0 ^{j}	0.0^{j}
p	broomrape seed sample	0.00**	0.00**
p	medium	0.00**	0.00**
p	broomrape seed sample *medium	0.00**	0.00**

Broomrape germ length and distance of germinated broomrape seed from sunflower root

There were differences between germ lengths in tested samples. The significant influence to the germ length had medium, seed sample and the interaction of these factors. The germ length ranged from 0.17-1.2 mm (Table 2).

Average germ length on medium with bio-agent Trifender plus roots of NS-H-111 was significantly shorter in all tested broomrape samples and in general (Tables 2 and 3). These results indicate that used bio-agent has the effect not only on germination rate, but also on germ length. In two tested samples 20/09 and 38/09 germ length on the medium with roots of resistant hybrid NORH-34 was not significantly different from those on medium with Trifender. Measuring lengths of broomrape germs showed that the longest were those on media with roots of NS-H-111 and HO-B-2.

Broomrape seeds were placed on different distances from sunflower root. After 7 days, the distance between every germinated broomrape seed and sunflower roots was measured during the period 14-28 days (Table 2 and 3). The average distance of the germinated seeds and sunflower roots for all media was 11.2 mm after 7 days and 12.3 mm in the period from 14 to 28 days (Table 2). It could indicate that broomrape seed which was closer to the sunflower root germinated earlier. In the first evaluation period nutritive medium and seed sample had the significant influence to the distance of the germinated seed, while there was no significant difference in distances in the second period (14-28 days) (Table 2). Average distance of germinated broomrape seeds from sunflower roots on medium with Trifender was the shortest. There is no significant difference in distance between germinated seeds and sunflower roots on the other tested media (Table 3).

Table 2. Germ length and distance of germinated broomrape seed from sunflower root

Broomrape sample	medium	Germ length (mm)	Average distance of germinated seed from sunflower root (mm)	
			after 7 days	after 14-28 days
2/09	GA+NS-H-111	0.9^{ab}	14.2^{ab}	14.0^{abcd}
	GA+HO-B-2	1.2^{a}	15.2^{ab}	17.7^{ab}
	GA+NORH-34	1.2^{a}	13.5^{ab}	12.4^{abcd}
	GA+T+NS-H-111	0.3^{b}	16.1^{a}	12.2^{abcd}
20/09	GA+NS-H-111	1.2^{ab}	7.1^{bcd}	12.0^{abcd}
	GA+HO-B-2	$0,7^{bcd}$	10.5^{abc}	5.8^{cd}
	GA+NORH-34	0.5^{cdef}	3.0^{cd}	10.9^{abcd}
	GA+T+NS-H-111	0.5^{cdef}	4.1^{cd}	8.4^{abcd}
31/09	GA+NS-H-111	1.0^{ab}	10.5^{abc}	13.4^{abcd}
	GA+HO-B-2	0.8^{abcd}	16.0^{a}	18.9^{a}
	GA+NORH-34	0.8^{abcd}	14.2^{ab}	14.3^{abcd}
	GA+T+NS-H-111	0.4^{ef}	8.8^{abcd}	13.3^{abcd}
38/09	GA+NS-H-111	1.0^{ab}	15.9^{a}	18.2^{a}
	GA+HO-B-2	1.0^{ab}	13.8^{ab}	13.8^{abcd}
	GA+NORH-34	0.4^{def}	8.8^{abcd}	10.1^{abcd}
	GA+T+NS-H-111	0.2^{f}	1.3^{d}	3.5^{d}
53/09	GA+NS-H-111	1.0^{ab}	12.6^{ab}	12.2^{abcd}
	GA+HO-B-2	0.9^{abc}	15.9^{a}	12.3^{abcd}
	GA+NORH-34	1.0^{ab}	13.8^{ab}	15.7^{abc}
	GA+T+NS-H-111	0.2^{f}	8.3^{abcd}	7.0^{bcd}
	average	***0.8***	***11.2***	***12.3***
p	broomrape seed sample	0.049*	0.00**	0.10ns
P	medium	0.00**	0.00**	0.06ns
P	broomrape seed sample *medium	0.02*	0.10ns	0.37ns

Table 3. Germ length and average distance of germinated broomrape seed from sunflower root on different nutritive media

medium	Germ length (mm)	Distance of germinated broomrape seed from sunflower root on different nutritive media (mm)	
		after 7 days	after 28 days
GA+NS-H-111	1.1^{a}	12.0^{a}	14.0^{a}
GA+HO-B-2	0.9^{ab}	14.3^{a}	13.7^{a}
GA+NORH-34	0.8^{b}	10.6^{ab}	12.7^{ab}
GA+T+NS-H-111	0.3^{c}	7.7^{b}	8.9^{b}
p	0.00**	0.005**	0.07 ns

DISCUSSION

According to our previous investigations water agar medium with gibberellic acid and with the presence of sunflower roots, was a good medium for testing of broomrape seed germination *in vitro* (Maširević et al., 2011). Our results that the best germination rate of broomrape seed was obtained in the presence of roots of susceptible sunflower hybrids are in the accordance with results of Glijin et al. (2011). These authors recorded the best broomrape seed germination on exudates of two differential lines for the broomrape race G and emphasized that germination ability is strictly dependent on the root exudate. Broomrape seeds germinate only in presence of sunflower roots as we obtained in our trials. Fernández-Aparicio et al. (2008) pointed out the fact that some sunflower genotypes have poorer stimulating effect on germination of broomrape seed. Differences in genotypes could be caused by the quantity of stimulating substances in the root exudate or by the different production of germinate inhibitors, but also by the combination of these two factors. Variability in receptors for the stimulating substances in the parasite could affect the germination.

A number of secondary metabolites which induce germination of parasitic plants belong to the group of strigolactones (Bouwmeester et al., 2003). The recent results of Joel et al. (2011) and Raupp and Spring (2013) have been indicated that *O. cumana* may recognize its natural host exclusively by sesquiterpene lactones or by compounds of yet unknown chemical nature. Eizenberg et al. (2004) reported that resistant genotypes stimulate broomrape seed germination as well as susceptible, but the difference is that parasite haustorium stopped with development after penetration into the resistant plant

tissue. However, Wegmann (2004) in his investigations underlined the importance of stimulators and presence of germination inhibitors in resistant genotypes. Our findings showed that susceptible genotypes induced significantly higher germination rate than resistant ones. That is in the accordance with the results obtained by Serghini et al. (2001) who reported that broomrape seed had approximately half germination in the presence of resistant sunflower genotypes than in susceptible. However, Labrousse et al. (2004) indicated that there is no correlation in the resistance of tested lines and germination of broomrape seed. Also Echevarrıa-Zomeño et al. (2006) reported that *O. cumana* seed germination is unlikely to account for the differences in resistance. Different germination response of broomrape seed samples as we obtained in the trail could be also due to different origin of broomrape samples. Joel et al. (2011) reported about the differences in the germination response of the various *O. cumana* populations to sunflower root exudates. Results presented by Fernández-Aparicio et al. (2012) shows that resistance based on low induction of broomrape seed germination do exist in faba bean germplasm.

Close vicinity of broomrape seed to sunflower root has very important role in stimulation of broomrape germination. Joel et al. (2011) reported that at a distance of 1–2 cm from the sunflower roots germination is normally lower than between roots. These findings are in accordance with the results from our trial where broomrape seeds which were closer to the sunflower roots germinated earlier.

In our experiments carried out in the control conditions bio-agent on the basis of *T. asperellum* affected not only broomrape seed germination, but also decreased germ length. Fan et al. (2003) tested 23 bio-agents known as resistance inducing agents in order to control *O.cumana* in sunflower under greenhouse conditions. However, these authors reported that bio agent based on *T. harzianum* insignificantly increased the infestation of *O.cumana* in comparison to the control. The fungal biocontrol agent *Trichoderma asperellum* has been recently shown to induce systemic resistance in plants through a mechanism that employs jasmonic acid and ethylene signal transduction pathways (Shoresh et al., 2006). Bioagent Trifender in concentration of 5% had high antagonistic effect to *Phytium aphanidermatum* and *S.sclerotiorum in vitro* (up to 100%) (Mihajlović et al., 2013).

According to our results broomrape seed germination in the presence of roots of different sunflower hybrids could be used as one of the method in the preliminary testing of genotypes with different level of resistance to the broomrape. The effect of bio-agent Trifender on sunflower broomrape should be further tested in green house and field in conditions of artificial inoculations.

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SU-01, a novel germination stimulant for root parasitic weeds from sunflower

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ABSTRACT

Root exudates of sunflower line 2607A exhibited germination inducing activity on seeds of the root parasitic weeds *Orobanche cumana*, *O. minor*, *O. crenata*, *Phelipanche aegyptiaca* and *Striga hermonthica*. Bioassay-guided purification led to the isolation of a novel germination stimulant. The FT-MS analysis indicated that the molecular formula of the stimulant is $C_{20}H_{24}O_6$. Detailed NMR studies revealed that the stimulant is a strigolactone-like compound with a methylfuranone group connected to a C_{15} part via an enol ether bridge. The stimulant production in aquaculture increased when sunflower plants were grown hydroponically in tap water. Supplementation with either phosphorus or nitrogen reduced stimulant production. Costunolide, a sesquiterpene lactone, already identified in sunflower root exudates as a germination stimulant for *O. cumana*, was detected at a higher concentration in a well-nourished medium than nutrition-deficient media, suggesting the contrasting contribution of the strigolactone and the sesquiterpene lactone to germination induction of *O. cumana* under different soil fertility levels.

Key words: Germination – *Orobanche* – *Striga* – strigolactone – sunflower

INTRODUCTION

Root parasitic weeds of the genera *Striga*, *Orobanche* and *Phelipanche* adversely affect many important food crops. The germination of root parasitic weed seeds depends on chemical signals released from host and non-host plants into the rhizosphere. Since the identification of strigol (Cook et al., 1972), a series of analogous compounds have been isolated. Strigol and analogous compounds, generally known as strigolactones, share a common structure composed of a tricyclic lactone connected via an enol ether bridge to a methylfuranone group. Strigolactones were recently identified as a branching factor for arbuscular mycorrhizal fungi and a new class of plant hormones that play a key role in the regulation of plant architecture (Ruyter-Spira et al., 2013). The root holoparasitic plant *O. cumana* presents a serious constraint to sunflower (*Helianthus annuus* L.) production in Southeast Europe, the Middle East and Southwest Asia (Parker, 2013). Four sesquiterpene lactones, dehydrocostus lactone (DCL) (Joel et al., 2011), costunolide, tomentosin and 8-epixanthatin (Raupp and Spring, 2013) isolated from sunflower root exudates, were identified as germination stimulants for *O. cumana* seeds. Yoneyama et al. (2011) reported the detection of signals by LC-MS/MS corresponding to the strigolactones 5-deoxystrigol and alectrol in sunflower root exudates. However, fractions eluted at their respective retention times did not induce germination of *O. minor* seeds. Considering the biological importance of strigolactones as an exogenous factor for arbuscular mycorrhizal fungi and an endogenous plant hormone as well as their extensive distribution in the plant kingdom (Ruyter-Spira et al., 2013), production of strigolactones by sunflower is logical. It was observed that sunflower root exudates induced germination of *S. hermonthica* seeds whereas the commercially available sesquiterpene lactones DCL and costunolide did not. Failure of DCL and costunolide to induce germination of *S. hermonthica* led to probing sunflower root exudates for non-sesquiterpene lactone germination stimulant(s). This paper reports isolation and identification of a novel strigolactone-like molecule from sunflower root exudates, its germination inducing activity on seeds of several root parasitic weeds and the effects of phosphorus and nitrogen on its production.

MATERIALS AND METHODS

Chemicals

DCL and costunolide were purchased from Wako, Osaka, Japan and Extrasynthese, Genay, France, respectively. GR24 was synthesized according to the method of Mangnus et al. (1992).

Plant materials

Sunflower seeds from line 2607A were obtained from Dobrudja Agricultural Institute, Bulgaria. Seeds of *O. cumana* and *O. minor* were collected from plants parasitizing sunflower in Dobrich region, Bulgaria and red clover in Yokohama, Japan, respectively. Seeds of other parasitic weeds *O. crenata*, *P. aegyptiaca*, *S. hermonthica*, and *S. gesnerioides* were supplied by Dr. Daniel Joel, Newe-Ya'ar Research Center, Israel, Dr. Yaakov Goldwasser, The Hebrew University of Jerusalem, Israel, Prof. Abdel Gabar Babiker, Sudan University of Science and Technology, Sudan, and Dr. Satoru Muranaka, International Institute of Tropical Agriculture, Nigeria, respectively.

Germination bioassay

The germination assay was conducted as reported by Sugimoto et al. (2003). The surface sterilized seeds were pretreated (conditioned) for 8–12 days at 30 ºC for *S. hermonthica* and at 23 ºC for other parasites on 8 mm glass fiber filter paper disks (*ca*. 50 seeds each) placed on distilled water-saturated filter paper. Aliquots (20 μL) of dilution series of test solutions were assayed by applying them to the conditioned seeds on 8-mm disks. The treated seeds were incubated at the same temperature as conditioning and were microscopically examined for germination (radicle protrusion) after 24 to 72 h.

Hydroponic culture and collection of root exudates of sunflower

Fifteen sunflower seedlings were grown hydroponically in a 30 L tank filled with 40% Long-Ashton nutrient solution in which phosphate and nitrogen concentrations were adjusted to 0.13 mM and 5 mM, respectively. The medium was continuously circulated with a pump, and sunflower root exudates of 2 to 8-week-old seedlings were collected by adsorbing on activated charcoal (12 g) as described previously (Ueno et al., 2011). The charcoal was collected every week and soaked in 80 mL of acetone for a couple of days at 4ºC. After evaporating acetone, the residual aqueous solution (*ca*. 20 mL) was treated three times with EtOAc of a total volume of 20 mL, and the organic layer was washed with 0.2 M K_2HPO_4 (pH 8.3), dried over Na_2SO_4 and concentrated in vacuo.

Isolation of germination stimulants

The concentrated EtOAc extracts of sunflower root exudates obtained from 28 replications were combined and subjected to column chromatography on silica gel (20 g) with a stepwise elution of *n*-hexane/EtOAc (100:0–0:100, 10% step, 50 mL x 2 in each step) and subsequent MeOH to give fractions 1-23. Distinct germination inducing activity toward *O. cumana* and *O. minor* was detected in fractions 9 and 10 (40% EtOAc) and 16 (70% EtOAc). Germination inducing activity toward *S. hermonthica* was in fraction 10. Fraction 10, the most active, was concentrated in vacuo and subjected to reversed-phase HPLC on a 250 x 20 mm i.d., 5 μm, CAPCELL PAK C18 UG120 S5 (Shiseido, Yokohama, Japan) with 60% MeOH in H_2O at 5 mL/min with monitoring at 235 nm. The active ingredients toward *S. hermonthica* were eluted at 24 min and 43 min. The stimulant eluted at 43 min showed the ion at *m/z* 361 in parent ion scan analysis in LC-MS/MS and the fragment ions at *m/z* 233 and 97 in daughter ion scan analysis of *m/z* 361. The stimulant eluted at 24 min showed the ion at *m/z* 377. The conversion of the stimulant of *m/z* 361 to that of *m/z* 377 was observed in the purification process, indicating that the latter is an artifact, probably the oxidized product of the former. Accordingly, the purification work was concentrated on the stimulant eluted at 43 min. The stimulant eluted at 43 min was subsequently subjected to chiral HPLC on a 250 x 10 mm i.d., 5 μm, CHIRALPAK IC (Daicel, Osaka, Japan) with 50% EtOH in *n*-hexane at 1 mL/min with monitoring at 235 nm. The stimulant eluted at 41 min was collected, concentrated, and passed through a silica gel column with 25% EtOAc in $CHCl_3$.

Extraction of sesquiterpene lactones together with the strigolactone-like compound

In each of small containers filled with 40% Long-Ashton nutrient solution (1 L), four sunflower seedlings were grown with a pump for aeration for 13 days. The solution was replaced by tap water, tap water supplemented with 1.33 mM Na_2HPO_4, tap water supplemented with 5 mM NH_4NO_3 or 40% Long-Ashton nutrient solution and plants were allowed to grow for 3 days prior to collection of culture filtrates. The culture filtrates were subsequently subjected to liquid-liquid partitioning using EtOAc three times with a total volume of 500 mL. The EtOAc was evaporated and the residue, dissolved in a small amount of hexane and $CHCl_3$, was roughly purified by silica gel column chromatography (10 x 3 mm i.d.) with

30% EtOAc in $CHCl_3$ (10 mL). The residue, dried, dissolved in acetonitrile (100 µL) was analyzed for sesquiterpene lactones and the strigolactone-like compound using LC-MS/MS.

RESULTS AND DISCUSSION

Isolation and structural determination

Bioassay-guided purification of sunflower root exudates by silica-gel column chromatography led to separation of active fractions that induced germination of *S. hermonthica* seeds. Fraction 10, the most active, was found to contain a novel germination stimulant with a molecular mass of 360 on LC-MS/MS analysis. The stimulant was purified with reversed-phase HPLC, followed by chiral HPLC to give an almost pure compound henceforth referred to as SU-01. The FT-MS analysis of SU-01 afforded the sodium adduct ion at *m/z* 383.1457 $[M+Na]^+$ calculated for $C_{20}H_{24}O_6Na$. Detailed NMR analysis of SU-01, including ^1H-^1H COSY, HMBC, HMQC, NOE, suggested the presence of a methylfuranone ring, a common structural component of strigolactones as the D-ring. Other resonances suggest a strigolactone-like structure with the D-ring connected to a C_{15} part via an enol ether bridge. The CD spectrum of SU-01 showed a positive and negative Cotton effect around 220 nm and 255 nm, respectively, suggesting that SU-01 has *R* configuration at C-2'. Research to confirm the proposed structure of the stimulant is ongoing.

Response of seeds of root parasitic weeds to SU-01 and sesquiterpene lactones

The germination inducing activities of SU-01 on seeds of *O. cumana*, *O. minor*, *O. crenata*, *P. aegyptiaca*, *S. hermonthica* and *S. gesnerioides* are shown in Fig. 1. SU-01 exhibited a broader spectrum of activity than DCL and costunolide. SU-01 strongly elicited germination of *O. minor*, followed by *S. hermonthica*. Seeds of *O. cumana*, *O. crenata* and *P. aegyptiaca* exhibited much weaker responses to SU-01. On the other hand, DCL and costunolide induced germination of *O. minor* and *O. cumana* seeds with a much weaker response of the latter. These sesquiterpene lactones were not effective on the other parasite seeds tested in this study.

Effects of phosphorus and nitrogen on the production of SU-01 and costunolide

As has been well documented, strigolactones function as a branching factor for arbuscular mycorrhizal fungi and therefore their biosynthesis is promoted in host plants under low phosphorus conditions. Nitrogen deficiency is also reported to affect the production of strigolactones (Yoneyama et al., 2012). In sunflower root exudates, substantial amount of costunolide was detected while SU-01 content was below the detection limit under conditions of high phosphorus and high nitrogen. Removal of phosphorus or nitrogen drastically reduced the production of costunolide. On the other hand, increase of SU-01 was observed under conditions lacking both phosphorus and nitrogen. It should be noted that DCL content was negligible under any aquaculture conditions tested in our experiments. If root parasitic weed seeds respond only to strigolactones, they have little chances to germinate in soils with high fertility. The extended responsiveness to sesquiterpene lactones may be an adaptation of *O. cumana* to ensure germination and parasitization to sunflower under a broad range of soil fertility.

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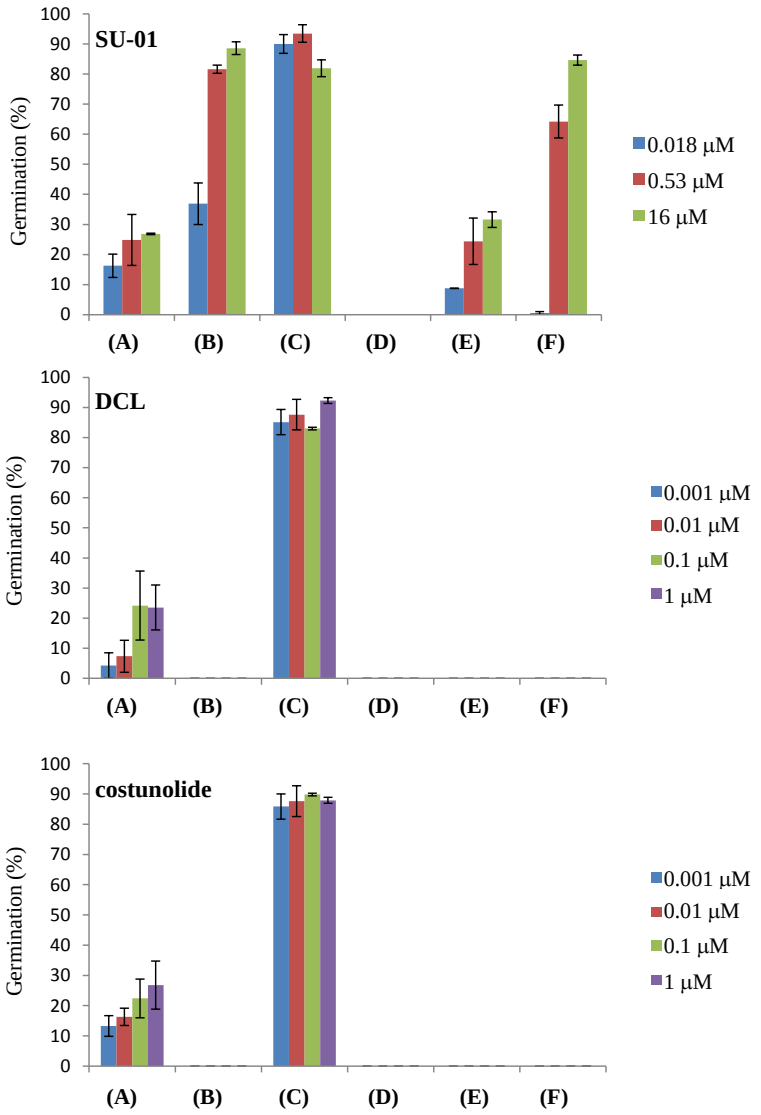

Fig. 1. Effects of SU-01, DCL and costunolide on germination of seeds of *O. cumana* (A), *S. hermonthica* (B), *O. minor* (C), *S. gesnerioides* (D), *P. aegyptiaca* (E), and *O. crenata* (F). Data are shown as mean ± S.E. (n=3).

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Diagnosis of the infection of sunflower by *Orobanche cumana* using multicolour fluorescence imaging

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ABSTRACT

Orobanche cumana is an holoparasite and thus totally dependent on sunflower for fixed carbon. Initial stages of the infection occur in the first weeks after sowing and are critical for the establishment of a continuum between the host and the parasite vascular system. From that moment the parasite obtains its supply of water, mineral nutrients and assimilates from the host plant. Alterations of plant photosynthesis can be detected using remote sensing techniques for detection of fluorescence emitted by plants. One of these indirect techniques is multicolour fluorescence imaging. In this work we assessed the early infection of sunflower by *O. cumana* using multicolour fluorescence imaging and we inferred physiological processes affected in sunflower plants infected by the parasite. Ten germinated seeds of the inbred line NR5 were inoculated with population LP2013 of *O. cumana*. The same number of not inoculated seeds was used as control. Sunflower was planted in pots with soil mixture and grown in greenhouse at 12-22ºC for six weeks. Multicolour fluorescence imaging was conducted three, four and five weeks after inoculation. The two first pairs of fully expanded leaves of each sunflower plant were imaged, and, for each measure date, five fluorescence variables in inoculated plants were compared to those in the control. Three weeks after inoculation, when symptoms of infection were still not observed, decreased levels of blue and green fluorescence and increased far-red fluorescence were observed in leaves of the inoculated plants. In subsequent measures four and five weeks after inoculation, when inoculated plants displayed symptoms of infection by *O. cumana*, differences of fluorescence between inoculated plants and the controls were the same and statistically supported. Our results show an increase in total chlorophyll content of sunflower plants infected by *O. cumana* which is probably related to the need of higher photosynthetic activity in order to supply the parasite with photosynthate. Biochemical mechanisms underlying photosynthesis impairment must be further investigated. The results obtained three weeks after inoculation show that multicolour fluorescence imaging can detect fluorescence differences in inoculated sunflower at early time. Therefore, this technique can be used as a diagnostic tool for early detection of genotypes of sunflower which are susceptible or resistant to *O. cumana*.

Key words: Early detection – *Helianthus annuus* L. – multicolour fluorescence – photosynthesis – secondary metabolites

INTRODUCTION

Orobanche cumana Wallr. is a parasitic plant that establishes in the root of *Helianthus* spp., including the crop species *H. annuus*. The attachment of seeds of *O. cumana* to the roots of sunflower and the establishment of the nutritional structure or haustorium are critical initial stages of the infection that result in the underground development of bulbous tubercles. During the time these infective processes take place (approximately in the first month after sowing), a continuum between the host and the parasite vascular systems is formed. The parasite then obtains its supply of water, mineral nutrients, and assimilates from the host plant. Because of the absence of functional chloroplasts, *O. cumana* is defined as an holoparasite, and is thus totally dependent on sunflower for fixed carbon.

Parasitic plants, including *Orobanche* spp., reduce the growth of their hosts through competition for water and nutrients, C transfer from host to parasite, and physiological dysfunction of the host plants (Stewart and Press, 1990). The rate of transpiration of parasitic angiosperms is often higher than that of host species. This maintains a water potential gradient towards the parasite, facilitating the transfer of organic and inorganic solutes to it (Blamey et al., 1997).

The infection of *O. cumana* in sunflower is commonly assessed by visual observation of symptoms, which appear in the crop around flowering and show as non specific water stress. Prior to that, the infection of sunflower by *O. cumana* is indicated by the presence of tubercles in the roots of the plant or by the emergence of parasite stems aboveground. The development of macroscopically detectable tubercles or, later, of emerged holoparasite stems depend on inoculum density of *O. cumana* and also on growth conditions of the host. It can take place in 26 d after inoculation (dai) for detection of underground

tubercles (García-Carneros et al., 2014) or 35 dai in the case of initial emergence of broomrape stems (Molinero-Ruiz et al., 2008).

Water stress and alterations of plant photosynthesis caused by either pathogen infection or drought can be early detected using remote sensing approaches using fluorescence emitted by plants in the infrared or the visible regions of the spectrum. One of these techniques, known as multicolour fluorescence imaging, is based on the detection of fluorescence in the blue, green, red and far-red regions and, although indirect, it is a highly sensitive, non-destructive and non-subjective tool to study mainly the activity of the secondary metabolism. Analyses of multicolour fluorescence imaging and multispectral imaging have been successfully applied for the detection of pathogen infection in plant species (Dammer et al., 2011; Calderón et al., 2013). The objective of this work is to assess the infection of sunflower by *O. cumana* in early stages using multicolour fluorescence imaging and to infer physiological processes affected in the infected sunflower plants.

MATERIALS AND METHODS

The population LP2013 of *O. cumana* was inoculated onto the susceptible sunflower inbred line NR5 following previous methodology (García-Carneros et al., 2014). Ten seedlings were transplanted into soil mixture infested with *O. cumana* at an inoculum density of 0.02 mg of parasite seeds/g soil. Ten germinated seeds were transplanted to un-infested soil mixture and used as controls. Plants were grown in glasshouse at 12-22 ºC for six weeks and watered as needed. At the end of the experiment sunflower plants were uprooted to visually assess the presence of nodules of the parasite in the roots.

Multicolour fluorescence imaging was conducted starting at horizontal development of the first pair of true leaves in the plants, which occurred three weeks after inoculation (wai). Thereafter measures were taken 4 and 5 wai. In each plant all upper true leaves were simultaneously imaged for 120 sec using an Open FluorCam FC 800-O (Photon Systems Instruments, Brno, Czech Republic). Images were captured always approximately at the same day time and with plants having similar water status. Each measure day, and prior to imaging, plants were visually assessed. Control and inoculated plants were confirmed to have the same number of similarly developed true leaves. The Fluorcam7 software was used to analyze the images. For each measure date, mean values of fluorescence at 440 nm (blue), 520 nm (green), 680 nm and 740 nm (red) as well as the ratios 440/740 (blue normalized to far-red) and 520/740 (green normalized to far-red) were considered. When clear trends of the variables were observed the robustness of the data was statistically assessed by analysis of variance (ANOVA) according to a complete randomized design. The effect of treatment (control or inoculation) was analysed for individual pairs of true leaves with one measure per leaf (replication); between four and eight replications were considered.

RESULTS

No significant differences on fluorescence emission were observed between the two pairs of fully expanded leaves present in the plants. Therefore, and although all the pairs were analysed, only results for the first pair of leaves are presented. Three weeks after inoculation none of the inoculated sunflower plants displayed visual symptoms of infection. One week later, 4 wai, symptoms consisting on reduced height of plants, and small size of leaves, appeared in inoculated plants. The time course of the five fluorescence variables measured in control *vs.* inoculated plants is presented in Fig. 1.

In the first measurement date (3 wai), decreases of the blue and green fluorescence were observed in the inoculated plants (132 and 360 respectively) as compared to the controls (149 and 415 respectively). Conversely, they showed higher far-red fluorescence than the controls (1275 and 875 respectively). The same trends than those of blue and green fluorescence were obtained for the ratios 440/740 and 520/740 (Fig. 1).

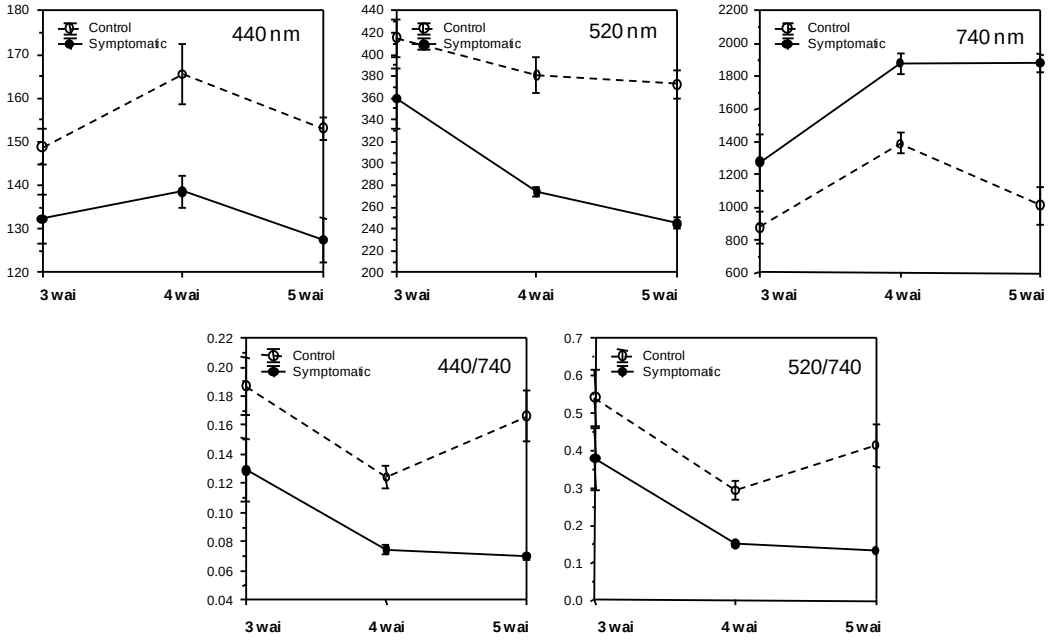

Fig. 1. Time course of fluorescence variables measured in the first pair of leaves of control plants and symptomatic plants of sunflower infected by *Orobanche cumana*.

One week later, differences of fluorescence parameters between symptomatic plants and the controls were clear and statistically supported. Leaves of symptomatic plants presented a significant lower fluorescence than the one of the control plants at 440 nm (138, $p \leq 0.0032$) and 520 nm (274, $p < 0.0001$). Far-red fluorescence of leaves was significantly higher ($p < 0.0001$) in symptomatic sunflower than in the controls (1879 and 1389 respectively) (Fig. 1). Similar results were obtained at final time; significant ($p < 0.0001$) decreases of blue and green fluorescence were measured in the first pair of leaves in symptomatic plants (127 and 245 respectively) as compared to control plants (153 and 373 respectively). Leaves fluorescence at 740 nm was significantly higher in symptomatic plants than in the controls: 1282 and 1011 respectively. Differences between symptomatic plants and the controls were confirmed at both dates (4 and 5 wai) by normalized measures: 440/740 and 520/740 (Fig. 1).

DISCUSSION

Low levels of blue and green fluorescence were obtained early in inoculated plants and kept lower than those in the controls along the time. A decrease in blue and particularly green fluorescence intensity suggests a decrease in the accumulation of secondary metabolites in the host plant (see review by Buschmann and Lichtenthaler, 1998). In parasitized plants the quantitative modification of secondary metabolites can be the result of a decrease in metabolic activities in favour of an increased photosynthesis (Barón et al., 2012). An increase in chlorophyll content was suggested by the increase in far-red fluorescence (emitted by chlorophyll *a*) which was early detected in inoculated sunflower. We suggest the increase in total chlorophyll content might be related to an increase in the content on photosystems in the thylakoid, possibly elicited by the need of higher photosynthetic activity in order to supply the parasite with photosynthate.

Therefore, our results show that *O. cumana*, which depends on the host plant for carbohydrates, affects sunflower secondary metabolism although it is likely that holoparasitic plants interact with host photosynthesis mostly by manipulating the source-sink interactions in the plant (Barón et al., 2012). Altering carbon fixation have been reported to have a variety of consequences, such as leaf mass reduction in tomato plants infected by *O. aegyptiaca* (Barker et al., 1996) or increased carbon allocation and delayed leaf senescence in normal-sized leaves of tobacco plants infected by *O. cernua* (Hibberd et al., 1998). The effect of the infection of *O. cumana* on the growth and development of sunflower has been explained by reductions in biomass, shoot dry weight, height and head diameter, but significant reductions in biomass have not been observed until initiation of parasite emergence aboveground (Alcántara et al., 2006). The biochemical mechanisms underlying the photosynthesis impairment in parasitized sunflower plants during initial stages of parasitism by *O. cumana* must be further investigated.

Multicolour fluorescence imaging allowed us to discriminate infected from non-infected plants at an early time (3 wai), when symptoms of the infection by *O. cumana* were not visually detected in sunflower. Our work is not only the first application of the multicolour fluorescence imaging for the analysis of how the parasitism of *O. cumana* affects secondary metabolism of sunflower, but it also constitutes the evidence that this technique can be used as a tool for early diagnosis of infection of the host by the holoparasite. Because early stages of infection are the most efficient targets for genetic resistance of *H. annuus* against the root parasite (Serghini et al., 2001) and genetic resistance remains the most important measure for controlling sunflower broomrape (Fernández-Martínez et al., 2010), one of the applications of this technique might be the early diagnosis of resistance and/or susceptibility of genotypes of sunflower to *O. cumana*.

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Agroecology of broomrape *Orobanche cumana* distribution in five continents

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ABSTRACT

The factors associated with the distribution of parasitic weed *Orobanche cumana* Wallr. (Broomrape), limiting sunflower production in Europe and the surrounding continents of Asia and Africa, have not been adequately investigated. The goals of this study were to broaden the understanding of environmental factors associated with broomrape' distribution in Europe, and to predict suitable habitats based on environmental factors which would be vulnerable to invasion and establishment of broomrape in North and South America. A robust agroecological database included 35 quantitative parameters associated with trials conducted by ten public research organizations from five continents. The database consisted of 117 sites (habitats), Europe (79), Africa (3), Asia (6), and Americas (29), equally distributed between invaded and non- invaded habitats. Environmental parameters analyzed using an ANOVA and PCA showed that all sunflower areas from Europe were vulnerable to broomrape attack. The parasitic weed develops in soil and climate environmental conditions similar to non-invaded areas. Its expansion to nearby areas of the Africa and Asia continents seems to indicate a broadening of the environmental conditions to which the parasitic weed can tolerate. Habitat conditions of sunflower crop areas in South America showed more similarity to invaded habitats than North America. The absence of the ability to predict actual broomrape distribution based on environmental factors enforces the need to use cultural practices of crop rotation and genetic resistance in infected areas. Strict phytosanitary controls need to be enforced to prevent the inadvertent introduction into non-infected areas and its spread in infected areas.

Key words: Crop-weed complex – parasitic weed – plant invasion – sunflower diseases

INTRODUCTION

The sunflower (*Helianthus annuus* L) parasitic weed *Orobanche cumana* Wallr. (Broomrape), native to the actual territory of Russia, is today widely distributed in all of Europe and surrounding regions of Asia Minor and Africa. There is a high incidence of this weed in the Black Sea region (Antonova et al., 2009), territories of Serbia (Masirevic and Medic-Pap, 2009), Romania (Pricop et al., 2011), Turkey (Dermici and Kaya, 2009), and Spain (Fernández Martínez et al., 2010). The weed has also been reported in Israel, Asia (Eizenberg et al., 2003), and Tunisia, Africa (Amri et al., 2012).

The weed is absent in the centre of origin of sunflower in North America and in the main crop production areas of South and North America. The risk of invasion seems to be high due to the small size of broomrape seed that could adhere to seed pericarp, and the high amount of sunflower seed exchange with Europe. This is of concern since seed trade intensity is highly associated with plant invasions (Nuñez and Pauchard, 2010). The strict phytosanitary controls, or limitations of abiotic habitat parameters associated with plant distribution and invasion could have limited their naturalization in the Americas (Kolar and Lodge, 2001).

Plant invasions dynamics could be explained and predicted by biogeographical parameters and environmental factors (Hierro et al., 2005; Radosevich et al., 2005; Jones, 2011; McGone et al., 2011). The soil biology, chemical and physic parameters are usual determinants of plant distribution and the composition of vegetal community (Dodd et al., 2002; Reinhart and Callaway, 2006) and texture affects the composition of a vegetal community. The seed germination and plant development of broomrape on sunflower is a highly climatic dependent process (Song et al., 2005; Ephrath and Eizenberg, 2010).

The information about environmental factors associated with broomrape distribution on a global scale is deficient. In a previous explorative study, we failed to explain the absence of *Orobanche* in some habitats of Serbia based on abiotic factors (Miladinović et al., 2012). Also, we did not find any good

environmental factors that explained their absence in Argentina (Cantamutto et al., 2012). From a biological invasion perspective, this could mean vulnerability for invasion in all non-invaded regions. The goals of this study were to broaden the understanding about the environmental factors associated with broomrape distribution in Europe, and to predict based on these environmental factors which habitats would be vulnerable to invasion and establishment of broomrape in North and South America.

MATERIALS AND METHODS

A robust agroecological information database collected for the sunflower crop included 35 quantitative parameters associated with trials conducted by ten public research organizations from five continents (Table 1). The database comprised 117 sites (habitats) from Europe (79), Africa (3), Asia (6) and Americas (29) with data provided or published by person(s) responsible for each trial or experiment. Geographical parameters were obtained from site information of 111 sites, while the other six were obtained from Google Earth (http://www.google.com.ar/intl/es/earth/). Physical and chemical parameters of the top 0-15/20 cm soil layer were obtained by analysing representative samples from experimental fields according to international soil test rules for 113 sites, or by official soil cartography for four sites. Climatic parameters were measured at meteorological stations located in the experimental field (21), estimated from website sources (60) from the nearest city (www.worldclimate.net; www.tutiempo.net) at a distance less than 15 km away, or based on a province mean (36). Climatic monthly means corresponds to 10-year average (88), and when a complete decade of data was not available, monthly means were estimated from the year with complete data closest to the observed year (29). The monthly climatic values were ordered with the planting month as the centre of each series. *Orobanche cumana* attack of the sunflower crop was observed in each experimental field and classified broomrape infested or non-infested.

InfoStat (2008) statistical software was used to analyse the data set in two steps. First, an ANOVA and Kruskal Wallis analysed the quantitative data from Europe to compare broomrape invaded (52) and non-invaded habitats (27). Several multivariate principal component analyses (PCA) were performed on the complete data set, and then reducing the number of parameters with the criteria to optimize the variance explained by the first two principal components, PC1 and PC2. In the second step, all quantitative data from the five continents was used to perform PCA using the following groups; A-invaded habitats at the world level (57), B-non-invaded habitats of Europe, C- non-invaded habitats of South America (17), D- non-invaded habitats of North America (12), and E- non-invaded habitats of Asia, as other four independent groups. Means distributions of the more explicative parameters were graphically compared using box-plots.

RESULTS

The geographic location of broomrape in Europe was not different from sites without the parasitic weed (Table 2). The calcareous level of the soil was significantly higher (P=0.05*) in invaded habitats than in non-invaded habitat (2.7 ± 4.6 vs. 5.5 ± 5.9 %, respectively), but also showed a high overlap between both. An ANOVA did not indicate any difference between the invaded and non-invaded sites based on the other 31 quantitative environmental parameters (Data not show).

PCA biplots of all geographic and soil parameters of sunflower habitats in Europe explained 52.4% of the total variability, but did not separate broomrape invaded from non-invaded habitats (Data not shown). Four climatic parameters explain 85.4% of the environmental variability observed in these habitats, but did not discriminate between broomrape invaded and non-invaded sunflowers areas (Fig. 1).

The same climatic parameters used in of Fig. 1 explained 76.0 % of the variability observed in the sunflower habitats from all continents (Fig. 2). Broomrape invaded habitats' distribution from Europe, Asia (2), and Africa (3) shown by the biplot overlapped with the non-invaded habitats of Europe and a fraction of habitats from South America. The habitats from North America were mainly distributed in the bottom negative quadrant outside of the 95% confidence ellipse of the invaded habitats. More than a three-quarter of the habitats from South America falls into the invaded biplot sector. The inclusion of other environmental factors in the PCA, such as soil texture or P availability did not increase the explained variability or separate the invaded from the non-invaded habitats on the five continents (Data not shown).

Table 1: Environmental parameters used to characterize the sunflower habitats across five continents.

Acronym	Unit	Explanation
		Geographic
ALT(mosl)	m over sea level	Elevation
LAT	Absolute decimal degrees	Latitude
LON	Absolute decimal degrees	Longitude
		Soil physical and chemical
CLAY	%	Clay soil content
LOAM	%	Loam soil content
SAND	%	Sand soil content
OM	%	Organic matter content
pH	--	Soil acidity
Pavai	ppm as P	P availability
N	%	Total N content
Kavai	ppm as K	K availability
CoCa	%	CO_2Ca incrustations
		Climatic
PLMOMT	°C	Planting month (PLM) mean month temperature (MT)
PLM+/-*N*MT	°C	MT *N* months +/- PLM
PLMR	Mm	Mean rain (MR) of PLM
PLM+/-*N*R	Mm	MR *N* months +/- PLM
		Climatic integrative
CoolestM	°C	MT of the coolest month
HottestM	°C	MT of the hottest month
Rainmm	Mm	Total year rain
PLM-35MT	°C	Average MT five to three months before PLM
PL+26SMT	°C	Sum MT two to six months after PLM
PLTrimMT	°C	Average MT of the three months around PLM
CIR(-1/6)	Mm	Accumulated rain -1 to 6 months after PLM

Table 2: Geographic localization of *Orobanche cumana* in Europe.

	Broomrape in sunflower		ANOVA
Parameter[1]	Absent	Present	P
ALT(mosl)	113.3	117.2	0.8245
LAT	43.5	43.1	0.5314
LON	21.8	24.3	0.0894

[1]See parameter acronyms and units in Table 1.

Extremes values of box-plot distributions of geographical environmental parameters, soil texture, and chemical composition from invaded habitats comprises a fraction of the distribution of non-invaded habitats from four continents (Figs. 3a, b, d). The integrated mean monthly temperatures during the sunflower reproductive period (PL+26SMT) calculated for Asia and North America did not overlap with the extreme values of the invaded habitats and were not included like those from Europe and South America (Fig. 3c).

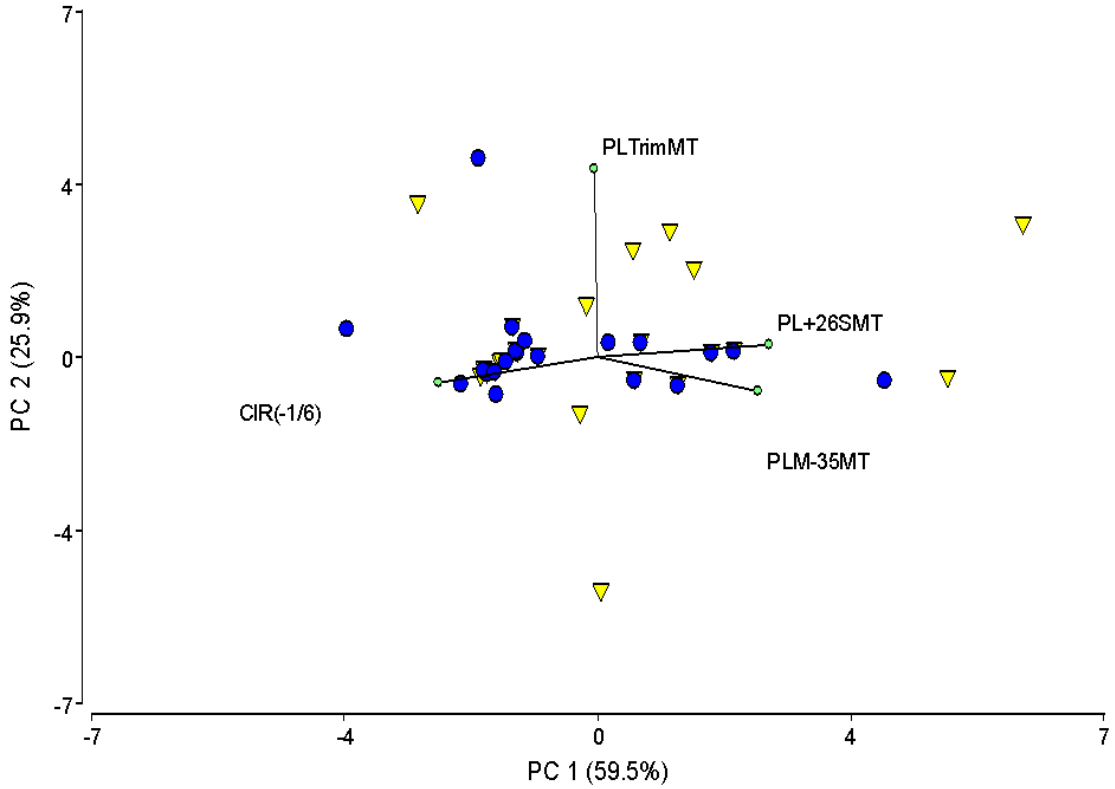

Fig. 1: PCA biplot of climatic parameters associated with the sunflower crop in Europe. Broomrape absent habitats = circle; present = triangle. (See parameter acronyms in Table 1)

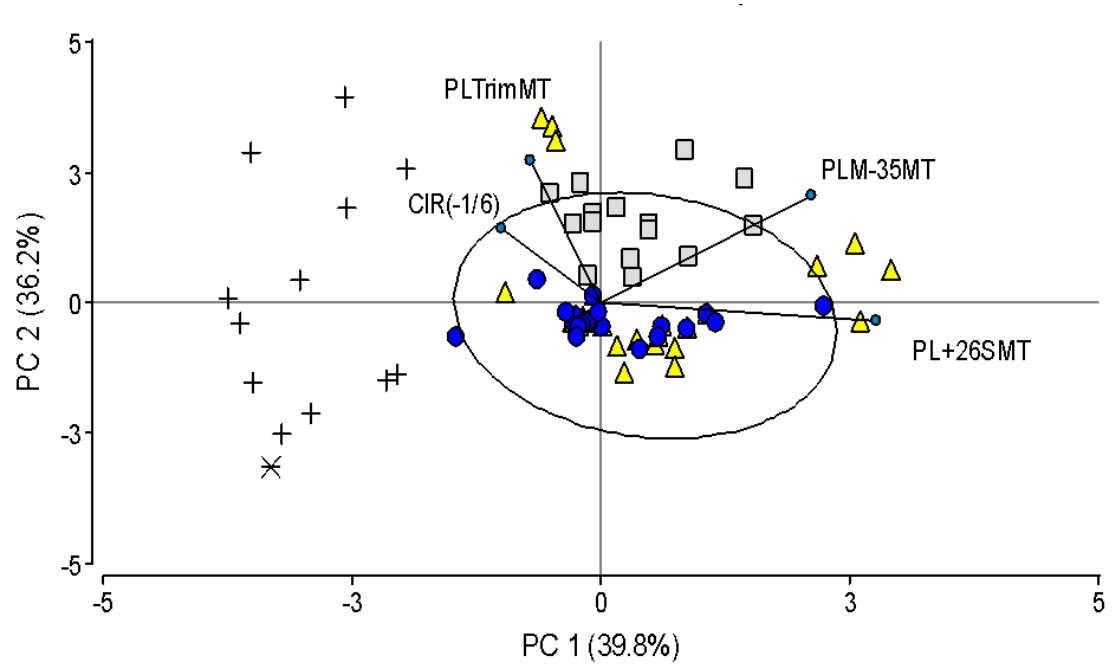

Fig. 2: PCA biplot of environmental parameters associated with the sunflower crop of broomrape invaded from three continents (triangle), or without the weed in Europe (circle), Asia (star), South America (square) and North America (cross). Predicted invasion ellipse for broomrape at the 95% confidence level (See parameter acronyms in Table 1).

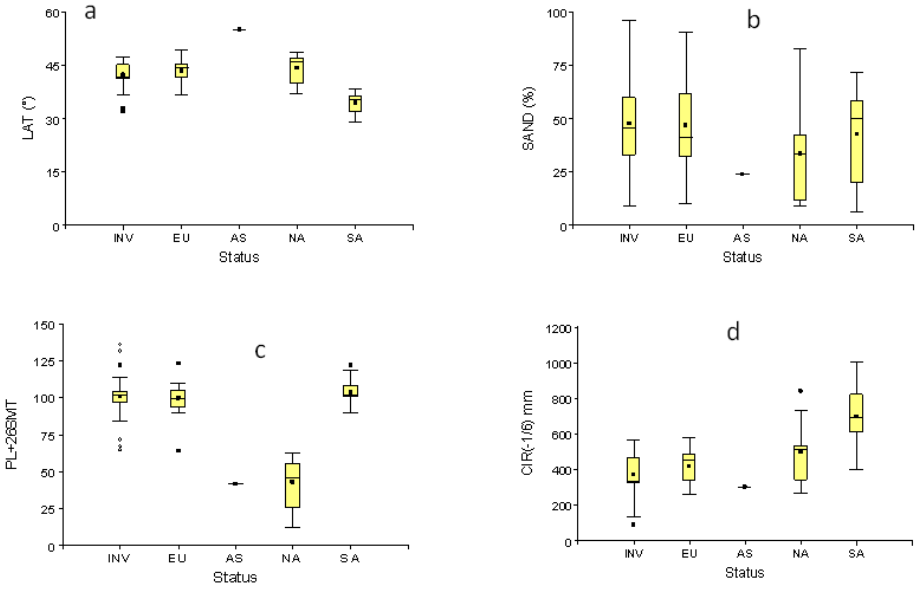

Fig. 3: Box-plot of selected parameters associated with the status of broomrape distribution in invaded habitats on four continents (INV) and not invaded habitats from Europe (EU), Asia (AS), South America (SA) and North America (NA) (See parameter acronyms in Table 1).

DISCUSSION

The 35 environmental parameters studied fail to explain the broomrape geographic distribution on the studied continents. In Europe, only the calcareous soil content was different between the invaded and non-invaded habitats, but the values strongly overlapped between both types.

Environmental parameters studied demonstrated that all crop areas of Europe were vulnerable to broomrape attack. The parasitic weed develops in soil and climate environmental conditions similar to non-invaded areas in Europe (Fig. 1). Its expansion to nearby areas in the Africa and Asia continents (Eizenberg et al., 2003; Amri et al., 2012) seems to indicate a broadening of the environmental conditions which the parasitic weed can tolerate. Habitat conditions of sunflower crop areas in South America could be more exposed to broomrape invasion because it showed more environmental similarities to invaded habitats of the world that North America (Fig. 2).

The absence of effects of soil physic or chemical parameters on broomrape distribution lessen the potential value of artificial soil modifications by means of manipulating organic matter, fertilizers or chemicals applications. Breeding for broomrape resistance is considered a successful tool against this weed (Kaya el at., 2012), but a strict crop rotation combined with genetic resistance should be used to limit the severity and spread of this noxious weed in the more affected areas of Europe (Gorbatchenko et al., 2011). The broad environmental conditions under which the parasitic weed develops (Fig. 3) reinforces the use of these cultural practices in invaded habitats and the application of strict phytosanitary controls to limit the seed movement into non-infected habitats.

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Microbiological characterization of the rhizosphere of sunflower (*Helianthus annuus* L.) infected by broomrape (*Orobanche cumana Wallr.*)

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ABSTRACT

The quantitative variations in the rhizospheral microflora of broomrape (*Orobanche cumana* Wallr.) infected sunflower plants were estimated in two soil types. The numbers of seven ecolo-trophic groups of soil microorganisms, associated with nitrogen and carbon transformations in the soil, were determined. Quantitative microbial characterisations of infected sunflower plants were juxtaposed with those of non-parasitised plants. Although microbial communities' structure was conserved, definite trend to decrease in the quantity of all groups of microorganism was observed. The effect of the factor broomrape was detected approximately or above 30%, in the distinct trophic groups microorganisms. The presence of the parasite had most extreme impact on microfungi numbers. The processes of soil depletion and intensive immobilization of organic nitrogen and are clearly pronounced. The results support the opinion on the soil microorganisms' significance as biotic component in the ongoing processes in host–parasite systems.

Key words: Broomrape – soil microorganisms – sunflower

INTRODUCTION

Sunflower broomrape (*Orobanche cumana* Wallr.) is considered one of the major pest in agriculture worldwide. The parasite currently infects approximately 160 Mha in the Mediterranean, South Europe and Southeast Asia, which is limiting factor in sunflower production (Gagne et al., 2000). Critical hindrance in broomrape control is the quick proliferation of new and more virulent races, which overcome the tolerance and resistance in sunflower hybrids (Wegmann et al., 1991). Over 65 %, corresponding to 11 Mha, of the arable lands in Europe are affected, while 18% of those are infected with new races. For the present, seven races of *O. cumana* Wallr. are identified in Bulgaria (A, B, C, D, E, F and G) last three of which, highly aggressive (Batchvarova, 2004; Georgiev et al., 2012).

Despite the significant achievements in the field of *Orobanchaceae* research, many questions are still unanswered. Especially challenging are the specific interactions in parasite - host systems, along with the multitude of factors involved. The latest trends in the exploration of characteristics of the different systems are focused on the role of soil-borne microorganisms as biotic component (Ter Borg, 1986; Wegmann, 1986; Amsellem et al., 2001; Rodríquez-Conde et al., 2004; Vurro et al., 2009). According to some authors, that presence of microorganisms is required to initiate germination in certain broomrape species (Petzoldt, 1979). There is underlying assumption, that that root exudates of some host plans contain not the germination inductors themselves, but their precursors which are later transformed into active compounds by the symbiotic microorganisms or enzymes produced by them (Antonova. & ter Borg, 1996; Pérez-de-Luque et al., 2001; Bouwmeester et al., 2003; Cardoso, 2011). Cases, in which soil microorganisms inhibit the germination of some broomrape species, along with others, where microorganisms induce germination in absence of a host, are previously described (Christeva and Naumova, 1998; Zonno and Vurro, 2002; Fernández-Aparicio et al., 2011). Considerable progress is registered in the field of arbuscular mycorrhiza (AM) research (Bouwmeester et al., 2007; Marschner and Timonen, 2004). The beneficial effect of AM on plants development is widely recognised, but its versatile role in ecosystems operation is still to be acknowledged (Akiyama et al., 1995; Azcon, 1996). Several microbiological species are currently tested as control agents of economic important broomrapes (Gururaj and Mallikarjunaiah, 1995; Hoagland, 2001; Sauerborn, 2001; Saadoum et al., 2001; Mayer, 2006; Zermane, 2005; Sun, 2011).

It is well established, that in plant rhizospheres biochemical processes associated with the nutrient cycle are continuously performed. Typical microbial communities with dynamic structures and ecologo-trophic links are developed in different plant rhizoshpheres, in strictly regulated interdependent relations. (Alexander, 1991; Frankenberg and Arshad, 1995; Atlas and Bartha, 1997; Werner, 2001). In this regard, the ongoing processes in the infected plants rhizosphere are not well defined, and the specificity of every distinct parasite- host system requires careful examination. Rhizosphere microorganisms may offer new

prospects for more efficient control, and on the other hand of detailed clarification of germination mechanisms, host specialisation in different region of *O. cumana* invasion, the formation of new aggressive races, host tolerance overcome and etc.

The aim of this study is to build quantitative microbiological characterisation of the rhizosphere of sunflower plants infected by broomrape (*O. cumana* Wallr.).

MATERIALS AND METHODS

The field experiments were conducted on sunflower crops grown on two different soil types, with following characteristics:

I. Haplic Vertisols - with organic matter content (by Turin) -2.3%; available nitrogen content (by Kjeldahl) - 0.1%; mobile forms of phosphorus (P_2O_5 – according to Egner-Reem) - 6.2 mg/100g soil; available potassium (by Milcheva) -29.2 mg/100g soil; Soil reaction (pH in H_2O) - 7.5.

II. Chromic Cambisols - with organic matter content (by Turin) -1.51%; available nitrogen content (by Kjeldahl) - 0.08%; mobile forms of phosphorus (P_2O_5 – according to Egner-Reem) - 2.75 mg/100g soil; available potassium (by Milcheva) -27.5 mg/100g soil; Soil reaction (pH in H_2O) - 7.2.

Soil samples in four replications, were collected from host rhizosphere in florescence of the parasite and the host for microbiological analyses. Uninfected sunflower plants in close proximity with the infected specimens were collected for control.

The microbiological analyses were executed according to Koh's method – culture of diluted soil suspensions on specific for every trophic group microorganisms nutrient media, in three replications (Koleshko, 1991). Population densities of the following groups of microorganisms were determined: Autochthonous microorganisms – on soil extract agar, with 10 days incubation period; Oligotrophic microorganisms – on diluted soil extract agar, with 10 days incubation period; Actinomycetes – on starch–ammonium agar, with 7 days incubation period; Microfungi – on Chapek agar, with 7 days incubation period; Ammonifying microorganisms – on meat–peptone stock agar, with 5 days incubation period; Mineral nitrogen assimilating microorganisms – on starch–ammonium agar, with 7 days incubation period; Aerobic nitrogen-fixing bacteria of the genus *Azotobacter* – on Ashby agar, with 7 days incubation period. The most probable number technique is used to estimate microbial population sizes per gram absolute dry soil (MPN/g a.d.s), with confidence level 0.05. Two indices, characterizing the structure of microbial communities were calculated – oligotrophic , which represents the oligotrophic: autochthonous microorganisms ratio; and mineralization – immobilization, which is the nitrogen assimilating microorganisms:ammonifying microorganisms ratio.

The data were subjected to two-factor analysis of variance. For each trophic group microorganisms the effects (ηx^2) of the factors: soil type (A) and broomrape (B) and the level of their statistical significance (p) according to the Fisher's criterion (F) were determined (Plochinskiy, 1980).

RESULTS AND DISCUSSION

The analysed indicative microbiological indices depict a set of organotrophic microorganisms, which can be generally divided to: soil carbon converting and soil nitrogen converting microorganisms. These groups are highly descriptive for the quantitative and qualitative status of the microbial communities as a whole, their ability to perform the basic conversion of biogenic elements in the soil, and their impact on plants nutrition (Alexander, 1991; Atlas and Bartha, 1997).

The present research found Chromic Cambisols in general poorer in microbial population. Despite of the soil type, the results demonstrated trend towards decrease in population density in all examined groups of microorganisms in the presence of broomrape (Table 1).

When building characterizations of microbial biomass in the soil, the numbers of autochthonous and oligotrophic microorganisms are considered representative for its quantitative status. In this case, very distinctive differences in these numbers are found in the rhizosphere of broomrape infected and uninfected sunflower plants. The registered reduction in density of autochthonous microorganisms is 75.80% in Haplic Vertisols and 71,75% in Chromic Cambisols. There was a significant drop in the oligotrophic microorganisms numbers also - 70.41% in the former soil type, and 66.91% in the latter. The observed changes were statistically significant, with confidence level $p=0.99$. The estimated effect of the factor broomrape on the numbers of autochthonous microorganisms was 39.99%, and of the factor soil type - 63.47% ($F_{exp.} > F_{tab.} = 4.57$). The oligotrophic microorganisms were affected by the presence of broomrapes by 37.29%, and by the soil type - 65.83%.

Table 1. Quantities (MPN/g a.d.s.) of the studied trophic groups microorganisms in the rhizosphere of sunflower-*O. cumana* systems

Variants/ soil types	Trophic groups microorganisms						
	Autochthonous	Oligotrophic	Actinomycetes	Microfungi	Ammonifying	Nitrogen assimilating	*Azotobacter*
I. Haplic Vertisols sunflower - control	1.71E+09 ± 8.13E+16	1.85E+09 ± 2.27E+17	4.27E+08 ± 3.13E+16	63335 ±161421712	6.24E+08 ±3.26E+16	8.18E+08 ± 2.18E+17	3E+08 ±2.79E+16
I. Haplic Vertisols sunflower + *O. cumana*	4.13E+08 ± 2. 93E+16	5.48E+08 ± 2.39E+16	7.05E+07 ± 1.90E+15	36388 ± 21033266	5.17E+08 ±3.49E+16	2.72E+08 ± 2.45E+16	2E+06 ±5.26E+14
II. Chromic Cambisols sunflower - control	51321818 ± 8.72E+14	46614482 ± 5.58E+14	4408532 ± 2.34E+11	74182 ± 2,13E+08	14046060 ± 9.69E+12	16753177 ± 1.82E+13	2956922 ±1.49E+11
II. Chromic Cambisols Sunflower + *O. cumana*	1400856 ±4.76E+13	15425238 ± 4.48E+13	1418316 ± 2.11E+11	41730 ± 3.45E+08	1016545 ± 3.74E+11	6750931 ± 3.64E+12	742355 ±3.72E+11

The features of microfungal and actinomycetal populations are broadly applied for evaluation of the soil biological properties. These microorganisms possess powerful and varied physiological means for organic compound conversion, and are key sections in microbial communities.

Beside their role as important determinants of rhizosphere microbial community structure and functional biodiversity, they are also substantial source of biologically active substances and have major part in mineralization processes in soil (Werner, 2001).

In the rhizosphere of broomrape infected sunflower plants, the numbers of microfungi populations, in both soil types, were lowered by more than 40%. The differences between infected and control hosts were statistically significant (p=0.99), and the effect of the factor broomrape (89.92%) was found bigger the effect of the factor soil type - 78.28%. Particularly notable was that this was the only index, which values are greater in Chromic Cambisols, were reduction in their number in Haplic Vertisols was by tens of thousands /g a.d.s.

The observed decline in quantities in the other hyphal group microorganisms - actinomycetes in the presence of broomrape, was by 83.47% in Haplic Vertisols, and by 67.83% in Chromic Cambisols. The differences were statistically significant, and the effect of the both factors was shown to be respectively 33.44 % for broomrape, and 51.30% for soil type.

The second group of microbiological indices describes some key transformations of nitrogen compounds in the soil. The possibility for nitrogen entrance through fixation could be depicted using the quantities of the free living aerobic bacteria of the genus *Azotobacter*. The decrease of their numbers in the rhizosphere of infected plants is clearly pronounced - over 90% in Haplic Vertisols, and over 70% in Chromic Cambisols. The differences were found statistically significant at confidence level p=0.99. The effect of broomrape factor was 29.9%, and of soil type - 38.1%.

The role of the ammonifying microorganisms is to complete the organic compounds mineralization and to produce available for the plants ammonium. Their quantity in infected plants rhizosphere was also altered. The difference with the control hosts in Haplic Vertisols was slight - 17.16%, while in Chromic Cambisols was very high - over 90%. The effect of the factor broomrape is shown to be 30.62%, whereas of the factor soil type - 86.84%.

The microbes, assimilating the mineral forms of nitrogen are immobilizing it in their own biomass, and thus provide dynamic reserve of fixed nitrogen, and at the other hand appear to be plants competitors for it. The numbers of these microbes were reduced by 60% in the presence of broomrape in both soil types. The effect of the factor broomrape on nitrogen immobilizing microorganisms were detected 27.65%, and of the factor soil type (59.7%).

The microbial communities' stability and function relies on the equilibrium between the different trophic groups. In ecological research on the soil-borne microflora, structural indices such as the oligotrophic and the mineralization–immobilization index are used to trace the specific interrelations and to predict changes in direction of certain processes. Estimated values of the oligotrophic index showed slight increase in broomrape infected plants, in both soil types . The detected minor differences to the controls are statistically significant at p=0.99. The effects of both factors were found approximately 50%.

More notable and diverse aberrations, due to soil type, are marked in the values of mineralization–immobilization index. Its levels were lower than optimal (1) in the control, as well as in infected plants rhizosphere in Haplic Vertisols, which demonstrated predominant mineralization processes. In Chromic Cambisols, the index' levels were many times higher, hence indicated intensified immobilization

processes. The effect of the factor (ηx^2) broomrape was found to be 34 %, while of the soil type was 42.19% (Table 2).

Table 2. Values of the Oligotrophic Index and Mineralization-Immobilization Index of microbial communities

Variants/ soil types	Oligotrophic index	Mineralization–Immobilization index
I. .Haplic Vertisols sunflower - control	1.073	1.128
I. .Haplic Vertisols sunflower + *O. cumana*	1.666	0.443
II. Chromic Cambisols sunflower - control	1.321	1.309
II. Chromic Cambisols sunflower + *O. cumana*	1.431	8.492

CONCLUSIONS

The microbiological characterization of broomrape - infected sunflower plants demonstrated definite trend towards decline of biodiversity at the population level, with conserved microbial communities' structure. Although the influence of the factor soil type was found to be greater, the factor broomrape definitely altered the population densities of the examined microorganisms. Its effect was detected approximately or above 30% in the distinct trophic groups soil microorganisms. The microfungi numbers were most extremely varied.

The results are in accordance with trends outlined by previous researches on population structure of rhizospheral microflora of different broomrape hosts (Hristeva et al., 2013; Naumova et al., 2004).
The data support the opinion of the soil microorganisms' significance as biotic component in the ongoing processes in host–parasite systems.

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Genetic studies in sunflower broomrape

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ABSTRACT

Much research has been conducted to identify sources of genetic resistance to sunflower broomrape (*Orobanche cumana* Wallr.) and to study their mode of inheritance. However, studies on the parasite have been scarce. This manuscript describes three genetic studies in sunflower broomrape. First, the inheritance of the absence of pigmentation in a natural mutant of this species with yellow plant colour phenotype was studied. Plants of a mutant line were crossed by plants of a normally pigmented line and the F_1, F_2, and F_3 generations were evaluated. The results indicated that plant pigmentation is controlled by a partially dominant allele at a single locus. Second, the unpigmented mutant was used to evaluate outcrossing potential of the species. Two experiments in which single unpigmented plants were surrounded by pigmented plants were conducted under pot and field conditions. The cross-fertilization rate was estimated as the percentage of F_1 hybrids in the progenies of unpigmented plants, which averaged 21.5% in the pot and 28.8% in the field experiment. The results indicated that, under the conditions of this study, the species was not strictly self-pollinated. Finally, the inheritance of avirulence was studied in crosses of plants from inbred lines of races E and F, by evaluating the F_1 and F_3 generations on the differential line P-1380 carrying the race-E resistance gene Or_5. The results suggested that race E avirulence and race F virulence on P-1380 are allelic and controlled by a single locus, which confirmed the gene-for-gene theory for the *O. cumana*-sunflower interaction.

Key words: Avirulence – cross-fertilization – genetic studies – hybridisation – inheritance – virulence

INTRODUCTION

The *O. cumana*-sunflower parasitic system is characterized by a clear differentiation of physiological races of the parasite and a host-parasite interaction that generally fits the gene-for-gene model. This contrasts with other crop-*Orobanche* associations, in which the genetic control of the avirulence of the parasite and the resistance of the crop plant is generally under quantitative genetic control (Pérez-Vich et al., 2013). In recent years, increasingly virulent races have appeared and spread into large areas of the main sunflower producing countries in the Old World, jeopardizing sunflower production (Škorić et al., 2010; Antonova et al., 2013; Molinero-Ruiz et al., 2014). Understanding race evolution in *O. cumana* requires basic studies on its mating system and the inheritance of avirulence/virulence mechanisms.

The genus *Orobanche* encompasses species that are largely pollinated by insects and species which are mainly self-pollinated (Musselman et al., 1981). Sunflower broomrape is considered a predominantly autogamous species on account of its flower morphology (Satovic et al., 2009) and population structure, generally characterized by marked among-population differences and low intrapopulation genetic diversity (Gagne et al., 1998; Pineda-Martos et al., 2013; Molinero-Ruiz et al., 2014).

Even though other forms of genetic control have been reported, the occurrence of resistance mechanisms to broomrape controlled by single dominant genes is common in sunflower (Vrânceanu et al., 1980; Sukno et al., 1999; Velasco et al., 2012). A full demonstration of the occurrence of a gene-for-gene relationship in the sunflower-*O. cumana* interaction requires studying the inheritance of avirulence in the parasite.

This paper summarizes several recent genetic studies on *O. cumana*, in particular the inheritance of a natural mutation resulting in absence of anthocyanin pigmentation, the use of this mutation for estimating the rate of cross-pollination under experimental conditions, and the inheritance of avirulence in crosses between *O. cumana* plants of races E and F.

MATERIALS AND METHODS

Detailed description of the materials and methods used in these genetic studies can be found in the original papers reporting the inheritance of the unpigmented plant trait (Rodríguez-Ojeda et al., 2011), the rate of cross-fertilization (Rodríguez-Ojeda et al., 2013a), and the inheritance of avirulence (Rodríguez-Ojeda et al., 2013b). In short, a spontaneous mutant lacking anthocyanin pigmentation was identified in a population collected in Central Spain (Fig. 1).

Fig. 1. Mutant plant of *Orobanche cumana* lacking anthocyanin pigmentation.

The inheritance of the trait was determined by studying the phenotypic segregation of the F_1, F_2, and F_3 generations from the cross of plants of the mutant with normally pigmented plants. Also, the mutant trait was used for estimating the rate of cross fertilization taking advantage of the intermediate phenotype of F_1 hybrids. Individual mutant plants were surrounded by normally pigmented plants at a minimum distance of around 15 cm under open pollination conditions in both pot and field experiments. The rate of cross fertilization was estimated as the percentage of hybrid plants in the progenies of mutant plants. For the study of avirulence, inbred lines of races E and F were developed and plants of both races were reciprocally crossed. The study was based on the avirulence/virulence reactions observed at the F_1 and F_3 generations on the race E-resistant differential line P-1380 (Vrânceanu et al., 1980).

Inheritance studies were conducted using manual emasculation and pollination (Fig. 2) and plant isolation (Fig. 3) as described by Rodríguez-Ojeda et al. (2010).

Fig. 2. Manual emasculation of *Orobanche cumana*

Fig. 3. Isolation of *Orobanche cumana* plants with microperforated plastic bags.

RESULTS AND DISCUSSION

The study of the inheritance of the unpigmented plant trait indicated that plant pigmentation is controlled by a partially dominant allele at a single locus (Rodríguez-Ojeda et al., 2011). The intermediate phenotype of the F_1 hybrid (Fig. 4) allowed the use of the mutant trait as a morphological marker for the study of the rate of cross-fertilization in *O. cumana*. The average cross-fertilization rate was 21.5% in the pot and 28.8% in the field experiment (Rodríguez-Ojeda et al., 2013a). It is important to note that these figures were obtained under experimental conditions using individual mutant plants surrounded by many normally pigmented plants, but they do not necessarily reflect the actual cross-fertilization rate under specific natural conditions, which should be determined on a case-by-case basis. In any case, the results demonstrated that the species is not strictly self-pollinated and some extent of cross-pollination can be expected, which has important implications for understanding population structure in *O. cumana*. We observed that cross pollination was mainly conducted by small insects of the Hymenoptera, with a length from around 3 to 9 mm. Some of them were identified as belonging to the Halictidae family (Rodríguez-Ojeda et al., 2013a). Finally, the study of the inheritance of avirulence in crosses between plants of races E and F indicated that race E avirulence and race F virulence on the differential line P-1380 are allelic and controlled by a single locus, which confirmed the gene-for-gene theory for the *O. cumana*-sunflower interaction.

Monogenic inheritance of avirulence/virulence in *O. cumana* is important for understanding race evolution in this parasitic weed, since new races may appear as the result of single mutation events. This is particularly relevant for an species in which single plants produce thousands of seeds. Recently, Pineda-Martos et al. (2013) and Molinero-Ruiz et al. (2014) identified two distinct gene pools of *O. cumana* in Spain, both of them containing low genetic diversity but populations classified as either race E or race F, suggesting that race F mutations occurred independently in both gene pools.

Because of the simple inheritance of avirulence/virulence and the existence of a relatively high rate of cross fertilization in *O. cumana*, recombination of different avirulence genes may play an important role in race evolution if populations with different virulence genes get into contact. In this sense, Pineda-Martos et al. (2013) documented the presence of heterozygotes in a few Spanish populations of *O. cumana* in which individuals of the two predominant gene pools were present.

Fig. 4. F_1 *Orobanche cumana* plant with intermediate pigmentation identified in the progenies of a mutant plant under open pollination conditions.

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Genetic similarity and differences between *Orobanche cumana* Wallr. populations from Russia, Kazakhstan and Romania assessed using SSR markers

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ABSTRACT

Broomrape (*Orobanche cumana* Wallr.) is an obligate parasite of higher plants, which affects sunflower in many countries, cultivating this crop. For the past decades it is noted the formation of new highly virulent biotypes of broomrape and their spreading to other areas. In our work we studied the molecular genetic diversity of broomrape populations of *O.cumana*, parasitizing on sunflower in Russia, Romania, and Kazakhstan, by using codominant microsatellite markers.During cluster analysis the broomrape populations divided into two clusters, regardless of their racial composition. One cluster grouped 19 samples from Russia and Kazakhstan, and the other 5 populations from Romania. The genetic distance between clusters according to Nei was 0.137. AMOVA analysis revealed that 22% of genetic variability was due to differences among the gene pools and 78% was due to differences within the gene pools. Pairwise comparisons made using Wright's statistics showed that the differences between these two gene pools are sufficient (Fst = 21.9%) to state the existence of a small genetic differentiation between them. Descriptive population genetic statistics for each of the two pools showed that the broomrape populations from the former Soviet Union countries are characterized by a higher level of intrapopulation diversity than the populations from Romania. Molecular genetic differences between broomrape populations parasitizing on sunflower on the post-Soviet territory and in Romania were insignificant. Possible reasons for these results are being discussed.

Key words: Genetic diversity – molecular characterization – *Orobanche cumana* – SSR markers – sunflower

INTRODUCTION

Broomrape (*Orobanche cumana* Wallr.) is an obligate parasite of higher plants parasitizing on the roots of sunflower. In severe cases of strong broomrape infestation yield losses of sunflower can reach up to 100%. In Russia, as well as in the other countries, widespread intensification of sunflower cultivation as a high-yielding crop in the last decade led to the formation of highly virulent biotypes of broomrape, overcoming the resistance of cultivated assortment (Antonova et al., 2012).

The researches on the evolution of the parasite and the population genetic structure are important for understanding the mechanism of its development in different countries and for developing a long term strategy of control over it (Fernández-Martínez et al., 2012; Škorić et al., 2010).

The molecular genetic differences between the populations of *O. cumana* from different countries have already been studied (Ciuca et al., 2004; Atanasova et al., 2005; Benharrat et al., 2002; Gagne et al., 1998; Pineda-Martos et al., 2013; Molinero-Ruiz et al., 2013). By using RAPD PCR technology it has been shown that the populations of *O. cumana* from Bulgaria, Spain, Romania, and Turkey have low intrapopulation variability, and very little gene exchange appears to occur between different geographic regions (Gagne et al., 1998). The weak polymorphism of the populations of *O.cumana* from Spain, Yugoslavia and Romania is shown (Ciuca et al., 2004). The existence of two distant gene pools in two provinces in Spain is revealed, one is in Cuenca province, and another one in Guadalquivir Valley. Within each gene pool, both inter- and intrapopulation variability were extremely low (Pineda-Martos et al., 2013). The molecular analysis among highly virulent populations of *O. cumana* identified four clusters, respectively, grouping populations from Central Spain, Hungary, South Spain and Turkey. The genetic homogeneity within parasite populations was confirmed, since no molecular differences were found within them (Molinero-Ruiz et al., 2013).

Molecular genetic differences of the populations of *O.cumana*, affecting sunflower in different regions of Russia and Kazakhstan, have not yet been studied. The purpose of our research is to analyze the molecular genetic diversity between broomrape populations, spread on the territory of Russia, and to compare them with the populations from Romania and Kazakhstan by using codominant microsatellite markers.

MATERIAL AND METHODS

The seeds of *O.cumana* from 24 populations were collected in 2012 and 2013 on sunflower fields of the Krasnodar, Stavropol, Rostov, Volgograd, and Saratov regions of Russia, as well as Romania and Kazakhstan, and were stored at a temperature of -18 ºC (Table 1).

Table 1. The characteristics of seed sample of *O.cumana*, used for the analysis

№	Place of collecting of broomrape seeds	Country	Collection year	Races, prevailing in seeds sample
V1	Krasnodar region, Krylovskiy district	Russia	2012	G
V2	Krasnodar region, Yeyskiy district	Russia	2013	F,G
V3	Stavropol region, Trunovskiy district	Russia	2012	F,G
V4	Rostov region, Azovskiy district, DOS VNIIMK	Russia	2012	E,F,G
V5	Rostov region, Milyutinskiy district	Russia	2012	G
V6	Rostov region, Azovskiy district, v. Krugloe	Russia	2012	G
V7	Rostov region, Bokovskiy district	Russia	2012	F,G
V8	Rostov region, Zernogradskiy district	Russia	2012	F,G
V9	Rostov region, Matveevo-Kurganskiy district	Russia	2012	F,G
V10	Rostov region, Matveevo-Kurganskiy district	Russia	2012	G
V11	Rostov region, Bokovskiy district	Russia	2012	F,G
V12	Rostov region, Bokovskiy district	Russia	2012	F,G
V13	Rostov region, Zernogradskiy district	Russia	2012	F
V14	Rostov region, Zernogradskiy district, stud farm	Russia	2012	F,G
V15	Rostov region, Kagalnitskiy district	Russia	2012	G
V16	Saratov region, Sovetskiy district	Russia	2013	E,F,G
V17	Volgograd region, Alexeevskiy district	Russia	2013	F,G
V18	Volgograd region, Novoanninskiy district	Russia	2013	F,G
V19	Shemonaikhinskiy district, Ust Kamenogorsk	Kazakhstan	2012	D
V20	Medgidia	Romania	2013	G
V21	Calarasi	Romania	2013	G
V22	Tulcea	Romania	2013	G
V23	Urziceni	Romania	2013	D
V24	Lunca	Romania	2013	G

For artificial inoculation with broomrape, sunflower plants of VNIIMK 8883 variety were cultivated in a growth chamber in plastic boxes with a capacity of 10 kg of a mixture of soil with river sand in the ratio 3:1. This variety has never been bred for resistance to broomrape. Broomrape seeds were applied to soil mixture at the rate of 100 mg per 1 kg. Twenty sunflower seeds were sown per each box. Watering was carried out at the drying up of topsoil. The operating regime of chamber: 16-hour photoperiod at a temperature of 25-27 ºC at daytime and 20 ºC – at night. 50 days after the emergence of seedlings, the plants were dug out of the pots, the root system was washed with water, and tubercles, stems and inflorescences of broomrape were collected for analysis. Fresh-cut tissue was stored at a temperature of -80 ºC.

DNA was extracted from frozen tissues by the method of Doyle and Doyle (1987) with our modifications. A mixture of 5-10 individual plants of equal weight was taken from each population for DNA extraction of broomrape.

PCR-amplifications were used with 10 ng of genomic DNA in 25 µL reactions. Each 25 µL of reaction volume contained 67 mM tris-HCl, pH 8.8; 16.6 mM $(NH4)2SO_4$; 1.5-3.0 mM MgCl2; 0.01 % Tween 20; 0.2 mM deoxynucleoside triphosphates, 10 µM primer and 1.0 unit Tag DNA polymerase (Moscow, Sibenzim). DNA amplification was performed in a thermal cycler S1000™ (BioRad, USA). The amplification conditions: initial denaturation at a temperature of 96 ºC for 2 minutes followed by 30 cycles in accordance with temperature-time mode: annealing at a temperature of 60 ºC for 40 sec., elongation at 70 ºC for 1 minute, denaturation at 94 ºC for 30 sec, and final elongation for 2 minutes. 15 SSR (simple sequence repeat) primers were used that were selected in work (Pineda-Martos et al., 2013).

Amplification products were resolved by electrophoresis in 8% polyacrylamide gel in 1xTBE buffer at 230 V constant, using VE-20 (Helicon, Russia) vertical camera. Subsequent staining was performed with ethydium bromide. Visualization of electrophoresis results in UV and their documentation was provided by using the system of digital video documentation BIO-PRINT (Vilber Lourmat, France). A 100-bp DNA ladder (Thermo Fisher Scientific Inc., Lithuania) was used as a standard molecular weight marker to get an approximate size of DNA fragments. Calculation of fragments size after electrophoresis was scored manually with the aid of Bio-Capture software (Vilber Lourmat, France).

To determine the differences between the broomrape samples, data of PCR analysis were processed by Ward's method. To do this, amplified fragments were scored for the presence (1) or absence (0) of homologous bands. Data were compiled into a binary data matrix. Cluster analysis was performed using a program package (Statistica 6.0). Geometric distances were calculated using Euclidean distance. Descriptive population genetic statistics (number of alleles per locus *Na*, effective number of alleles *Ne*, polymorphism *P*, observed *Ho* and expected *He* heterozygosity, Shannon's Information Index *I*), as well as analysis of molecular variance AMOVA, F statistics, Nei Genetic Distance and Nei Genetic Identity were calculated by using Gen AlEx 6.5 program (Peakall and Smouse, 2012). Probability P for Fst, Fis and Fit is based on 999 permutations across the full data set. The Analysis of Molecular Variance (AMOVA) procedure follows the methods of (Excoffier et al. 1992), calculation of Nei's standard genetic distance between pairs of populations was performed by Nei (Nei, 1972).

RESULTS AND DISCUSSION

Among the 15 primers initially assayed, 9 were selected since they produced consistent polymorphisms. As a result of amplification of 9 microsatellite loci 21 alleles were detected from 24 broomrape populations, from 2 to 4 alleles per locus (Table 2).

Table 2. The characteristics of microsatellite loci, used for evaluating genetic diversity in 24 *O.cumana* populations

Locus	Size	Alleles
Ocum-52	114, 131	2
Ocum-59	90, 100	2
Ocum-70	127, 120,130	3
Ocum-81	72, 90	2
Ocum-87	132, 136, 134,138	4
Ocum-108	144, 152	2
Ocum-141	186, 191	2
Ocum-196	192, 197	2
Ocum-197	104, 113	2

The dendrogram resulting from the Ward analysis of the SSR data set distinguished two well-differentiated clusters among the 24 samples of *O. cumana* (Fig. 1). Cluster I grouped the 19 samples, collected on the territory of Russia and Kazakhstan, regardless of racial composition. Cluster II combined broomrape populations, collected in different regions of Romania.

Cluster I was divided into 2 subclusters with a union distance of 5 units. Subcluster Ia combined the populations originating from Krasnodar and Stavropol regions, and Bokovskiy and Zernogradskiy districts of the Rostov region. Subcluster Ib grouped the populations from Kazakhstan, Saratov and Volgograd regions, and Matveevo-Kurganskiy, Azovskiy, Kagalnitskiy and Milyutinskiy districts of the Rostov region. Subclusters combination did not depend on the geographical origin or the level of virulence of the population, since the populations with different virulence have been grouped together (Table 1, Fig. 1). For example, broomrape populations from Bokovskiy and Zernogradskiy districts of the Rostov region combined in subcluster Ia are quite far from each other (about 200 km). Broomrape from Kazakhstan, the most geographically distant from the populations of Russian group (a distance over 2.000 km and different soil and climatic conditions), is located in the one subcluster with the populations from

Rostov, Volgograd, and Saratov regions. The level of genetic differentiation between the main clusters was quantified by calculating Nei genetic distances (Nei, 1972). Nei Genetic Distance and Genetic Identity Values were 0.137 and 0.872, respectively.

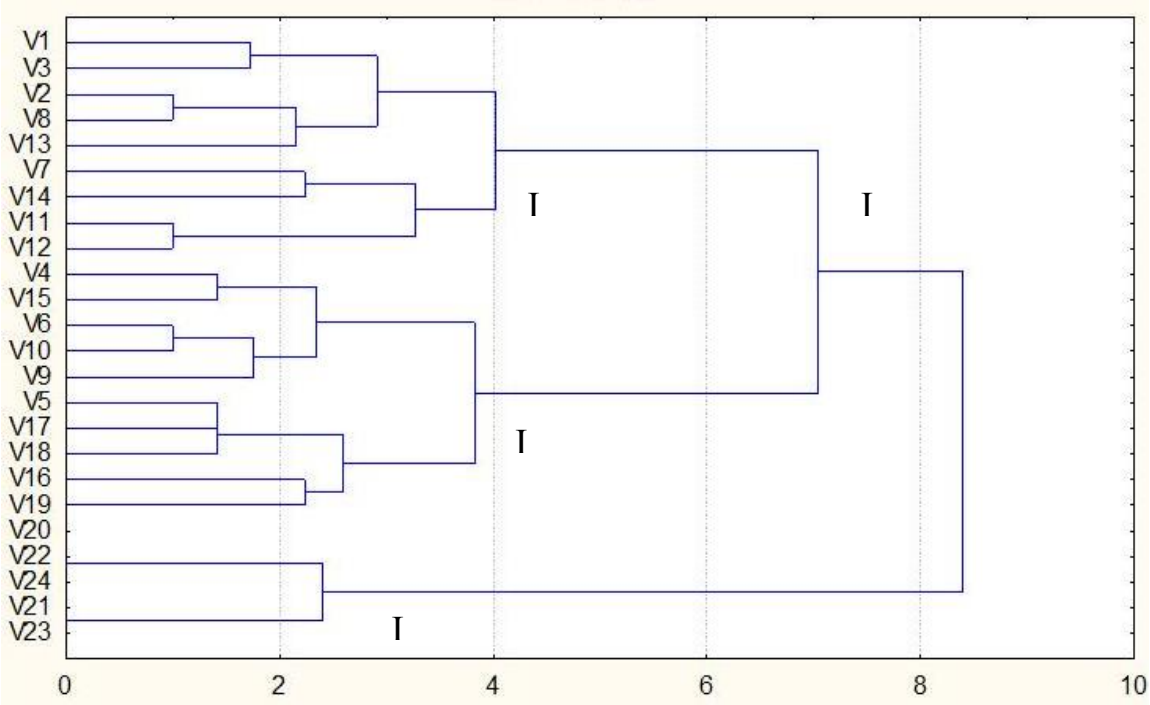

Fig. 1. Ward's method dendrogram based on Euclidean distances between 24 populations of *Orobanche cumana* (see Table 1), collected in different regions of Russia, Kazakhstan and Romania using 9 SSR markers.

All 5 broomrape samples from Romania were virtually identical. Percentage of polymorphic loci was 11.11. The populations originating from Russia and Kazakhstan were more polymorphic, with percentage of polymorphic loci of 100.00. For example, allelic variation of the five populations from Romania and of the five populations from Russia at the Ocum-87 SSR locus is shown in Fig. 2

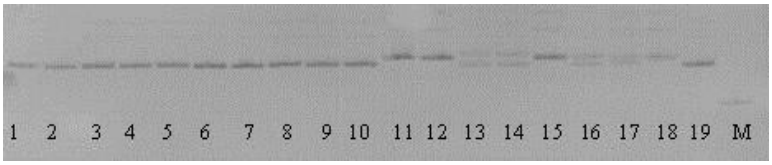

Fig. 2. Allelic variation of the Ocum-87 SSR locus of ten *Orobanche cumana* populations. 1,2 - Medgidia, Romania; 3,4 - Calarasi, Romania; 5,6 – Tulcea, Romania; 7,8 -Urziceni, Romania; 9,10 - Lunca, Romania; 11,12 – Saratov region, Sovetskiy district, Russia; 13,14 – Krasnodar region, Yeyskiy district, Russia; 15,16 - Volgograd region, Alexeevskiy district, Russia; 17,18 - Volgograd region, Novoanninskiy district, Russia; 19 - Rostov region, Azovskiy district, v. Krugloe, Russia; M – DNA fragment 100 bp long.

To determine mean descriptive population genetic statistics, the analysis of molecular variance AMOVA and F statistics of populations of *O.cumana* were combined by geographical origin. Next, the parasite populations originating from Russia and Kazakhstan are indicated as Pop1, and the populations from Romania are indicated as Pop2. Descriptive diversity statistics for populations from the two main gene pools showed significant variation for number of alleles per locus, number of alleles with frequencies pi >5%, effective number of alleles, observed heterozygosis and expected heterozygosis (Table 3). The average number of alleles per locus ranged from 1.11 in Pop2 to 2.33 in Pop1. The effective number of alleles ranged from 1.05 in Pop 2 to 1.77 in Pop 1. Pop1 showed the highest heterozygosis value (0.44), whereas Pop 2 showed heterozygosis value of 0.05. The values for the mean expected heterozygosis ranged from 0.03 in Pop2 to 0.41 in Pop1. Diversity analysis within the genetic pools using Shannon's

Information Index revealed the highest diversity values in Pop1 (0.63) and the lowest in Pop2 (0.05) (Table 3).

Table 3. Mean descriptive population genetic statistics for each of the two populations of *O. cumana* followed by standard deviation values

Population	Na	Na Freq. > 5%	Ne	I	Ho	He
Pop1	2,33±0,23	2,11±0,26	1,77±0,11	0,63±0,07	0,44±0,06	0,41±0,04
Pop2	1,11±0,11	1,11±0,11	1,05±0,05	0,05±0,05	0,04±0,04	0,03±0,03

Na: No. of Different Alleles; Na (Freq >=5%): No. of Different Alleles with a Frequency >5%; Ne: No. of Effective Alleles; I: Shannon's Information Index; Ho: Observed Heterozygosity; He: Expected Heterozygosity

The great intrapopulation diversity of broomrape in Russia and Kazakhstan in comparison with Romanian seems natural. Here, many populations are at large distances from each other (from 200 to 2000 km), whereas in Romania they are not enough distant geographically.

AMOVA analysis revealed that 22% of genetic variability was due to differences among the populations and 78% was due to differences within the populations (Table 4).

Table 4. Analysis of molecular variance in two groups of populations of *O. cumana*

Source of variation	df	Sum of squares	Variance components	% Variation	P
Among populations	1	8,552	0,443	22%	<0,001
Within populations	24	38,500	1,604	78%	
Total	25	47,042	2,047	100%	

When pairwise differences among populations were checked, it was confirmed that there was sufficient differentiation between Pop1 and Pop2 (Fst = 21,9%; Table 5) to suggest the existence of a small genetic differentiation of these two pools. The small value of Fis (-0,019) indicates the deficit of mean heterozygosis in each population, and Fit, which characterizes the deficiency or excess of mean heterozygosis in the group of populations, has an average value (0.205). In our case, the populations from Romania have virtually no heterozygosis, thereby reducing the Fis.

Table 5. Comparisons of pair of *O. cumana* populations (F-Statistics) as a measure of population differentiation due to genetic structure

F-Statistics	Value	Probability (P)
Fst	0,219	0,001
Fis	-0,019	0,577
Fit	0,205	0,012

The gene pools of broomrape parasitizing on sunflower in the former Soviet Union countries and Romania have much in common. There are almost no unique alleles in the studied populations (except the alleles 120 and 130 bp long in locus Ocum-70 of broomrape samples from Trunovskiy district, Stavropol region, Russia), high genetic similarity (0.872) of gene pools and Fst (21.9%).

Our results showed that broomrape populations from the indicated three countries are grouped into clusters according to the country of origin, regardless of their racial composition. At the same time, one of the clusters combines the parasite populations from the countries of the former Soviet Union. This fact can be explained by the following. According to our data, the broomrape from Kazakhstan is characterized by weak virulence and does not overcome the resistance gene *Or4* in sunflower. It suggests that this broomrape population is represented by seeds preserved in the soil since the USSR time. We believe that the similarity between the Kazakh and Russian broomrape populations and their intrapopulation polymorphism is based on genetic diversity and similarity of sunflower open pollinated varieties, which were cultivated in the former Soviet republics. The isolation of the USSR territories from the assortment of foreign countries for many years formed here the peculiarity of gene pool of both sunflower and its parasite. And this certain peculiarity had not yet been neutralized by a modern free exchange of seed material between countries. Years of parasitizing on open pollinated varieties can explain the fact that the gene pool of broomrape from the former Soviet Union countries is characterized by a higher level of intrapopulation diversity than the populations of Romania.

We believe that a small variability in the genome of *O.cumana*, also described by several authors (Ciuca et al., 2004; Gagne et al., 1998) may be due to the fact that the habitat of this parasitic plant is metabolites of sunflower plant, while the external factors (climate, soil, etc.) can affect the parasite only

indirectly through its host. Considering the fact that broomrape parasitizes on sunflower for a little more than 100 years, it is too early to expect its high variability.

In conclusion, this research revealed the existence of two poorly differentiated gene pools of *O.cumana*: Russian-Kazakh and Romanian. Broomrape populations from the former Soviet Union are more polymorphic and have a larger intrapopulation genetic diversity than the populations from Romania.

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Phylogenetic relationships and genetic diversity among *Orobanche cumana* Wallr. and *O. cernua* L. (Orobanchaceae) populations in the Iberian Peninsula

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ABSTRACT

Orobanche cumana is found in the Iberian Peninsula as an allochthonous species parasitizing exclusively sunflower, in contrast to the closely related species *O. cernua*, which is an autochthonous species that only parasitizes wild Asteraceae hosts. Ten *O. cumana* populations were collected in the two traditional areas of sunflower broomrape occurrence, the Guadalquivir Valley, Southern Spain (six populations) and Cuenca province, Central Spain (four populations). Twelve *O. cernua* populations were collected on wild hosts across its natural distribution area in Southeastern Spain. Genetic relationships within and between both sets of populations were studied using a subset of 50 robust and co-dominant SSR markers from *O. cumana*. The results supported the taxonomic separation of the two species and the existence of two distant genetic groups for *O. cumana*, one in Guadalquivir Valley and another one in Cuenca province. The inter- and intra-population variability was extremely low for *O. cumana*, whereas the overall genetic diversity was much higher for *O. cernua*. The genetic structure of *O. cumana* populations probably reflects a founder effect, with the two genetically distant groups deriving from separate introduction events. The high degree of genetic differentiation observed in *O. cernua* is mainly explained on the basis of restricted gene flow due to ecological barriers together with the occurrence of a predominantly self-pollinating mating system. Complementary diversity studies on both species in its current distribution area are required for understanding global genetic variability and evolutionary characteristics of the parasitism.

Key words: Genetic diversity – *Helianthus annuus* − microsatellite markers − *Orobanche cernua* – *Orobanche cumana* – sunflower broomrape

INTRODUCTION

Orobanche cumana Wallr. (sunflower broomrape) is a holoparasitic plant that parasitizes sunflower roots. It is present in sunflower crops in many countries around the world, especially in Central and Eastern Europe, Spain, Turkey, Israel, Iran, Kazakhstan, China (Ŝkorić et al., 2010), and more recently in new areas such as France (Jouffret and Lecomte, 2010). *Orobanche cumana* was first described in the Iberian Peninsula parasitizing confectionary sunflower (*Helianthus annuus* L.) crops in 1958 in Toledo province (Díaz-Celayeta, 1974). The presence of *O. cumana* in oilseed sunflower fields was observed later in wide areas of Cuenca province in Castilla-La Mancha region (Central Spain) and the Guadalquivir Valley in Andalucía (Southern Spain) (González-Torres et al., 1982). Since them, *O. cumana* has spread over the whole sunflower cultivation regions, comprising new and traditional areas of Castilla-León (Northern Spain), Castilla-La Mancha and Andalucía, causing severe yield losses in sunflower crops (Alonso et al., 1996; Fernández-Martínez et al., 2012).

The closely related species *O. cernua* L. was observed for the first time near Aranjuez (Central Spain) (Loefling, 1758). The species is mainly distributed in the North- and the South- East of the Iberian Peninsula, and is only found in the wild, in arid areas of degraded, xerothermic scrub, parasitizing different species of the Asteraceae, being most frequently found on plants of the genus *Artemisia* (Pujadas-Salvà and Velasco, 2000). *Orobanche cumana* and *O. cernua* have been traditionally considered as very closely related taxa (Pujadas-Salvà and Velasco, 2000). Several studies based on different molecular markers systems, such as RAPDs (Katzir et al., 1996; Paran et al., 1997; Román et al., 2003) or ISSRs (Benharrat et al., 2002), as well as those based on ecological, morphological and biochemical data (Pujadas-Salvà and Velasco, 2000) or seed morphology analysis (Plaza et al., 2004), clearly support the taxonomic separation of *O. cumana* and *O. cernua* and the treatment of both taxa as different species.

Specific and joint studies on genetic diversity and phylogenetic relationships among both *O. cumana* and *O. cernua* species growing in the Iberian Peninsula based on a larger number of populations could be of interest to clarify the relationships between the two species. Coupled with this, alternative markers

such as simple sequence repeat (SSR) markers, which are reproducible, neutrally evolving, multiallelic and co-dominant, are needed to enable more powerful genetic analyses in the genus *Orobanche*. A recently developed collection of SSR markers and resources are available for molecular research in *O. cumana*, which proved to be highly transferable to *O. cernua* (Pineda-Martos et al., 2014a). Accordingly, the objective of this research was to study genetic diversity in a large set of *O. cumana* and *O. cernua* populations from the Iberian Peninsula using a subset of the newly SSR markers reported.

MATERIALS AND METHODS

Plant material

Ten *O. cumana* populations were collected from 1989 to 2008 in different sunflower fields located across the main traditional distribution areas of sunflower broomrape in Spain − Cuenca province in Central Spain and Guadalquivir Valley in Southern Spain − (Table 1). The populations (seed or plant tissue, as indicated in Table 1) were collected by the authors with the exception of populations SE01 and CO06 from Southern Spain, and populations CU12, CU05, and CU07 from Central Spain, which were kindly provided by Dr. J. Fernández-Escobar (Koipesol Semillas S.A., Sevilla, Spain). Those populations in which only seeds were collected were multiplied as described in Pineda-Martos et al. (2013). In addition, twelve populations (plant tissue) of *O. cernua* were collected during the years 2000-2006 in their natural distribution area in Southeastern Spain, parasitizing *Artemisia barrelieri* Besser, *A. glutinosa* J. Gay ex Besser, and *Launaea lanifera* Pau (Asteraceae). Fresh tissue samples from individual broomrape plants of each population were frozen at −80°C, lyophilized and ground individually.

SSR analyses

The *O. cumana* and *O. cernua* populations were genotyped in previous studies (Pineda-Martos et al., 2014a, 2014b) with a set of 50 *O. cumana* SSR markers (Table 2) showing high quality. Despite the samples were pooled for each population, no complex banding patterns were observed and SSR amplification products for each population consisted in one single band (allele) in *O. cumana* and one single band or two bands in *O. cernua*. Accordingly, the bands were scored as homozygous or heterozygous patterns, although this did not represent individual genotypes, but homogeneity or heterogeneity among the individuals bulked within each population. Marker informative values such as the total number of alleles (NA) and polymorphism information content (PIC), were calculated as implemented in PowerMarker version 3.25 software package (Liu and Muse, 2005) (Table 2).

Analysis of bands was done following the shared-alleles method. Bands with the same mobility were considered identical, scored as present (1) or absent (0), and compiled into a binary data matrix. Cluster analysis based on the similarity matrix and Dice index was performed using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method of NTSYSpc, Numerical Taxonomy System, version 2.21r (Exeter Software, Setauket, NY, USA). Monomorphic markers were excluded from the analysis. The cophenetic correlation coefficient was computed and Mantel's test was performed.

Table 1. Identification and collection details of the *Orobanche cumana* and *O. cernua* populations from the Iberian Peninsula used in this study

Population	*Orobanche* spp.	Collecting site and method employed†	Year	Host	*n*
O. cumana populations from Southern Spain (Guadalquivir Valley)				Sunflower hosts	
IASCum-1	*O. cumana*	Andalucía, Córdoba, Córdoba. PT	2008	Confectionary	15
IASCum-2	*O. cumana*	Andalucía, Sevilla, Écija. PT	2008	Oilseed	15
IASCum-3	*O. cumana*	Andalucía, Sevilla, Osuna. PT	2008	Oilseed	15
Boro-13	*O. cumana*	Andalucía, Sevilla, Écija. S	2002	Oilseed	15
SE01	*O. cumana*	Andalucía, Sevilla, El Coronil. S	1989	Confectionary	21
CO06	*O. cumana*	Andalucía, Córdoba, La Carlota. S	2001	Oilseed	20
O. cumana populations from Central Spain				Sunflower hosts	
IASCum-4	*O. cumana*	Castilla-La Mancha, Cuenca, Villarejo de Fuentes. PT	2008	Oilseed	15
CU12	*O. cumana*	Castilla-La Mancha, Cuenca, Palomares del Campo. S	2008	Oilseed	20
CU05	*O. cumana*	Castilla-La Mancha, Cuenca, La Almarcha. S	1996	Oilseed	20
CU07	*O. cumana*	Castilla-La Mancha, Cuenca, Carrascosa del Campo. S	1996	Oilseed	20
O. cernua populations from Southern Spain				Wild hosts	
Boro-37	*O. cernua*	Andalucía, Almería, Níjar, Lucainena. PT	2006	*Launaea lanifera*	9
Boro-38	*O. cernua*	Andalucía, Almería, Tabernas, Venta los Yesos. PT	2006	*Artemisia barrelieri*	15
Boro-39	*O. cernua*	Andalucía, Almería, Níjar, Lucainena. PT	2006	*A. barrelieri*	5
Boro-40	*O. cernua*	Andalucía, Almería, Níjar, Huebro. PT	2006	*L. lanifera*	2
Boro-41	*O. cernua*	Andalucía, Almería, Albox, El Saliente Alto. PT	2006	*A. glutinosa*	1
Boro-42	*O. cernua*	Andalucía, Almería, Cabo de Gata, Vela Blanca. PT	2006	*L. lanifera*	7
Boro-43	*O. cernua*	Andalucía, Jaén, Jódar. PT	2006	*A. barrelieri*	7
Boro-44	*O. cernua*	Andalucía, Jaén, Cabra del Santo Cristo. PT	2006	*A. barrelieri*	8
Boro-45	*O. cernua*	Andalucía, Jaén, Cabra del Santo Cristo. PT	2000	*A. barrelieri*	10
Boro-46	*O. cernua*	Andalucía, Granada, Sierra de Parapanda. PT	2000	ND	1
Boro-47	*O. cernua*	Andalucía, Granada, Sierra de Parapanda. PT	2000	ND	6
Boro-48	*O. cernua*	Andalucía, Granada, Salobreña. PT	2003	*A. barrelieri*	8

†PT: plant tissue; S: seed.
ND: not determined.
n: number of individuals analyzed.

RESULTS AND DISCUSSION

Orobanche cumana and *O. cernua* have traditionally been considered closely related species. Putt (1978) suggested the possibility that *O. cumana* developed from a single population of *O. cernua* after the sunflower crop began to have economic importance in Russia in the nineteenth century. Although *O. cumana* has often been regarded as a variant of *O. cernua*, Joel (1987) and Jacobsohn et al. (1991) clearly differentiated the two species based on morphological differences and host. Subsequent molecular studies clearly supported the distinction between *O. cumana* and *O. cernua* (Katzir et al., 1996; Paran et al., 1997; Benharrat et al., 2002; Román et al., 2003). The results reported by Katzir et al. (1996) revealed identical diagnostic markers in *O. cumana* samples, supporting the hypothesis that these populations were different from those of *O. cernua* collected from the wild. These results suggest that the two species are genetically different, which has been supported by Pujadas-Salvà and Thalouarn (1998), and by studies of morphological, phenological, ecological and biochemical characters performed in both species by Pujadas-Salvà and Velasco (2000).

SSRs are currently considered the markers of choice in many areas of molecular genetics, due to their co-dominance and high level of polymorphism, even between closely related species. A valuable set of 217 SSR markers has been isolated from *O. cumana* and characterized in diverse populations of this species and its closely relative *O. cernua* (Pineda-Martos et al., 2014a). In the present research, a subset of 50 selected SSR markers transferred from *O. cumana* was used to evaluate the evolutionary relationships and genetic characteristics in the genetic makeup of these *Orobanche* species.

Table 2. *Orobanche cumana* SSR markers and its diversity parameters in the study of 22 *Orobanche* spp. populations collected in the Iberian Peninsula

SSR marker†	*O. cumana* populations from Southern and Central Spain collected on sunflower (*N*=10)		*O. cernua* populations from Southern Spain collected on wild Asteraceae (*N*=12)	
	NA†	PIC†	NA	PIC
Ocum-002	1	0.0000	2	0.2533
Ocum-004	1	0.0000	4	0.5665
Ocum-005	1	0.0000	3	0.4342
Ocum-006	1	0.0000	4	0.6713
Ocum-009	0	–	3	0.4992
Ocum-012	1	0.0000	4	0.6992
Ocum-014	1	0.0000	5	0.7036
Ocum-015	1	0.0000	5	0.4828
Ocum-028	1	0.0000	5	0.6539
Ocum-037	2	0.3648	5	0.5590
Ocum-042	1	0.0000	6	0.7087
Ocum-045	1	0.0000	5	0.6192
Ocum-046	1	0.0000	4	0.6218
Ocum-052	2	0.3648	7	0.8278
Ocum-063	2	0.3648	3	0.4992
Ocum-066	1	0.0000	3	0.4186
Ocum-067	1	0.0000	3	0.3633
Ocum-069	1	0.0000	3	0.5669
Ocum-070	2	0.3648	4	0.5593
Ocum-075	2	0.3648	6	0.6085
Ocum-076	1	0.0000	3	0.5045
Ocum-080	1	0.0000	4	0.4760
Ocum-085	2	0.3648	6	0.6955
Ocum-087	2	0.3648	4	0.5350
Ocum-089	1	0.0000	6	0.7879
Ocum-092	2	0.3648	3	0.3680
Ocum-094	2	0.3648	5	0.6890
Ocum-123	1	0.0000	3	0.4491
Ocum-124	1	0.0000	4	0.5182
Ocum-129	1	0.0000	2	0.2533
Ocum-140	1	0.0000	2	0.2392
Ocum-141	2	0.3648	3	0.4491
Ocum-144	1	0.0000	2	0.3047
Ocum-149	1	0.0000	1	0.0000
Ocum-163	1	0.0000	2	0.3457
Ocum-167	1	0.0000	3	0.4102
Ocum-168	1	0.0000	6	0.7456
Ocum-174	2	0.3648	3	0.4491
Ocum-176	1	0.0000	5	0.7560
Ocum-180	1	0.0000	3	0.3633
Ocum-185	1	0.0000	2	0.2392
Ocum-187	1	0.0000	2	0.2392
Ocum-192	1	0.0000	1	0.0000
Ocum-196	2	0.3648	3	0.4491
Ocum-197	2	0.3648	3	0.4361
Ocum-198	1	0.0000	3	0.4102
Ocum-205	1	0.0000	7	0.7209
Ocum-206	2	0.3648	3	0.4491
Ocum-215	1	0.0000	3	0.4442
Ocum-216	2	0.3648	8	0.7983
Mean	1.28	0.1117	3.78	0.4968

†SSR characteristics (primer sequences, annealing temperatures and product length) and its amplification quality are reported in Pineda-Martos et al. (2014a); NA: number of alleles; PIC: polymorphism information content.
N: number of populations within the group.

Cluster analysis resulted in a dendrogram with a high cophenetic value (r = 0.9952, P < 0.001) that separated the populations of both species into two main clusters, corresponding with the two species analyzed (Fig. 1). *Orobanche cumana* populations clustered together at similarity values of 0.68 or higher, while *O. cernua* populations clustered together at similarity values of 0.32, with the exception of populations Boro-46 and Boro-47. *Orobanche cumana* populations were grouped into two main groups; one group contained all the populations from Southern Spain (provinces of Córdoba and Sevilla), and a second one contained all the populations from Central Spain (province of Cuenca) (Fig. 1). *Orobanche cernua* populations were separated into five different groups. These groups comprised four populations from the South-West of Almería province, three populations from the South of Jaén province, one

population from the South of Granada province, two populations from the Central area of Almería province and two populations from the Central area of Granada province (Fig. 1).

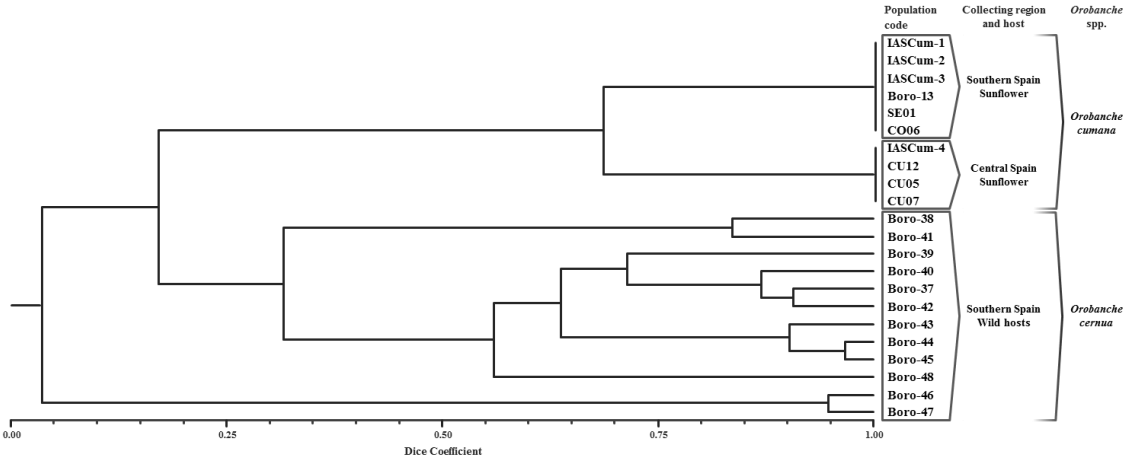

Fig. 1. UPGMA dendrogram based on Dice similarity matrix between ten populations of *Orobanche cumana* and twelve populations of *O. cernua* collected in the Iberian Peninsula obtained with 48 SSR polymorphic markers (see Table 1 for additional population details).

In the Iberian Peninsula, *O. cumana* is not found in the wild, but exclusively within sunflower fields (Pujadas-Salvà and Velasco, 2000). The great genetic separation between populations of Cuenca and the Guadalquivir Valley suggests that they may derive from seed introductions from different areas. It is also interesting to note that genetic diversity observed in *O. cumana* was considerably lower than in *O. cernua*, despite the geographically proximal populations used in the *O. cernua* set. Gagne et al. (1998) concluded that *O. cumana* populations from different geographical origins were genetically very similar, pointing to a monophyletic origin. The high genetic differentiation observed between the five groups of *O. cernua* populations, suggested the presence of effective ecological barriers preventing gene flow between the populations together with the occurrence of a predominantly self-pollinating mating system.

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Genetic diversity of *Orobanche cumana* and *Orobanche cernua* populations as revealed by variability of Internal Transcribed Spacers1/2 of ribosomal cistron and ribulose-bisphosphate carboxylase pseugene

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ABSTRACT

The sunflower broomrape - *Orobanche cumana* (Wallr) parasitizes on roots of sunflower plants and is a serious constraint on sunflower production, causing yield losses of up to 60%. The variability of Internal Transcribed Spacers1/2 of ribosomal cistron (ITS1/2) and ribulose-bisphosphate carboxylase pseugene (RbcL) in 32 samples of *O. cumana* and 4 samples *O. crenata* collected from different European locations were studied. The results showed that *O. cumana* can be differentiated from *O. cernua*, by single C/T transition located in ITS2 (rel. position 423). Rubisco large subunit in *O cumana* differs from *O. cernua* with two transversion: T/G (rel. position 15) and A/C (rel. position 84). The genetic diversity observed in *O. cumana* was lower than in *O. cernua*. When comparing the ITS and rbcL sequences isolated from *O. cernua* two single SNPs were found that can discriminate different origins. Both ITS1/2 and rbcL sequences isolated from *O. cumana* however were completely homogeneous, despite the fact that samples were collected form very distant locations: from Volgograd, Russia to the East to Spain to the West. This observation is in favor of hypothesis that the move of *O. cumana* from wild hosts on sunflower was a single act that occurred once and all invasive races are descendants from ancient Caucasus population. Probably genes related to *O. cumana* aggressiveness should be identified and used for molecular markers to determine genetic relationships within and among *O. cumana* populations.

Key words: Broomrapes – molecular markers – molecular phylogeny – *Orobanche cernua* – *Orobanche cumana*

INTRODUCTION

The sunflower broomrape - *Orobanche cumana* (Wallr.) parasitizes on roots of sunflower plants and is a serious constraint on sunflower production, causing yield losses of up to 60% (Parker, 2009). *O. cumana*. originates from wild habitats in Southern Russia and around the Black Sea coast: Romania, Turkey and Bulgaria (Parker and Riches, 1993). It forms there isolated wild population parasitizing mainly Artemisia spp. (Venkov and Bozoukov, 1994). The first reports of *O. cumana* parasitizing sunflower came from Saratov region in Russia in 1890 (Morozov, 1947). The research on the problem started more than 100 years ago (Parker and Riches, 1993) and the breeding of resistant varieties has resulted in the first sunflower varieties resistant to a race A of *O. cumana*, developed by Plachek in 1918 (Morozov, 1947). A few years later Ždanov (in 1926) identified a new *O. cumana* race - B in Rostov area and developed a number of sunflower varieties resistant to it (Morozov, 1947). Sunflower selection for broomrape resistance so far produced some significant results. Dominant genes for resistance against *O. cumana* races A, B, C, D, E, and F have been found and incorporated into cultivated sunflower genotypes. In the last few years, however new broomrape populations have been discovered in several different countries (Romania, Russia, Turkey, Spain and Ukraine). None of the existing commercial hybrids resistant to races A, B, C, D, E, and F have proven resistant to these new populations of the *O. cumana* (Parker, 2009).

The taxonomy status of *O. cumana* is also subject of debate. According to some authors *O. cumana* is conspecific with *Orobanche cernua* (Teryokhin, 1997). However other authors argued that *O. cumana* and *O. cernua* are closely related, but yet distinct species (Katzir et al., 1996; Pujadas-Salvà and Velasco,

2000). Molecular analyses can provide information about taxonomy status and intraspecific genetic variations of *O. cumana*. Other important question is: "what the races are?": physiological adaptations or there is a genetic background determining the races existence. Recently Pineda-Martos and co-authors (2013 a, b) found 78 SSR primers that produced reproducible results and allowed them to distinguish *O. cumana* from *O. cernua.* In addition they were able to discriminate among *O. cumana* isolates by geographic origin and host. Molecular diagnostics however, works better when data from random amplification of DNA targets can be compared with sequence data from specific DNA targets. In this sense, we used sequences of two genetic markers: the first one is a fragment of ribosomal cistron including 3' end of gene encoding 18S rRNA - Internal Transcribed Spacer1 (ITS1) – gene for 5,8S rRNA – ITS2 – and 5' end of gene encoding 26S rRNA. The second is fragment of plastid pseudogene encoding ribulose-bisphosphate carboxylase (RbcL). Both sequences have been used widely before for molecular taxonomy studies of Orobanchaceae representatives (Schneeweiss et al., 2004; Weiss-Schneeweiss, 2006; Wolfe et al., 1992; Benharrat et al., 2000). Here we present our study on variability of ITS1/2 and RbcL in 32 samples of *O. cumana* and 4 samples of *O. crenata* collected from different European locations.

MATERIALS AND METHODS

Plant materials: *O. cumana* samples used in this investigation originate from different regions in Europe (table 1). Fourteen samples were supplied as isolated total DNA from Instituto de Agricultura Sostenible (IAS-CSIC), Cordoba, Spain, twelve samples were provided from seeds collections of the University of Novi Sad, Serbia, eight from seeds collections of Agrobioinstitute, Sofia and two samples from seed collection of Agricultural Research & Development Institute Fundulea, Fundulea, Romania.

Primers design: The annotated in NCBI sequences of *Orobanche cernua* / *cumana* ITS1/5,8S/ITS2/26 S region (AY209234, AY911235, DQ310015, KC800810, AY209233, AY209232, AY209231) were processed by Vector NTI 10.0 software for multiple allayment and finding conservative regions and next consensus sequences were used for design of primers by and Primer 3 plus software.

We used similar approach to design primers for *Orobanche cernua* / *O. cumana* Rubisco large subunit (rbcL) pseudogene (Accession: U73968, AY582189, AY582188, AF090349). Primers were purchased Metabion AG, Germany. Upon arrival they were dissolved in DNAse free water up to 100 µmol concentration and stored at -20 ºC until use. Before use 10 µmol aliquots were prepared.

Isolation of total (genomic and chloroplast) DNA: Seeds came as pools by locations. About 50 mg of each seeds sample were grind to fine powder in pre-cooled by liquid nitrogen mortar and pestle. The powder was quickly transferred in pre-cooled microcentrifuge tube and the DNA was isolated by Analytic Yena kit following the standard protocol.

PCR reaction conditions: Approximately 150 ng DNA template was taken from each sample and mixed in 200 µL PCR tube with 1 µL of each primer (10 $mmol.L^{-1}$ concentration), 25 µL PCR master mix (Fermentas, Cat № K0171) and 21 µL DNAse-free water (supplied with the master mix kit). The PCR tubes were places TC-512 THERMAL CYCLER (Techne) PCR apparatus and PCR amplification was carried-out by using the following program: initial DNA melting at 94 ºC – 5min; next 35 cycles of 94 ºC – 45 s; 57 ºC –45 s; 72 ºC – 2 min 30 s and final extension at 72 ºC for 10 min.

Purification of the PCR products: Initially PCR products were separated by agarose gel electrophoresis. For this purpose each sample was mixed with 5 mL of loading dye (Fermentas, Cat № R0611), loaded onto 1% agarose gel containing 0.5 mg/mL ethidium bromide (final concentration) covered with 0,5 X TBE buffer and separated by applying 7 volts per cm electrical currency. The size of the products was determined by comparison with DNA ladder (Fermentas GeneRuler Cat № SM0311). The PCR products were visualized by UV light and documented by BIO-VISION+3026.WL system (Vilber Lourmat). Next the band containing separated PCR the products were sliced out of the gels by clear surgical blades and isolated from the agarose by QIAquick Gel extraction kit (Qiagen, Cat № 28704) according to the original protocol. Purified PCR products were sent for sequencing to GATC – Biotech AG, Cologne, Germany.

Data analysis: Initially the sequences were subjected to online analyses in NCBI database to confirm that the isolated sequences are indeed those of interest using the nblast algorithm of Altschul et al. (1997). The multiple alignments of obtained sequences, phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 (Tamura et al., 2013).

RESULTS

Primer design

The universal ITS1 primers available elsewhere had poor performance during our initial experiments and therefore we decided to design our own primers based on annotated in NCBI sequences for *O. cumana* and *O. cernua*. Multiple sequence alignments provided information about conservative regions in annotated sequences. The multiple sequence alignment of *Orobanche cernua* / *O. cumana* ITS1/5,8S/ITS2/26 S region in presented as illustration on Fig. 1.

Table 1. List of the studied samples

№	*Acronym*	*Species*	*Geographic origin, year collected, (host if different from sunflower)*
1.	IAS cum 1	*O. cumana*	Andalucía, Córdoba, Spain, 2008
2.	IAS cum 2	*O. cumana*	Andalucía, Sevilla, Ecija, Spain, 2008
3.	IAS cum 4	*O. cumana*	Castilla-La Mancha, Cuenca, Villarejo de Fuentes, Spain, 2008
4.	SE 01	*O. cumana*	Andalucía, Sevilla, El Coronil, Spain 1989
5.	Co 06	*O. cumana*	Andalucía, Cordoba, La Carlota, Spain 2001
6.	Cu 05	*O. cumana*	Castilla-La Mancha, Cuenca, La Almarcha, Spain, 1996
7.	Cu 07	*O. cumana*	Castilla-La Mancha, Cuenca, Carrascosa del Campo, Spain, 1996
8.	Cu 12	*O. cumana*	Castilla-La Mancha, Cuenca, Palomares del Campo, Spain, 2008
9.	Boco 34	*O. cumana*	Balchik, Bulgaria, 2006 (*Artemisia maritima*)
10.	Boco 35	*O. cumana*	Gorun-Tyulenovo, Bulgaria, 2006 (*Anthemis arvensis*)
11.	Boro 37	*O. cernua*	Andalucía, Almeria, Nijar-Lucainena, Spain, 2006 (*Launaea lanifera*)
12.	Boro 38	*O. cernua*	Andalucía, Almeria, Venta de Los Yesos, Spain, 2006 (*A. barrelieri*)
13.	Boro 43	*O. cernua*	Andalucía, Jaen, Jodar, Spain, 2006 (*A. barrelieri*)
14.	Boro 44	*O. cernua*	Andalucía, Jaen, Cabra Santo Cristo, Spain, 2006 (*A. barrelieri*)
15.	Barolo	*O. cumana*	Backa topola, Vojvodina, Serbia, 2012
16.	Barolo - Ro	*O. cumana*	Backa topola, Vojvodina, Serbia, 2012
17.	Beluga	*O. cumana*	Backa topola, Vojvodina, Serbia, 2012
18.	Or5	*O. cumana*	Sombor, Vojvodina, Serbia, 2012
19.	Or5+	*O. cumana*	Sombor, Vojvodina, Serbia, 2012
20.	24/11	*O. cumana*	Sivac, Vojvodina, Serbia, 2011
21.	31/11	*O. cumana*	Sombor, Vojvodina, Serbia, 2011
22.	32/11	*O. cumana*	Sombor, Vojvodina, Serbia, 2011
23.	Negotin	*O. cumana*	Negotin, Bor District, Serbia, 2011
24.	Norh-34	*O. cumana*	Novi Sad, Vojvodina, Serbia, 2011
25.	VRSAC	*O. cumana*	Novi Sad, Vojvodina, Serbia, 2009
26.	Sunfl-2010	*O. cumana*	Backa topola, Vojvodina, Serbia, 2010
27.	Volgograd	*O. cumana*	Volgograd, Volgograd District, Russia, 2002
28.	Krapets	*O. cumana*	Krapets, Dobrich District, Bulgaria, 2002
29.	Vratza	*O. cumana*	Vratza, Bulgaria, 2001
30.	Edirne	*O. cumana*	Edirne, Eastern Thrace, Turkey, 2003
31.	Zaporozhye	*O. cumana*	Zaporozhye, Southeastern Ukraine, 1998
32.	Gen. Toshevo	*O. cumana*	General Toshevo, Dobrich District, Bulgaria, 2008
33.	Izmir	*O. cumana*	İzmir, Anatolia District, Turkey
34.	Sadovo	*O. cumana*	Sadovo, Upper Thrace, Bulgaria
35.	PF 100 (Rom)	*O. cumana*	Fundulea, Romania, 2012
36.	1238/2 (Rom)	*O. cumana*	Fundulea, Romania, 2012

Fig. 1. Multiple alignment of annotated in NCBI sequences of *Orobanche cernua* / *cumana* ITS1/5,8S/ITS2/26 S region (AY209234, AY911235, DQ310015, KC800810, AY209233, AY209232, AY209231.

We used the consensus sequences to designed two sets of specific primers for each sequence by Primer3 plus (Table 2). The external sets of primers were used to isolate sequences, while the internal were used for control PCRs and sequencing.

Table 2. List of specific primers designed for isolation of ITS1/2 and RbcL sequences. Primers Fw1/Rev 1 are external toward Fw2/Rev2.

Primer name	*Sequence 5' → 3'*
18 S Fw1	ATAAAGCAGACCGYGAACATG
18 S Fw2	CGACTATATGGAAYTGTGGCG
26 S Rev1	AGAGCCCAACATGCAACACC
26 S Rev2	CGCAGTCGAAAGCACAAGTAG
RbcL Fw 1	CCTGCGTGATCTATGTCTGG
RbcL Fw 2	GCTCCATGGTATTCAAGTTTAAAGAG
RbcL Rev 1	TGCATTACGCTAAGGATGTCC
RbcL Rev 2	TCATTACGAGCTTGTACACATGC

Isolation of marker sequences

Newly designed primers for ribosomal gene cluster produces single reproducible bands for each sample with expected size:580 bp (Fig. 2). Isolation of fragments from Rubisco large subunit (rbcL) pseudogene was much more difficult probably due to the fact that we used as plant material dry seeds. The amount of chloroplast DNA in seeds of any species is negligible. Nevertheless we managed to isolate RbcL products with expected size from 31 out of 36 samples.

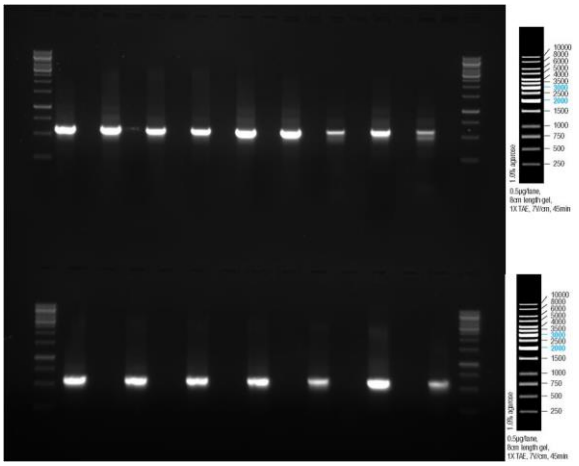

Fig. 2. The ITS1/2 products amplified by primers 18S Fw1 and 26S Rev1 were separated on 1% agarose gel containing 0.5 mg/mL ethidium bromide and visualized by UV light. The PCR products size was determined using 1 Kb Fermentas GeneRuler (Cat. № SM0311)

Sequences comparison and phylogenic analyses.

Initially we compared annotated by other authors sequences of ribosomal gene cluster. The sequences revealed clear separation of *O. cumana* from *O. cernua* by SNP located at relative position 423 (Fig. 3).

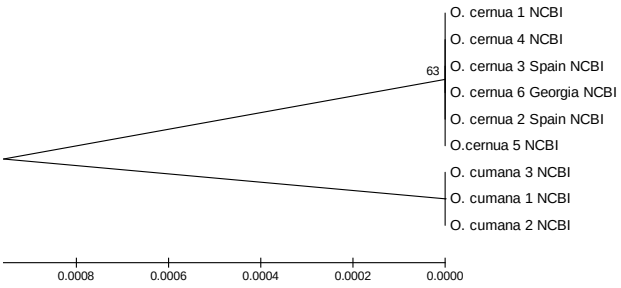

Fig. 3. Phylogenic tree build by annotated in NCBI sequences of *Orobanche cernua* / *cumana* ITS1/5,8S/ITS2/26 S region (AY209234, AY911235, DQ310015, KC800810, AY209233, AY209232, AY209231). Maximum likelihood was used, applying general time reversal model and uniform rate of substitution (Kimura, 1968). Phylogeny Test- Bootstrap method by 500 replications.

Next step was to compare our samples and officially annotated. The same algorithm was used. The final phylogenic is presented on Fig. 4. Similar approach was adopted also for comparison of annotated in NCBI and obtained by us sequences of Rubisco large subunit (rbcL) pseudogene. Because the annotated sequences were not sufficient for building separated tree, only one joint tree was produced (Fig. 5)

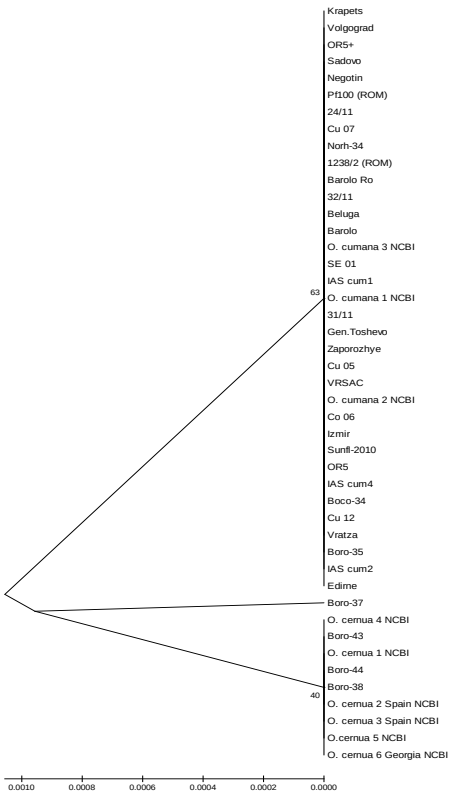

Fig. 4. Phylogenic tree build by experimental and annotated in NCBI sequences of *Orobanche cernua* / *cumana* ITS1/5,8S/ITS2/26 S region. Maximum likelihood was used, applying general time reversal model and uniform rate of substitution (Kimura, 1968). Phylogeny Test- Bootstrap method by 500 Replications.

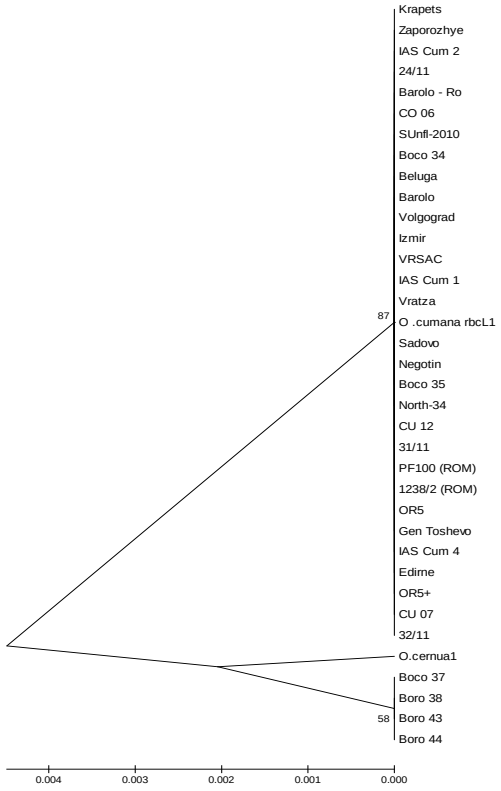

Fig. 5. Phylogenic tree build by experimental and annotated in NCBI sequences of *Orobanche cernua* / *cumana* partial Rubisco large subunit (rbcL) pseudogene (Accession: U73968, AY582189, AY582188, AF090349). Maximum likelihood was used, applying general time reversal model and uniform rate of substitution (Kimura, 1968). Phylogeny Test- Bootstrap method by 500 Replications.

DISCUSSION

The ITS1/2 regions of the ribosomal cistron are one of the most popular sequences for phylogenetic analyses at the generic and infrageneric levels in plants. The main feature that makes this region so widely used in molecular phylogeny and evolution studies is the combination of highly conservative (rRNA genes) and highly variable (ITS) regions (Moller and Cronk, 2001; Cruichshank, 2002). Other advantages of the use of ITS sequences are:

• The ITS1/2 regions is relatively short (500-800 nt).It is easily amplified by PCR using primers complementary to the conservative 18S and 26 S regions ITS-flanking
• ITS 1/2 regions are easily amplified even from diluted or degraded DNA samples.
• ITS 1/2 regions are highly variable – they could accumulate differences between closely related species and even between populations within a species.

As the ITS regions are not functional, the evolution of these sequences seem to occur according to the neutral model of Kimura, in which the genetic drift is the major driving force. Natural selection cannot operate on non-functional sequences because they don't have adaptive meaning for the organism, so the changes occurring in such sequences are random and accumulate mutations uniformly with the time (molecular clock). This makes neutral sequences useful markers for phylogeny analyses because the mutation rate reflects the divergence time between populations/species. (Kimura, 1968, 1985). Similar to the ITS 1/2 regions in non photosynthetically active plastids of broomrapes, Rubisco large subunit (rbcL)

is relaxed from evolutionary pressure and is a pseudogene. So we can expect both nuclear and plastid sequences to accumulate uniformly mutations.

Our experimental as well as those officially annotated in NCBI data demonstrated that *O. cumana* can be differentiated from *O. cernua*, by single C/T transition located in ITS2 (rel. position 423). Rubisco large subunit in *O cumana* differs from *O. cernua* with two transversion: T/G (rel. position 15) and A/C (rel. position 84). The genetic diversity observed in *O. cumana* was lower than in *O. cernua*. When comparing the ITS and rbcL sequences isolated from *O. cernua* two single SNPs were found that can discriminate different origins. Both ITS1/2 and rbcL sequences isolated from *O. cumana* however were completely homogeneous, despite the fact that samples were collected form very distant locations: from Volgograd, Russia to the East to Spain to the West. This observation is with agreement of earlier by study of Gagne et al. (1998). The authors also found *O. cumana* populations from different geographical origins to be genetically very similar, pointing to a possible monophyletic origin Gagne et al. (1998). Such assumption supports the hypothesis that the move of *O. cumana* from wild hosts on sunflower was a single act that occurred once and all invasive races are descendants from ancient Caucasus population. The high sequence similarity between *O. cumana* and *O. cernua* could mean that the separation of the two groups is a recent event. It is not possible however at this stage to draw conclusion whether they are specie or subspecies. Various molecular processes can impact ITS and RbcL sequences. Among the most prevalent complications is the existence in many plant genomes of extensive sequence variation, arising from ancient or recent array of duplication events, presence of pseudogenes at various states of decay, and/or incomplete intra- or interarray homogenization (Álvarez and Wendel, 2003). This is the reason why in their extensive review, Álvarez and Wendel (2003) recommend together with non functional sequences other single-copy functional nuclear genes to be used. Probably genes related to *O. cumana* aggressiveness should be identified and used for molecular markers. We assume the reported by Pineda-Martos et al. (2013a,b) SSRs flank such genetic regions and their sequencing can provide molecular tools to determine genetic relationships within and among *O. cumana* populations.

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Diversity of broomrape populations in Vojvodina

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ABSTRACT

Orobanche cumana is a parasitic weed that can cause a reduction in sunflower yield of up to 100% in infected areas. New races of broomrape spread due to an extensive use of broomrape resistant sunflower varieties. Up to date, only presence of races B and E was reported in Serbia. In this study, we collected broomrape dry stalks and used them for diversity studies. Broomrape stalks were collected from nine different locations in Vojvodina Province (northern Serbia) and used for diversity study with 7 RAPD markers. Five different clusters were identified in constructed dendrogram. Diversity study showed that there is little interpopulation genetic diversity between tested broomrape populations of Vojvodina. In addition, we found some differences in intrapopulation diversity among tested populations.

Key words: Diversity – *Orobanche cumana* – RAPD – sunflower

INTRODUCTION

Orobanche cumana Wallr. is a parasitic angiosperm that absorbs water and nutrients from host plant leading to a significant yield loss. It parasitizes not only on sunflower, but also on tomato, tobacco, etc. In contaminated fields, broomrape infestation leads to reduction of sunflower yield between 10 and 100% (Acimovic, 1998). *Orobanche* spp. were first reported in USSR at the end of the 19th century, while it has widely spread at the beginning of the 20th century (Acimovic, 1998). Broomrape seeds are extremely small in diameter and can be transferred easily from contaminated fields. Seed dissemination can be facilitated by humans, agricultural tools, crop seeds, etc. Since genetic resistance is the most efficient tool for controlling broomrape, extensive work on discovering and introducing resistance genes in cultivated sunflower was done (Jan and Fernández-Martínez, 2002; Škorić et al., 2010; Cvejic et al., 2012). This led to accelerated spreading of new, more virulent races.

In Serbia, sunflower is the main source of vegetable edible oil and therefore the most cultivated oil crop. Broomrape can mostly be found in the North of Serbia, in Vojvodina Province. *O. cumana* was first reported in 1951 (Acimovic, 1977), and is one of the biggest constraints in sunflower production in Vojvodina. Until the end of the 20th century race B was dominant in Serbia. In the mid-90s race composition changed and new broomrape race (E) was discovered (Mihaljcevic et al., 1996). Broomrape attacks have always been more severe in Backa in comparison to Banat and Srem (Mihaljcevic et al. 1996; Masirevic and Malidza, 2006).

Understanding genetic composition of broomrape populations is important for sunflower breeding programs, especially when new, more virulent races emerge. Until now, RAPD markers have widely been used for diversity studies in number of weeds (Xu et al., 2003; Zybartaite et al., 2011). RAPD markers were also used for determination of genetic diversity of *Orobanche* spp. populations (Gagne et al., 1998; Ciuca et al., 2004; Paran et al., 1997; Román et al., 2003; Atanasova et al., 2005; Molinero-Ruiz et al., 2013). Recently, Pineda-Martos et al. (2013) reported use of SSR markers for analysis of broomrape diversity between different populations from Spain, Turkey, Hungary and Romania. As in the last decades a change in racial composition was observed, the aim of this study was to genotype different broomrape populations in Northern part of Serbia, Vojvodina Province. This is especially important, since there have been reports of more virulent races of broomrape emergence in neighboring countries: Romania and Hungary (Pacureanu-Joita et al., 2008; Molinero-Ruiz et al., 2013). This is the first diversity study of different Vojvodina broomrape populations on molecular level that should give an insight into their genetic structure. We have tested 9 broomrape populations collected in Vojvodina Province in order to analyze population diversity.

MATERIALS AND METHODS

Dry broomrape stalks were collected from nine sites from different locations in Vojvodina Province: northern populations: Mol (individuals: Mol1-Mol5), Supljak (Sup1-Sup5), Senta (Sen1-Sen5), Sombor (Som1-Som5), Krivaja (Kri1-Kri5) and Zrenjanin (Zre1-Zre5); southwestern population: Erdevik (Erd1-Erd5); southeastern populations: Pancevo (Pan1-Pan5) and Zagajica (Zag1-Zag5) (Fig.1.).

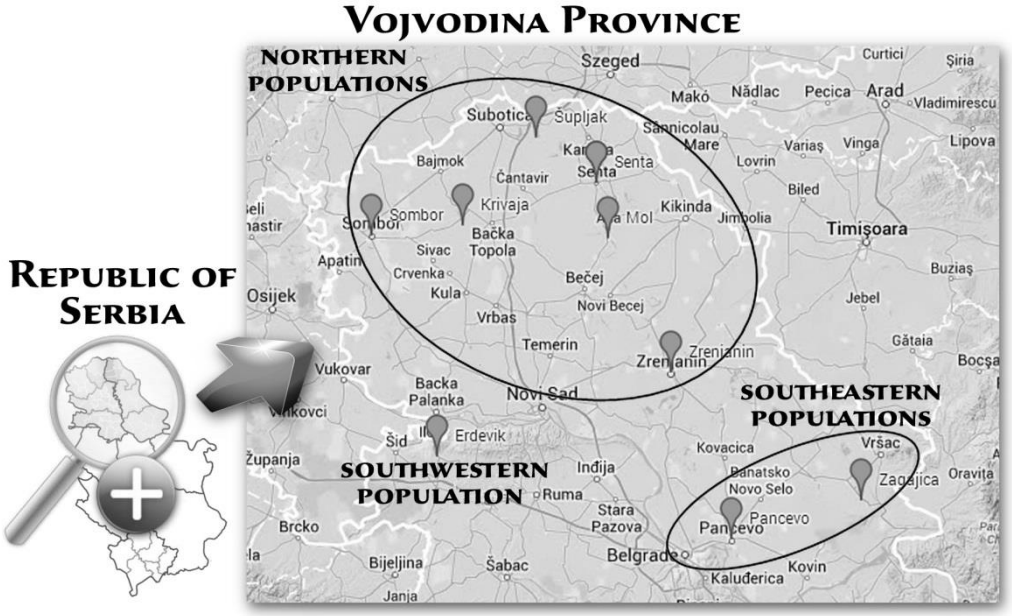

Fig.1. Locations of the collected broomrape populations (Google maps)

DNA was isolated from dry stalks by modified Rogers and Bendich method (1985) (Dimitrijević et al., 2013). Isolated DNA was amplified with 7 different RAPD UBC primers created at University of British Columbia, Vancouver (Table 1.). PCR amplification was performed according to Dimitrijevic et al. (2013) in TGradient Professional Standard, Biometra. PCR products were separated on 2% agarose gels/TBE buffer. Gels were visualized under UV light, by use of BIO-Print system (Vilber Lourmat, Marne la Velee, France).

Presence of amplified bands was scored with 1, while absence with 0 and binary matrix was created for UPGMA cluster analysis in STATISTICA12 (StatSoft, Inc., Tulsa, OK 74104, USA).

Table 1. Primers used for diversity study and their sequences

Primer	Sequence
UBC 268	AGG CCG CTT A
UBC 272	AGC GGG CCA A
UBC 299	TGT CAG CGG T
UBC 358	GGT CAG GCC C
UBC 363	ATG ACG TTG A
UBC 377	GAC GGA AGA G
UBC 383	GAG GCG CTG C

RESULTS AND DISCUSSION

Based on profiles obtained from amplification with 7 RAPD primers, dendrogram was generated by UPGMA method (Fig. 2.). Used primers were polymorphic and amplified 152 bands in total (between 13 and 32 bends per primer) (UBC 358 - 28, UBC 272 - 15, UBC 272 - 28, UBC 299 - 13, UBC377 - 22, UBC383 - 32, UBC363 - 14).

Present study of broomrape populations showed that populations from Vojvodina grouped in five clusters (I, II, III, IV and V). Within cluster I two sub-clusters were formed (Ia and Ib). All individuals of Zrenjanin and Sombor populations grouped within cluster I, while individuals of Erdevik population formed cluster III. Individuals of Krivaja population were grouped in four clusters (I, II, IV, V), while individuals of Pancevo population in three (I, IV, V). Populations of Zagajica, Supljak, Mol and Senta were found in two clusters: I and IV, I and V, I and II, I and II, respectively.

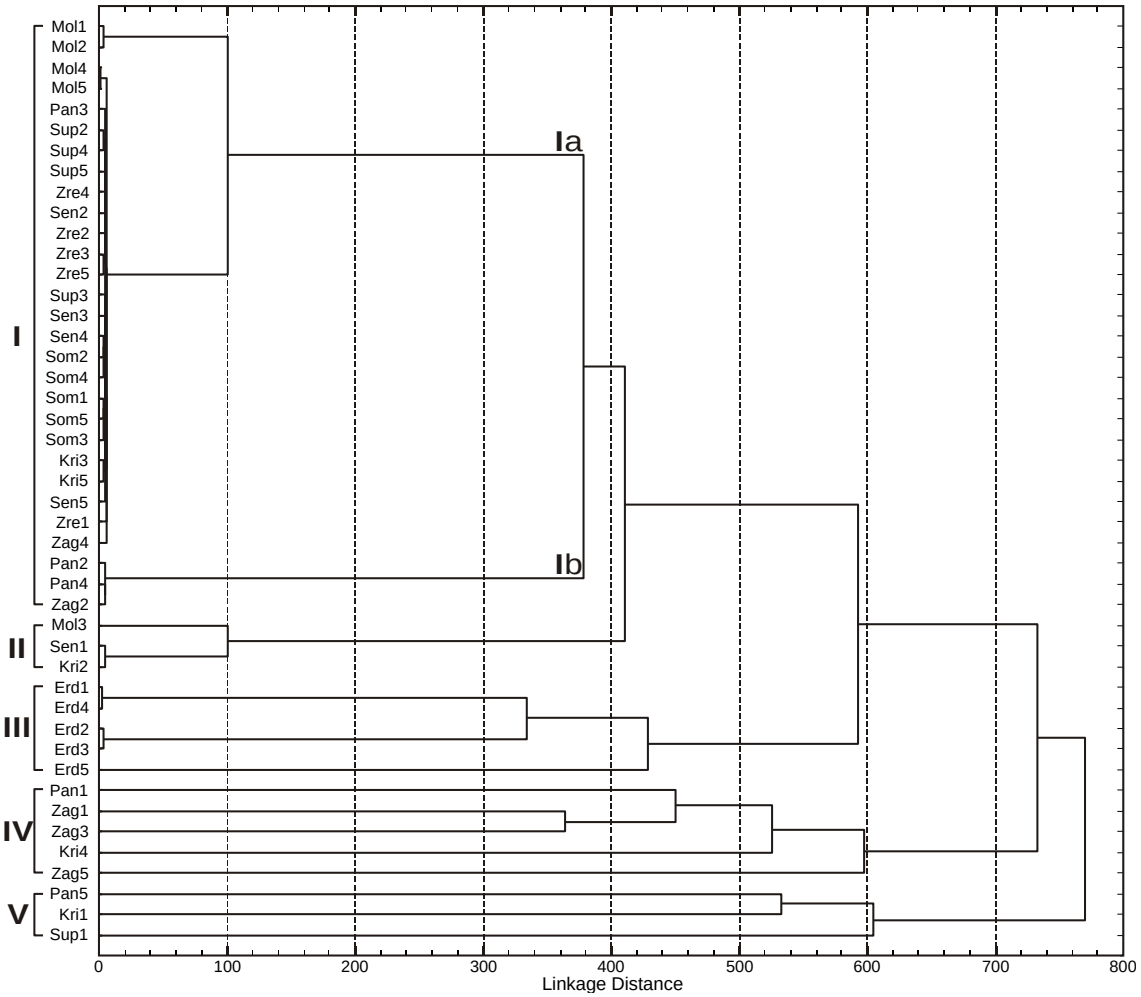

Fig.2. Dendrogram based on RAPD analysis of tested broomrape populations

We did not find clear connection between geographic and genetic distance of tested broomrape populations, since a few individuals of the southern populations were grouped with northern populations (cluster I), and vice versa (in cluster V). Molinero-Ruiz et al. (2013) established a clear connection between formed groups and geographical origin, while Pacureanu-Joita et al. (2012) found no correlation between genetic distance and geographic distance between different populations from Romania, Serbia, Moldova, Spain and Turkey.

Out of all tested populations, Krivaja population showed the largest diversity. Similar results were obtained by Mihaljcevic (1996) who found that out of twenty Vojvodina broomrape populations, populations from Krivaja and Ljutovo were the least homogenic. This was explained by the possibility of presence of, most likely, race D (Mihaljcevic, 1996).

Individuals of two populations from southeastern Vojvodina (Pancevo and Zagajica) were grouped with individuals of northern populations. This could be due to presence and genetic diversity of both races B and E, since there are reports of presence of both of these races in Serbia (Mihaljcevic, 1996; Dedic et al., 2009). The majority of individuals of Pancevo population were grouped within cluster I (in comparison to IV and V), posing as an intermediate between northern populations and Zagajica.

In this study, we observed differences in intrapopulation diversity between tested populations. The largest intrapopulation diversity was observed in Krivaja, while the lowest intrapopulation diversity was observed in Erdevik, Sombor and Zrenjanin populations. Similarly, Pineda-Martos et al. (2013) found little intrapopulation diversity in tested Spanish broomrape populations with a few populations showing great intrapopulation genetic diversity. In addition, differences in broomrape intrapopulation variation was observed by Castejón-Muñoz et al. (1991) who reported that out of five tested populations only one had greater intrapopulation diversity using isoenzymes and Gagne et al. (1998) who found little intrapopulation diversity in broomrape populations from Romania, Bulgaria, Turkey and Spain.

Present diversity study showed that there is little interpopulation genetic diversity between tested broomrape populations of Vojvodina. In addition, we found some differences in intrapopulation diversity

among tested populations. Since, in recent years broomrape is spreading thorough Serbia, future analysis will include more Serbian populations and use of newly reported SSR markers for diversity study.

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DIVO project: Study of *Orobanche cumana* genetic diversity

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ABSTRACT

Sunflower broomrape (*Orobanche cumana* Wallr.) is a holoparasitic plant that constrains sunflower production in many countries around the world (Škorić et al. 2010). The yield damage can be severe and impact 100% of the production. For almost a century, there has been a constant tug-of-war between sunflower breeders and *Orobanche cumana*, with frequent changes in which side has the upper hand (Škorić et al. 2010). Therefore, a better understanding of *O. cumana* genetic architecture and phylogeny may allow a better prediction of its evolution. The objective of DIVO project is to study the genetic diversity of *O. cumana* by collecting broomrape populations across Europe. The genetic diversity of *O. cumana* is assessed by a molecular approach combining whole exome sequencing and SNP genotyping. The first work package of the project is dedicated to the collect of broomrape populations across Europe. For this purpose, a set of 20 differential hybrids has been defined in order to discriminate broomrape populations for their aggressiveness. A total of seven countries (France, Spain, Hungary, Romania, Turkey, Ukraine, and Russia) with an average of 4 locations per country have been collected, feeding an 'Orobanchoteque' of more than 500 lots. The second work package aims to develop molecular tools for broomrape genetic characterization. Among the collected environments (combination of country x location), twelve *O. cumana* populations representing five different countries have been selected for whole exome sequencing. Sequencing results allows the detection of more than 400,000 SNP on 43,000 transcripts, with an important rate of heterozygous loci. After filtering for quality, minimum allele frequency and rate of heterozygosity a set of 1536 SNP have been selected for the genotyping of the 500 lots. The third work package is focus on the genetic diversity study based on the genotypic data produced in WP2. Preliminary results suggest that the pattern of genetic diversity mimic the different level of aggressiveness observed in field and/or in the high throughput screening platform developed and patented by Biogemma[1]. Finally, the main deliverable of the project will be a SNP kit (the smallest optimized one) for routine characterization and classification of broomrape populations. This SNP kit will be publicly available at the end of the project.

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[1]*Orobanche* high throughput screening platform is a technology protected by patent application EP 13306587.0

SESSION 3

Genetic resistance to sunflower broomrape

Genetic resistance to sunflower broomrape (*Orobanche cumana* Wallr.)

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ABSTRACT

Orobanche cumana Wallr causes important economic damage in sunflower production in a number of countries around the world. Historically, breeders have been successful in developing cultivars resistant to this parasite, but the introduction of new resistance sources has been followed by the appearance of new pathogenic races overcoming resistance. Sunflower selection for broomrape resistance makes use of different methods for testing breeding materials, looks for resistance sources, and has so far produced significant results. Dominant genes for resistance to races A, B, C, D, E, and F have been found and incorporated into cultivated sunflower genotypes. However, in the last fifteen years, new broomrape populations are being discovered for which very limited or none of the existing commercial hybrids have proven to be resistant. In this review, we will focus on recent contributions on breeding for broomrape resistance, including the identification and characterization of new sources and mechanisms of resistance, their the genetic and molecular characterization, and also recent research about important aspects on the parasite side. Additionally, we will discuss the need of long term strategies involving (i) the complete host-parasite system and the integration of classical and molecular approaches, (ii) international cooperation to establish a common designation of the new races of the parasite and genes for resistance in different countries, and (iii) a greater level of collaboration between breeders from public institutions and private companies, which will all contribute to the development of more durable sources of resistance.

Key words: Broomrape races – sunflower – genetic resistance – breeding – molecular markers

INTRODUCTION

Broomrape (*Orobanche cumana* Wallr.) is a parasitic angiosperm that has been causing a great deal of damage to sunflower production for more than a century. According to Morozov (1947), the first reports of broomrape in sunflower came from Saratov Oblast in Russia and date back to the 1890s. The same author mentions that the first sunflower varieties resistant to race A of broomrape were developed by Plachek (1921) at the Saratov breeding station. Morozov (1947) and Pustovoit (1966) both note that Ždanov (1926) identified a new broomrape race (B) in Rostov Oblast and soon after the discovery developed a number of sunflower varieties resistant to it. In the period that followed, according to Pustovoit (1966), a number of high-oil varieties resistant to race B were developed at the VNIIMK institute in Krasnodar, Russia that after played an important role in the spread of sunflower around the world.

Nowadays, broomrape causes great damage to sunflower production and new races of the pathogen appear frequently in Russia, Ukraine, Romania, Bulgaria, Turkey, and Spain. In addition, broomrape is also present in Serbia, Hungary, Moldova, Greece, Israel, Iran, Kazakhstan, China, Mongolia, and Australia, and possibly in a few other countries as well (Škorić et al., 2010). Sunflower breeders and geneticists have been successful in developing broomrape resistant cultivars but breeding programs are often based on a reduced number of dominant genes and resistance breakdown caused by the appearance of new virulent races that overcome all known resistance genes occurs (Fernández-Martínez et al., 2012). This situation has forced sunflower breeders to continuously search for new sources of resistance and/or using alternative methods of control. The objective of this paper is to make an overview of what has been achieved in sunflower genetics and breeding for *Orobanche* resistance so far and to describe the sources of this resistance as well as the breeding methods and directions employed in the field.

DISCUSSION

Occurrence of broomrape races

At the beginning of 20th century, broomrape spread across Russia significantly and endangered the mass production of sunflower. The first cultivar resistant to race A, Saratovski 169, was created by Placek (1921). In the years that followed, other cultivars resistant to race A were also produced (Kruglik A- 41; Zelenka and Fuksinka). As the mass production of sunflower spread quickly, it was followed by a new race, called B (Zhdanov, 1926). During 1925-1960, Pustovoit created in Russia highly productive cultivars, resistant to race B. After the cultivars resistant to race B, for years, nobody mentioned the occurrence of new races, even though the composition of races had changed. The existing cultivars were assumed to possess several genes for resistance to broomrape (races C and D). In 1970, Petrov (1970) announced the existence of a new race in Bulgaria (race C). At the same time, its existence was announced in Romania. Vrânceanu et al. (1980) clarified significantly broomrape racial situation and helped to detect dominant genes by establishing the existence of five races which were controlled by dominant genes: A (*Or1*), B (*Or2*), C (*Or3*), D (*Or4*) and E (*Or5*). Later studies confirmed the prevalence of races D and E in several countries such as Spain (Melero-Vara et al., 1989), Serbia (Masirevic and Medic-Pap, 2009), Turkey (Bulbul et al., 1991), and Bulgaria (Shindrova, 2006). New races appeared from the middle 1990s onward in several countries. Initially, all of them were named as race F, although the relationship between the different races F has not been studied. They have been reported in Spain (Alonso et al., 1996), Romania (Pacureanu-Joita et al., 1998), Turkey (Kaya et al., 2004), Bulgaria (Shindrova, 2006), Ukraine (Burlov and Burlov, 2010), and Russia (Antonova et al., 2013). Populations overcoming resistance sources to race F, named as races G and H, have been identified in most of these countries (Antonova et al., 2013; Kaya et al., 2009; Pacureanu-Joita et al., 2009a; Shindrova and Penchev, 2012). As mentioned for race F, no comparative studies have been conducted between races G and H reported in different countries. In China, *Orobanche cumana* has been present for a long time, with the identification of race A done by Bichun et al. (1996). Since then, new races have appeared in this country, but the race composition has not been reported.

Therefore, the current racial situation of broomrape populations from different countries remains unclear. There is not information on whether races under the same classification, reported in different countries, are the same or they differ for virulence. For this reason, collaboration to clarify the racial status of sunflower broomrape, to establish a common sunflower differential sets and molecular tools is becoming a top priority. In order to understand race evolution, knowledge of genetic structure and dynamics of *Orobanche cumana* populations is required. Molecular diversity among and within parasite populations from different geographical areas has been done (Gagne et al., 1998; Pineda-Martos et al., 2013; Molinero-Ruiz et al., 2014). The studies indicated that there are different pathogenic groups from the same geographical origin and provided useful information for developing molecular markers associated with geographical origin of *O. cumana*, which could be used as a diagnostic tool for new parasite introduction into growing areas where they might represent a threat to sunflower production.

A study by Joel et al. (2004) confirmed the importance of molecular markers for the study of sunflower broomrape. They found that RAPD patterns of DNA extracted from soil-borne *Orobanche* seeds is identical to that of DNA from vegetative plant material, provided that the seeds had not deteriorated. They also note that DNA of reasonable diagnostic quality could be extracted not only from tetrazolium-positive soil-borne *Orobanche* seeds but also from tetrazolium-negative seeds. This makes it possible to perform quick genetic analysis without having to wait for broomrape seeds to germinate or develop into plants.

Sources of broomrape resistance

Genes for resistance to broomrape races A, B, C, and D are present in varietal populations of sunflower developed in breeding programs from Saratov (former USSR), Krasnodar (former USSR), Odessa (Ukraine), Fundulea (Romania) and several other places. Some of these genes have been identified in certain wild species of the genus *Helianthus* and have been incorporated into cultivated sunflower genotypes by interspecific hybridization. A species of wild sunflower (*Helianthus tuberosus*) was first used as a source of *Orobanche* resistance (Vrânceanu et al., 1980; Škorić et al., 2010) as the donor of *Or* genes.

As new broomrape races were appearing, sources of resistance in cultivated sunflower became increasingly scarce. In Turkey, Gulya et al. (1994) found only 22 resistant entries in a field evaluation of 903 accessions, whereas in Spain, Domínguez et al. (1996) found 8 resistant and 33 segregating entries in the evaluation of 429 accessions of different origins for resistance to race E. Sources of resistance to the

latest races have been scarce in germplasm of cultivated sunflower, although valuable resistant germplasm has been identified in breeding programs conducted in Spain (Fernández-Martínez et al., 2004; Rodríguez-Ojeda et al., 2001), Romania (Pacureanu-Joita et al., 2004, 2009b), Turkey (Kaya et al., 2009), and Russia (Gontcharov et al., 2004; Gontcharov, 2009). In most cases, the germplasm exhibited vertical or qualitative genetic resistance. In addition, genetic sources of horizontal or quantitative genetic resistance have been developed (Pérez-Vich et al., 2005). In contrast, a high level of resistance to the newest races has been found in wild *Helianthus* species. Fernández-Martínez et al. (2000) tested for race F resistance 54 wild sunflower accessions (representing 27 perennial and four annual species) and 55 cultivated sunflower accessions. Most of the perennial species proved fully resistant to this race. The only exceptions were some populations of four of the wild perennials, which had a certain percentage of susceptible plants. Among the wild annual species, *H. anomalus* and *H. agrestis* were completely resistant, while *H. debilis* ssp. *cucumerifolius* and *H. exilis* segregated with regard to *Orobanche* resistance. Jan and Fernandez-Martinez (2002) employed interspecific hybridization to incorporate genes for resistance to race F from several wild species into cultivated sunflower, and developed four populations (BR1-BR4) resistant to this race from the wild sunflower species *H. maximilianii* Schrad, *H. grosseserratus* Mart., and *H. divaricatus* L (Jan et al., 2002). Where necessary, they used embryo culture and chromosomal doubling by colchicine in order to bypass the barriers and enable the transfer of desirable genes. Christov et al. (1992, 1998, 2009) have achieved outstanding results in identifying genes for broomrape resistance in the wild species of the genus *Helianthus* and incorporating them into cultivated sunflower genotypes. Especially important are the findings reported in Christov et al. (2009), which concern the detection of *Or* genes in 11 perennial wild sunflower species and their incorporation into elite cultivated sunflower lines by means of interspecific hybridization. Recently, resistance to a race classified as G has been transferred from *H. debilis* into cultivated sunflower (Velasco et al., 2012).

Sources of *Orobanche* resistance can also be found by the use of induced mutations. Venkov and Shindrova (1998) reported that they obtained a mutant with partial resistance to *O. cumana* using a 0.4% solution of the mutagen nitrosomethylurea.

Characterization of resistance

In parallel with the appearance of new broomrape races and sources of broomrape resistance, the genetics of resistance to this parasitic plant has been studied. As sources of resistance to races A and B were identified, it was also determined that resistance to broomrape was controlled by dominant genes. Burlov and Kostyuk (1976) and Pogorletsky and Geshele (1976) studied the genetic basis of *Orobanche* resistance and discovered that it was controlled by a single dominant gene, which they named *Or*. Vrânceanu et al. (1980) conducted extensive genetic research as part of his study of broomrape in Romania from 1976 to 1980. They established that there were five pathogenic races of this parasite and labeled them A, B, C, D, and E. They also identified a set of differential lines that had cumulative resistance to the five successive races, conferred by the dominant genes Or_1, Or_2, Or_3, Or_4, and Or_5, respectively. When race F subsequently appeared in Romania and resistance to it was discovered in the line LC-1093 (Or_6) by Pacureanu-Joita et al. (1998), this cycle of genetic research was completed.

The appearance of new broomrape races in Spain triggered a new cycle of large-scale genetic analyses. Dominquez et al. (1996) noted that there is a low frequency of genes for resistance to race E in cultivated sunflower and that this resistance is controlled by two dominant genes. Sukno et al. (1999) reported that resistance to race E was controlled by a single dominant gene (Or_5). Alonso (1998) noted that, the known dominant genes notwithstanding, resistance to *Orobanche* may be more complex than previously thought and that genes other than single dominant ones may also be involved. In some cases involving cultivated sunflower germplasm, resistance to race F is controlled by recessive genes. Thus, *Orobanche* resistance found in the lines P-96 and KI-534 is controlled by recessive alleles at two loci (Rodríguez-Ojeda et al., 2001; Akhtouch et al., 2002). The same recessive genes control resistance to race E in the line KI-534 (Rodríguez-Ojeda et al., 2001). Akhtouch et al. (2002) crossed lines resistant to race F with those that are susceptible to it and found segregation ratios of 1:15 [Resistant (R): Susceptible (S)] and 1:3 (R : S) in the F_2, and BC_1 generations, which in most cases indicates double dominant epistasis. Cases of segregation ratios of 3:13 (R : S) and 1:1 (R : S) were also recorded in the F_2s and BC_1s, which is indicative of dominant-recessive epistasis. Velasco et al. (2007) crossed a line resistant to race F (J1) with three susceptible lines and studied the inheritance of face F resistance, obtaining segregation ratios of 3:1, 13:3, and 15:1 (R + Moderately R : S) in the F_2, generations. These results indicated incomplete dominance of the Or_6 alleles and the presence of a second gene, Or_7, whose expression was influenced by the environment. The digenic model was confirmed through the evaluation of $F_{2:3}$ families.

Recently, Pacureanu-Joita et al. (2008) tested the latest, virulent race of broomrape from Romania through a cross between the resistant line AO-548 and the susceptible line AD-66 and segregation ratios of 15:1 (R:S) and 3:1 (R:S) were observed in the F_2 and BC_1 generations, respectively, indicating that the resistance in AO-548 is controlled by two independent dominant genes. In Spain, a single dominant gene controlling resistance to the most virulent race G in lines derived from interspecific crosses with *Helianthus debilis* subsp. *tardiflorus* has recently been reported by Velasco et al. (2012).

Most of the molecular research for characterizing broomrape resistance has been focused on mapping the Or_5 gene conferring resistance to races A to E. This gene has been mapped to a terminal, probably telomeric region of linkage group (LG) 3 of the sunflower genetic map (Lu et al., 2000; Pérez-Vich et al., 2004; Tang et al., 2003). The closest marker was identified at around 6 centimorgan downstream of Or_5 (Lu et al., 2000; Tang et al., 2003), but no flanking markers were found in the upper part of the LG. Márquez-Lema et al. (2008) identified a telomere-associated target region amplification polymorphism (TRAP) marker linked to Or_5, probably flanking the gene in the upper telomeric side. In addition to the major role of Or_5 in race E resistance, Pérez-Vich et al. (2004) also identified a quantitative component of the race E resistance determined by four quantitative trait loci (QTL) with minor effect associated with the number of broomrape shoots per plant. Imerovski et al. (2013) demonstrated that simple sequence repeat (SSR) markers of LG 3 were also strongly associated with resistance genes *Or2*, *Or4*, and *Or6*. For race F resistance, QTL analysis in a population derived from line P-96, for which phenotypic analysis suggested the presence of two recessive loci (Akhtouch et al., 2002), revealed the presence of six QTL with small to moderate effects on reducing the number of broomrape shoots per plant, three of them being non-race specific (Pérez-Vich et al., 2004). These results suggest that sunflower resistance to broomrape is controlled by a combination of qualitative, race-specific resistance effecting the presence or absence of broomrape and quantitative, non-race-specific resistance affecting the number of broomrape stalks per plant.

Mechanisms of sunflower resistance to broomrape

It is very important to know all the mechanisms involved in broomrape resistance (physiological, biochemical, mechanical, etc.) in order to be able to understand all aspects of this phenomenon. These resistance mechanisms have been studied for a long time. Thus, Morozov (1947) cites the results of Richter (1924) that indicated that broomrape susceptible sunflowers had root systems with a low pH, and those of Suhorukov (1930) concerning the link between peroxidase values and sunflower susceptibility to broomrape, according to which increased soil acidity increased peroxidase activity and the susceptibility of sunflower plants to *Orobanche*. According to Morozov (1947), Barcinskiy (1932, 1935) reported that sunflower root cells contain substances that stimulate the germination and development of broomrape seeds and seedlings. Long after that, Wegmann (1998), Alonso (1998), Matusova et al. (2004), and Honiges et al. (2009) also pointed out the importance of broomrape germination stimulants. Joel et al. (2011) identified the natural broomrape germination stimulant from sunflower roots exudates as a dehydrocostus lactone. Low exudation of germination stimulants by sunflower roots has been described as a preattachment resistance mechanism (Labrousse et al., 2001). Another preattachment resistance mechanism is the exudation by sunflower roots of seed germination inhibitors and/or inhibitors of radicle exoenzymes (Höniges et al., 2008). Phytoalexins, in particular 7-hydroxilated simple coumarins, have been suggested to play a defensive role by preventing broomrape germination and subsequent connection with sunflower roots (Serghini et al., 2001).

Mechanical barriers like lignifications of the cell wall by peroxidase-catalyzed reactions have been proposed as postattachemt resistance mechanisms (Höniges et al., 2008). Panchenko and Antonova (1975) concluded that the protective response of different sunflower cultivars came down to the accumulation of lignin and its precompounds in injured host cells, resulting in the haustoria losing the ability to supply themselves with water and nutrients from the host cells. Antonova and Borg (1996) reported that differences in peroxidase production can be used for interpreting the different virulence of races C and D. Also, a physical barrier by reinforcement of the host cell walls through suberization and protein cross-linking that prevents parasite intrusion has been described in sunflower genotypes resistant to race F (Echevarría-Zomeño et al. 2006). This mechanism was also observed for race E, but in this case cell wall was reinforced by means of callose depositions (Letousey et al., 2007).

Some of the previously mentioned studies have revealed the simultaneous occurrence of several resistance mechanisms in genotypes exhibiting complete resistance (Echevarría-Zomeño et al., 2006; Labrousse et al., 2001; Letousey et al., 2007). Labrousse et al. (2000, 2001, 2004) discuss different criteria for assessing *Orobanche* resistance and the different mechanisms by which such resistance operates. The authors were able to distinguish between three types of broomrape resistance in their work:

a) resistance acting at an early stage in broomrape development (*H. debilis* ssp. *debilis*), when broomrape seedlings were present on the sunflower root, but an impassable encapsulation layer blocked the intruding parasite, which then died; b) resistance found in the resistant line LR1, which involves two types of action: i) decreased stimulation of broomrape germination (a three-fold reduction compared to susceptible line 2603); and ii) rapid necrosis that appeared as early as stage 2 of parasite development; and c) resistance observed at a later stage of broomrape development in the line 92B6 (necrosis developing prior to broomrape flowering).

Louarn et al. (2012) found that arbuscular mycorrhizal fungi could produce inhibitors of *O. cumana* germination, and that this inhibitory effect seemed restricted to broomrape seeds.

Methods used for evaluating broomrape resistance

In order to attain their breeding goals and identify sources of broomrape resistance, sunflower breeders must develop a breeding strategy, decide on a breeding method, secure the necessary germplasm and differential lines for broomrape race identification, and choose the appropriate inoculation method. In the years in which races A through E were discovered, sunflower breeders tested their breeding materials in naturally infested fields, usually on plots that had been severely infested by broomrape the year before. This method is still employed by some breeders. However, this approach does not always produce reliable results due to the influence of environmental factors and an inadequate amount of broomrape seeds in the soil. In an effort to avoid this, breeders resorted to collecting broomrape seeds and to carrying out artificial infestation in the field experiments, either by incorporating the seed into the soil using basic tillage (Vrânceanu et al., 1980) or by inoculating individual plants in small pots to be transplanted into the field after 2-3 weeks in the growth chamber (Velasco et al., 2007). However, this method is prone to producing experimental errors too, caused primarily by the effects of environmental factors. Much more accurate results can be obtained by putting broomrape seeds into containers filled with a pre-prepared soil medium which are then placed in a controlled environment (growth chamber or greenhouse). Panchenko (1975) developed a screening method for assessing resistance to broomrape in greenhouse conditions during autumn and winter. This method was further honed by Grezes-Besset (1994), who made testing using plastic test tubes part of the procedure. The advantage of this technique is that it provides a higher level of reliability and makes it possible to test a large number of genotypes in a short period of time.

Different methods have been developed for evaluating sunflower physiological mechanisms of resistance, which include the evaluation of the underground broomrape development in Petri dishes assays covered with glass fiber paper (Echevarría-Zomeño et al., 2006) or in two-layer filter paper rolls (Antonova et al., 2011; Rodríguez-Ojeda et al., 2010), or the use of hydroponic co-culture (Labrousse et al., 2004).

Methods of sunflower breeding for resistance to broomrape

Breeding programs focused on the development of broomrape-resistant hybrids of sunflower are mainly based on single dominant *Or* genes. To ensure their success, the best way to go is to pick out an elite line and cross it with a source of *Or* genes, which should then be incorporated into the breeding material using certain techniques (recurrent cross-breeding together with screening for resistance in all BC generations). At the start of the program, the breeder must determine which race or races are present in the region for which the hybrids are being developed. A set of differential lines for races A, B, C, D, and E has been provided by Vrânceanu et al. (1980), while Pacureanu-Joita et al. (1998) have identified such a line for race F. As of yet, there are no public differential lines for the new, virulent races of broomrape that have appeared in the last few years.

Alternative breeding strategies are required to increase the durability of genetic resistance to broomrape. Continuous search for new sources of resistance is important. The most significant results are achieved by interspecific hybridization in which wild species of genus *Helianthus* are used as donor of the gene of resistance. Transferring resistance genes from annual wild species is accomplished rather easily with a conventional crossing scheme, but, from perennial species is generally more difficult, due to problems associated with early hybrid embryo abortion and sterility in F_1 and BC_1F_1 generations. Such problem can be overcome with using of embryo rescue and chromosome doubling of the F_1. Also, alternative breeding strategies involving vertical resistance should incorporate gene pyramiding, alternation of several forms of a hybrid with different *Or* genes, or mixtures of these different forms grown together. Finally, to get the best use of these major genes, they need to be backed-up by quantitative, non-race specific resistance. These strategies will require QTL analysis and development of molecular markers linked to major and minor resistance genes to ensure that they are simultaneously

introgressed during backcross, and a detailed characterization of the physiological mechanisms underlying genetic resistance.

CONCLUSIONS AND FUTURE PROSPECTS

Sunflower broomrape continues to be one of the most serious production constraints in many countries around the world. Breeders and geneticists have been successful in responding to the rapid changes in the race composition of broomrape. They have found genes for resistance to this parasitic plant and incorporated them into elite lines of cultivated sunflower, making it possible to develop *Orobanche*-resistant hybrids. Nowadays, in order to face the continuous broomrape expansion and virulence evolution, international cooperation including public and private Institutions is needed to establish (i) an international collection of broomrape populations that would be kept within the confines of a single institution and accessible to all users, with comprehensive evaluation of genetic diversity of this parasite across the main infected areas; (ii) a common designation of the new races of the parasite in different countries through a new set of differential lines for the new races that have appeared; (iii) a common designation of genes for resistance reported by different authors in different countries though allelic crosses and molecular mapping; and (iv) universal protocols (methods) for screening for resistance to broomrape in field and greenhouse conditions and at the molecular level, so that the findings of teams from different parts of the world can be compared to each other.

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Wild sunflower species as a genetic resource for resistance to sunflower broomrape (*Orobanche cumana* Wallr.)

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ABSTRACT

Broomrape (*Orobanche cumana* Wallr.) is a parasitic weed that causes economic damage in sunflower production in many countries, especially in Central and Eastern Europe, Spain, Turkey, Israel, Iran, Kazakhstan, and China. Genes for resistance to broomrape races A, B, C, D, and E are present in varietal populations of cultivated sunflower. Since broomrape is a highly variable parasitic weed, the breakdown of resistance is a frequent phenomenon, and multiple sources of resistance are needed to control the emerging races. Genes that confer resistance to races F, G, and H, and others that have not been assigned a race designation have been identified in wild sunflower species and incorporated into hybrid sunflower through interspecific hybridization. The USDA-ARS National Plant Germplasm System wild sunflower collection contains 2,239 accessions with 1373 annual accessions represented by 14 species and 866 perennial accessions represented by 39 species. Sunflower germplasm evaluations for resistance to broomrape races have demonstrated that the *Helianthus* species constitute a substantial reservoir of genes conferring resistance to new virulence races. The resistance to broomrape, including immunity reported in seven annual and 32 perennial species, provides breeders a broad genetic base from which to search for resistance to existing and newly emerging races.

Key Words: Broomrape – *Helianthus* – genetic resources – wild species – parasitic weed – genebank

INTRODUCTION

Broomrape, caused by *Orobanche cumana* Wallr., is a parasitic weed that infects sunflower roots causing severe crop losses in Central and Eastern Europe, including the Black Sea region, and the Middle East (Höniges et al., 2008; Pricop and Pacureanu, 2011; Fernández Martínez et al., 2010. The parasite has also been reported in Israel, Asia (Eizenberg et al., 2003), and Tunisia (Amri et al., 2012). It has also been observed in Australia, Mongolia, and China and is generally associated with drier climates.

Broomrape was first observed on sunflower in the Saratov District in Russia in the 1890s. Traditionally five races, A through E, have been controlled using resistance genes Or_1 through Or_5, respectively (Vrânceanu et al., 1980). The first sources of resistance to *Orobanche* found in the early sunflower breeding programs in the former USSR, Ukraine, and Romania originated from land races of cultivated sunflower. Since broomrape is a highly variable parasite, the breakdown of resistance is a frequent phenomenon, and multiple sources of resistance are needed to control the emerging races. Historically, sunflower breeders have been successful in developing broomrape resistant cultivars but breeding programs are often based on a few dominant genes and resistance breakdown caused by the appearance of new virulent races that has occurred frequently in recent decades (Fernández-Martínez et al., 2010). Unlike other host plant-*Orobanche* systems, most sources of resistance to *O. cumana* in sunflower have been based on vertical resistance mechanisms controlled by single dominant genes (Fernández-Martínez et al., 2012). This has led to a rapid breakdown of the resistance and subsequently to a continuous need for new resistance sources. Genes that confer resistance to race E, F, G, and H, and others that have not been assigned a race designation have been incorporated into hybrid sunflower through interspecific hybridization.

Genetic resources are the biological basis for global food security. Preservation of cultivars, landraces, and wild relatives of sunflower provides the basic foundation to promote and sustain sunflower production. The USDA-ARS, National Plant Germplasm System wild species collection is preserving the genetic diversity of the genus *Helianthus*, but at the same time making accessions available for screening for broomrape resistance. Examples of the extensive use of the wild species sources of race-specific broomrape resistance will be given.

MATERIALS

USDA-ARS NPGS sunflower collections. The mission of the USDA-ARS National Plant Germplasm System (NPGS) is to conserve genetically diverse crop germplasm and associated information, to conduct germplasm-related research, and to encourage the use of germplasm and associated information for research, crop improvement, and product development. The NPGS is united through the Germplasm Information Resource Network (GRIN) database, which serves as the portal for requesting available germplasm from the NPGS collections and as a resource for passport data and associated information for each accession.

Cultivated sunflower collection. The NPGS sunflower collections are maintained at the USDA-ARS, North Central Regional Plant Introduction Station (NCRPIS) in Ames, Iowa, USA. The cultivated sunflower germplasm collection was established at Ames, Iowa in 1948. This collection is a diverse assemblage of 1825 cultivated accessions from 59 countries. Cultivated sunflower is represented by a single species, *Helianthus annuus*. Currently, 92% of the cultivated accessions are available for distribution.

Wild sunflower species collection. The wild *Helianthus* species collection was established at Bushland, Texas in 1976. In 1985 it was transferred to NCRPIS, Ames, Iowa where it is currently housed. The wild species collection contains all 39 perennial species and 14 annual species (Schilling, 2006). The number of wild species accessions is 2239, of which 929 are wild *Helianthus annuus* accessions and 444 accessions represent 13 other wild annual *Helianthus* species. Thirty-nine perennial *Helianthus* species are represented by 866 accessions. Currently, 95% of the annual accessions are available for distribution, while 70% of the perennial species accessions are available. The number of accessions for each species is given in Tables 1 and 2.

Table 1. Infrageneric classification of annual *Helianthus* species and number of accessions in the USDA-ARS, NPGS sunflower genebank.

Section[1] (Chromosome number)	Species	Common name	Number accessions
Helianthus			
(2n=34)	*H. annuus* L.	Annual, Prairie	929
(2n=34)	*H. anomalus* Blake	Anomalous	6
(2n=34)	*H. argophyllus* T.&G.	Silver-leaf	51
(2n=34)	*H. bolanderi* A. Gray	Bolander's, Serpentine	14
	H. debilis		
(2n=34)	ssp. *debilis* Nutt.	Beach	12
(2n=34)	ssp. *cucumerifolius* (T.&G.) Heiser	Cucumber-leaf	11
(2n=34)	ssp. *silvestris* Heiser	Forest	22
(2n=34)	ssp. *tardiflorus* Heiser	Slow-flowering	9
(2n=34)	ssp. *vestitus* (Watson) Heiser	Clothed	3
(2n=34)	*H. deserticola* Heiser	Desert	21
(2n=34)	*H. exilis* A. Gray	Serpentine	30
(2n=34)	*H. neglectus* Heiser	Neglected	22
	H. niveus ssp. *niveus* (Benth.) Brandegee		
(2n=34)	ssp. *niveus* (Benth.)Brandegee	Snowy	1
(2n=34)	ssp. *tephrodes* (Gray) Heiser	Ash-Colored, Dune	11
(2n=34)	*H. paradoxus* Heiser	Pecos, Puzzle, Paradox	12
	H. petiolaris		
(2n=34)	ssp. *canescens* (A. Gray)E.E. Schilling	Gray	20
(2n=34)	ssp. *fallax* Heiser	Deceptive	31
(2n=34)	ssp. *petiolaris* Nutt.	Prairie	103
	H. praecox		
(2n=34)	ssp. *hirtus* Heiser	Texas	7
(2n=34)	ssp. *praecox* Englm. & A. Gray	Texas	8
(2n=34)	ssp. *runyonii* Heiser	Runyon's	26
Agrestes			
(2n=34)	*H. agrestis* Pollard	Rural, Southeastern	10
Porteri			
(2n=34)	*H. porteri* (A. Gray) J. F. Pruski	Confederate Daisy, Porter's	9

[1]Schilling and Heiser, 1981; Schilling, 2006.

Table 2. Infrageneric classification of perennial *Helianthus* species and number of accessions in the USDA-ARS, NPGS sunflower genebank.

Section[1] (Chromosome number)	Series	Species	Common Name	Number accessions
Ciliares	***Ciliares***			
(2n=34)		*H. arizonensis* R. Jackson	Arizona	2
(2n=68,102)		*H. ciliaris* DC.	Texas blueweed	32
(2n=34)		*H. laciniatus* A, Gray	Alkali	7
Ciliares	***Pumili***			
(2n=34)		*H. cusickii* A. Gray	Cusick's	23
(2n=34)		*H. gracilentus* A Gray	Slender	14
(2n=34)		*H. pumilus* Nutt.	Dwarfish	59
Atrorubens	***Corona-solis***			
(2n=102)		*H. californicus* DC.	California	22
(2n=34,68)		*H. decapetalus* L.	Ten-petal	30
(2n=34)		*H. divaricatus* L.	Divergent	28
(2n=102)		*H. eggertii* Small	Eggert's	13
(2n=34)		*H. giganteus* L.	Giant	26
(2n=34)		*H. grosseserratus* Martens	Sawtooth	48
(2n=68)		*H. hirsutus* Raf.	Hairy	12
(2n=34)		*H. maximiliani* Schrader	Maximilian	68
(2n=34)		*H. mollis* Lam.	Soft, Ashy	28
(2n=34)		*H. nuttallii* ssp. *nuttallii* T.&G.	Nuttall's	25
(2n=34)		*H. nuttallii* ssp. *rydbergii* (Britt.) Long	Rydberg's	12
(2n=102)		*H. resinosus* Small	Resinous	23
(2n=34)		*H. salicifolius* Dietr.	Willow leaf	19
(2n=102)		*H. schweinitzii* T.&G.	Schweinitz's	1
(2n=68, 102)		*H. strumosus* L.	Swollen, Woodland	33
(2n=102)		*H. tuberosus* L.	Jerusalem artichoke	92
Atrorubens	***Microcephali***			
(2n=34)		*H. glaucophyllus* Smith	White leaf	12
(2n=34)		*H. microcephalus* T.&G.	Small-headed	14
(2n=34, 68)		*H. smithii* Heiser	Smith's	7
Atrorubens	***Atrorubentes***			
(2n=34)		*H. atrorubens* L	Purple-disk	14
(2n=34)		*H. occidentalis* ssp. *occidentalis* Riddell	Few leaf, Western	5
(2n=34)		*H. occidentalis* ssp. *plantagineus* (T.& G.) Heiser	Few leaf, Western	12
(2n=102)		*H. xlaetiflorus* Pers.	Mountain	11
(2n=102)		*H. pauciflorus* Nutt. ssp. *pauciflorus*	Stiff	21
(2n=102)		*H. pauciflorus* ssp. *subrhomboides* (Rydb.) O. Spring	Stiff	17
(2n=34)		*H. silphioides* Nutt.	Odorous	15

[1] Schilling and Heiser, 1981; Schilling, 2006.

Table 2. (Cont'd) Infrageneric classification of perennial *Helianthus* species and number of accessions in the USDA-ARS, NPGS genebank.

Section[1] (Chromosome number)	Series	Species	Common name	Number accessions
Atrorubens (2n=34)	***Angustifolii***	*H. angustifolius* L.	Narrow leaf, Swamp	28
(2n=34)		*H. carnosus* Small	Fleshy	5
(2n=34)		*H. floridanus* A. Gray ex Chapman	Florida	9
(2n=34)		*H. heterophyllus* Nutt.	Variable leaf	17
(2n=34)		*H. longifolius* Pursh	Longleaf	3
(2n=34)		*H. radula* (Pursh) T.&G.	Scraper, Rayless	40
(2n=34)		*H. simulans* E. E. Wats.	Muck, Imitative	7
(2n=34)		*H. verticillatus* Small	Whorled	5
(2n=34		*H. winteri* Stebbins	Winter's	5

[1]Schilling and Heiser, 1981; Schilling, 2006.

A new endemic perennial species, *H. winteri* was discovered in the foothills of the Sierra Nevada Mountains, near Fresno in central California in 2012 (Stebbins et al., 2013). The new species is distinguished from the common wild annual *H. annuus* by its shrubby perennial growth habit, woody stem, and year round flowering. It grows on dry, steep rocky slopes with granitic soils with plant heights up to 4 meters.

DISCUSSION

The early wild species sources were introduced into susceptible sunflower from wild species, mainly *H. tuberosus* (Pustovoit et al., 1976). The early former Soviet Union cultivars and *H. tuberosus* were also important sources of resistance for the broomrape complex of races in Romania (Vrânceanu et al., 1980). Early reports of broomrape resistance were from cultivars 'Progress' and 'Novinka', which were developed using the "Group Immunity" breeding approach (Pustovoit and Gubin, 1974). Immunity to broomrape in lines derived from *H. tuberosus* was also described by Pogorietsky and Geshle (1976).

Several investigators (Fernández-Martínez et al., 2000, 2010; Nikolova et al., 2000; Bervillé, 2002; Škorić and Pacureanu-Joita, 2011; Christov, 2013; Antonova et al., 2011; Terzic et al., 2010) reported that sunflower germplasm evaluations for resistance to broomrape races have demonstrated that the *Helianthus* species constitute a substantial reservoir of genes conferring resistance to new virulence races. Resistance to races E, F, G and all subsequent races have been found in wild species of sunflower.

A new broomrape race, race F, discovered in Spain in 1995 that spread rapidly was capable of overcoming all previously effective resistance genes (Alonso et al., 1996). Sukno et al. (1998) reported that perennial *H. giganteus*, *H. laevigatus*, *H. pauciflorus*, and *H. resinous* have resistance to race SE194 from Spain. Hladni et al. (2009) described resistance to races E and F of broomrape in an *Rf* line derived from annual *H. deserticola* in Serbia.

High levels of resistance to races E and F have been found in the wild *Helianthus* species by Ruso et al. (1996) and Fernández-Martínez et al. (2000). They found resistance to races E and F in 29 perennial wild species, while very low levels were found in annual species, with only four of eight species evaluated showing some resistance to race F. Ruso et al. (1996) evaluated wild annual and perennial sunflower species' reactions to Spanish races and found two annual species, *H. anomalus* and *H. exilis*, that had resistance, and all 26 perennial species tested were resistant. Crossing perennial species with cultivated sunflower can be difficult, but with the use of embryo culture and chromosome doubling of the F_1s, amphiploids that facilitate the transfer of broomrape-resistant genes from the wild perennial species can be created. Using these techniques, amphiploids of perennial wild species *H. grosseserratus*, *H. maximiliani*, and *H. divaricatus* were produced that were resistant to race F (Jan and Fernández-Martínez, 2002) and led to the release of four germplasm populations resistant to race F, named BR1 through BR4 (Jan et al., 2002). 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). Pérez-Vich et al. (2002) studied the inheritance of resistance to race F derived from interspecific amphiploids with *H. annuus* and with two wild perennials, *H. divaricatus* and

H. grosseserratus. They suggested that the resistance is controlled by a single dominant gene. Upon reexamination by Velasco et al. (2006), however, the resistance of the sunflower germplasm J1 derived from *H. grosseserratus* proved to be digenic, the second gene being influenced by environmental factors. Petcu and Pacureanu (2011) reported that interspecific hybrids derived from *H. argophyllus* were resistant to races E and F in Romania.

Christov (2013) reported that 17 wild *Helianthus* species, perennial *H. tuberosus*, *H. pauciflorus* (=*rigidus*), *H. eggertii*, *H.* x *laetiflorus*, *H. decapetalus*, *H. hirsutus*, *H. divaricatus*, *H. giganteus*, *H. maximiliani*, *H. nuttallii* ssp. *rydbergii*, *H. salicifolius*, and *H. smithii*, and annual *H. annuus* (wild), *H. argophyllus*, *H. debilis*, *H. petiolaris* and *H. praecox* were resistant to broomrape races A to G in Bulgaria. Also in Bulgaria, resistance to broomrape (race not specified) was reported in different progenies of interspecific hybrids with *H. pumilus* by Nikolova et al. (2004). Diploid perennial species *H. divaricatus*, *H. giganteus*, *H. glaucophyllus*, *H. grosseserratus*, *H. mollis*, *H. nuttallii*, and *H. smithii* and their interspecific hybrids were reported to be resistant to broomrape by Nikolova et al. (1998).

However, a more virulent race (designated G) attacking cultivars resistant to race F was identified (Molinero-Ruiz and Melero-Vara, 2005; Škorić et al., 2010). Antonova et al. (2011, 2013) reported a high percentage of race H in the southern regions of the Russian Federation. Recently, resistance to race G has been transferred from annual *H. debilis* ssp. *tardiflorus* (Velasco et al., 2012). Cvejic et al. (2012) reported a new source of resistance to race G, and unnamed more virulent races in an inbred line derived from interspecific hybridization with *H. divaricatus* in Serbia. Inbred lines possibly resistant to race G were developed from crosses with *H. tuberosus*.

The interaction between *Orobanche* and the roots of wild sunflowers has been studied by Labrousse et al. (2001). Roots of an interspecific hybrid derived from *Helianthus debilis* ssp. *debilis* produced an impassable encapsulation layer that blocked the intruding parasite, which then died. Another interspecific hybrid from the same species showed reduced stimulation of broomrape seed germination and rapid necrosis at an early stage of parasite development. Resistance also occurred in an interspecific hybrid derived from *H. argophyllus* occurring mainly at stage four of the parasite development with no broomrape seed production observed, because necrosis occurred before the broomrape flowered.

Cultivated sunflower has a narrow genetic background and is deficient in genes for resistance to broomrape. The diversity of wild sunflower species in the USDA-ARS wild species genebank offers breeders a diverse genetic pool from which to discover unique genes for existing and emerging new races of broomrape.

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Broomrape (*Orobanche cumana* Wallr.) resistance breeding utilizing wild *Helianthus* species

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ABSTRACT

Wild *Helianthus* species possess valuable resistance genes for sunflower broomrape (*Orobanche cumana* Wallr.), especially the 39 largely underutilized perennial species. Resistance to race F has been transferred into a cultivated background via bridging of interspecific amphiploids. More recently, a single dominant gene resistant to race G was identified in annual *H. debilis* ssp. *tardiflorus* and transferred into cultivated HA 89. Interspecific crosses between wild annual *Helianthus* species and cultivated lines are relatively easy compared to those involving wild perennial species, which were made easier only after the development of embryo rescue techniques. Interspecific amphiploids resulting from colchicine treatment of F_1 hybrids provide bridging materials for transferring genes without relying on embryo rescue. Among the diploid, tetraploid, and hexaploid perennial species, the speed of gene utilization follows the ploidy level of diploids, tetraploids, and hexaploids due to the time consuming backcrosses required to eliminate the extra chromosomes in the latter two groups. In the development of pre-breeding materials, the retention rate of genetic material of the wild species is another concern with each additional backcross. For crosses involving tetraploid and hexaploid wild perennials, the use of 2n=51 chromosome F_1 or BC_1F_1 generation, as pollen source, could accelerate chromosome reduction to 2n=34 in BC_1F_1 or BC_2F_1, resulting in useful materials with fewer backcrosses for trait selection.

Key words: Sunflower – broomrape – interspecific gene transfer – interspecific amphiploids

INTRODUCTION

Sunflower broomrape (*Orobanche cumana* Wallr.) is a holoparasitic plant, which parasitizes sunflower roots and constrains sunflower production worldwide (Škorić et al., 2010). Vrânceanu et al. (1980) identified five broomrape races, A to E with a set of differential lines carrying corresponding major dominant resistance genes Or_1 to Or_5. *Orobanche* was not a major problem in sunflower production, being controlled by the Or_5 gene for over 20 years until the appearance of race F in Spain in the mid-1990s, making the gene ineffective (Alonso et al., 1996). Shortly afterward, race G was reported by Škorić et al. (2010). More recently germplasm resistant to race G was overcome in Romania (Pacureanu-Joita et al., 2009) and Turkey (Kaya et al., 2009), suggesting the appearance of a new race H. These rapid race shifts of *Orobanche* since the mid-1990s sent an alarming message to the sunflower community and all sunflower breeders who were in a tight race with the ever-evolving broomrape races, with the majority of the sunflower hybrids lacking the critically needed resistance genes to the new races.

Fortunately, resistance to *Orobanche* race F was identified in both cultivated and wild *Helianthus* sources with exceptional resistance in wild perennials. Race F resistance genes from four interspecific amphiploids (Amps) were transferred into a cultivated background with the resistance genes from one source suggesting a digenic model of resistance (Jan et al., 2002; Velasco et al., 2007). Subsequently, a single dominant race G resistance gene was transferred from *H. debilis* ssp. *tardiflorus* into a cultivated background (Velasco et al., 2012).

Wild *Helianthus* species have been shown to possess an abundance of valuable genes for improving cultivated sunflower, including broomrape resistance, but have long been neglected and underutilized, especially the perennials, due to the difficulties of crossing with cultivated sunflower. Major problems include cross incompatibility, F_1 sterility, and the time-consuming backcrossing to eliminate extra chromosomes when polyploid perennial species are used, as well as the concern of the loss of wild species genetic materials. However, embryo rescue techniques developed in the 1980s (Chandler and Beard, 1983) have made interspecific hybridization a routine process, coupled with improved greenhouse evaluation procedures that have enabled effective selection with speed and accuracy.

However, it took three years to obtain resistant BC_1F_2 and BC_2F_1 plants for the race G resistance, and even longer for the race F resistance utilizing interspecific Amps that required several cycles of backcrossing to reduce the chromosome numbers from 2n=68 to 2n=34, while selecting for resistant progenies (Jan et al., 2002; Velasco et al., 2012). As a general rule, diploid wild x diploid cultivated crosses produce adequate progenies for selection in BC_1F_1, BC_1F_2 or BC_2F_1, requiring the least amount of time of 2 to 3 years. Tetraploid wild x cultivated crosses require more time to reduce the 2n chromosomes from 68 to 34, with 2n=34 progenies starting to appear in BC_3F_1 generation at the earliest. Hexaploid wild x cultivated crosses take even longer, beginning to observe 2n=34 plants in approximately the BC_4F_1 generation.

Since wild perennials are known to be highly resistant to broomrape, proactive pre-breeding efforts should be considered before race shifts by focusing on the production of a large number of progeny families, since the wild species accessions can be screened for resistance and the corresponding progeny families can be quickly screened. Research is in progress to compare the effectiveness of producing progenies with maximum wild species genetic diversity with 2n=34 chromosomes for breeders' use utilizing different wild species sources. It is generally believed that progenies with fewer backcrosses retain more wild species genetic information, and therefore the diploid wilds would be the best candidates for pre-breeding. However, the tetraploids, hexaploids, and the interspecific Amps all have merit and value for interspecific gene transfer due to their large genome size with multiple genomes, as well as the improvement in backcrossing allowing for faster utilization in breeding for broomrape resistance.

During the past 10 years, we have conducted interspecific gene transfer for Sclerotinia stalk and head rot resistance from wild *Helianthus* species, with a focus on the utilization of the difficult-to-cross perennial species, which are mostly immune to Sclerotinia stalk rot and have high levels of resistance to head rot. Since the resistance to Sclerotinia is controlled by quantitative genes, the strategy has been to develop early generation backcross progenies with maximum genetic diversity from the wild parent(s) for selection in replicated field trials using artificial inoculation. Families with significantly higher resistance than the recurrent parents have been identified verifying the usefulness of this approach (Liu et al., 2014). A high level of resistance to broomrape in wild *Helianthus*, particularly the perennials, has been reported by Fernández-Martínez et al. (2008). To stay ahead of the expected evolution of new broomrape races, a similar approach to that used for the Sclerotinia resistance could be used by breeders to save time by using the already developed pre-breeding segregating families.

Previously, we successfully transferred resistance to broomrape race F from interspecific Amps involving perennial species, and race G resistance from annual *H. debilis* ssp. *tardiflorus*, while monitoring resistance in early generations. While utilizing tetraploid Amps, plants with 2n=34 to 51 chromosomes were observed in the BC_2F_1 generation, resistant plants with 2n=34 were self-pollinated, and BC_2F_2 plants were used to produce seeds for germplasm release (Jan et al., 2002). A resistance gene for race G was identified in *H. debilis* ssp. *tardiflorus* plants, crossed by HA 89, and BC_1F_1, BC_1F_2, BC_2F_1, and BC_2F_2 were used to demonstrate the monogenic dominant gene control of the resistance (Velasco et al., 2012).

MATERIALS AND METHODS

Plant materials

The following representative wild species groups will be used to demonstrate the germplasm development, including embryo rescue, the F_1s, backcrosses, and resulting lines for resistance selection; (1) wild diploid perennial species *H. salicifolius* and *H. occidentalis*, (2) wild tetraploid perennial *H. hirsutus*, (3) wild hexaploid perennial *H. californicus*, and (4) tetraploid interspecific Amps of *H. grosseserratus* x P21 and *H. nuttallii* x P21, and hexaploid interspecific Amps with *H. strumosus* x P21. Traditional crossing and backcrossing methods were used to transfer the genes from wild species, with the aid of embryo rescue technique for some crosses. Two oilseed maintainer lines, HA 410, HA 451, and a nuclear male-sterile line NMS HA 89 were used for backcrossing. Selected F_1 or BC_1F_1 progenies were used both as female and as the pollen parent for backcrosses in order to compare the efficiency of germplasm development, as well as for chromosome reduction to 2n=34 when polyploid wild *Helianthus* species were involved.

Embryo rescue and mitotic chromosome counts

Embryos from crosses were rescued six to eight days after pollination following Jan and Chandler (1989). Root tips were collected from two to three week-old seedlings for chromosome counts for each plant in

different generations following Liu et al. (2013). Slides were analyzed using a Zeiss Axioplan2 fluorescence imaging microscope (Jena, Germany).

RESULTS

Representative crosses involving wild species with different ploidy levels are shown in Table 1.

Table 1. The chromosome number and seed set percentage of F_1 and backcross progeny for selected crosses during development.

Parentage	F_1			The first backcross generation with 2n=34 plants observed			The backcross generation with most 2n=34 plants observed[a]		
	F_1 embryo %	2n	BC embryo /seed %	Generation	2n	Seed set %	Generation	2n	Seed set %
H. salicifolius× HA 410	5.13	34	1.31[b]	BC_1F_1[c]	34-36	9.6	BC_1F_1	34-36	9.6
H. salicifolius× HA 410[d]	-	34	3.53[b]	BC_1F_1	34	66.2	BC_1F_1	34	66.2
H. occidentalis× HA 410	17.93	34	0.62[b]	BC_1F_1[c]	34, 35	7.1	BC_1F_1	34, 35	7.1
H. occidentalis× HA 410[d]	-	34	11.09[b]	BC_1F_1	34	68.3	BC_1F_1	34	68.3
H. hirsutus× HA 451	14.13	51	0.65[b]	BC_3F_1 / BC_2F_2[c]	34-37	28.3	-	-	-
H. hirsutus× HA 451[d]	-	51	0.59[b]	BC_1F_1	34	73.2	BC_1F_1	34	73.2
H. californicus × HA 410	32.39	51	2.7	BC_4F_1	34-40	35.3	BC_4F_3	34-37	39.6
Amp *H. grosseserratus* / P 21 (2n=68) × HA 410	-	51	9.1	BC_2F_1	34-38	59.1	BC_2F_2/ BC_3F_1	34-35	67.9
Amp *H. nuttallii* 730/ P 21 (2n=68) × HA 410	-	51	1.1	BC_2F_1	34-37	50.7	BC_2F_2/ BC_3F_1	34-36	35.9
Amp *H. strumosus* /P 21 (2n=102) × HA 410	-	68	19.7	BC_2F_1	34-41	78.2	BC_2F_2/ BC_3F_1	34-36	62.1

[a]The plants with 2n=34 were selected for seed increase for field evaluation for Sclerotinia stalk or head rot.
[b]The percentage of embryos/florets in the BC_1 generation.
[c]The first generation with 2n=34 plants observed when the F_1s were used as the female parents in backcrossing with HA 410 or HA 451.
[d]The generation with the most 2n=34 plants observed when the F_1s were used as the male parents in backcrossing with NMS HA 89.

Crosses involving hexaploid *Helianthus* are represented by *H. californicus* x HA 410. Initial crosses were obtained relatively easily, with a high percentage of hybrid embryo formed (32.39 %), resulting in a large number of F_1 seedlings (388) after embryo rescue. The process of chromosome reduction from the 2n=68 to 2n=34 required repeated backcrosses, with 2n=34 chromosome progeny first observed in BC_4F_1, with sufficient progeny families with 2n=34 selected for trait selection with good seed set of 35.3%.

Crosses involving tetraploid *Helianthus* were represented by *H. hirsutus* x HA 451. It was moderately easy to produce F_1s via embryo rescue, with 14.13% embryo formation. However, the backcrosses using the F_1s as the female parent resulted in extremely low embryo formation (0.65%). The same low seed set problem continued for the subsequent backcrosses with no plants having 2n=34 in BC_1F_1. Three 2n=34 plants out of 27 BC_3F_1/BC_2F_2 plants were obtained with a range of 2n=34-37 chromosomes, and an average seed set of 28.3%. The long time needed to reduce the chromosome number cannot be ignored since it increased the probability of losing valuable genes. Alternatively, crosses of NMS HA89 x F_1 resulted in all 2n=34 BC_1F_1 progeny, with an average seed set percentage of 73.2%, which drastically shortened the breeding time when utilizing this tetraploid species.

Crosses involving diploid *Helianthus* were represented by *H. salicifolius* and *H. occidentalis* x HA 410. Interspecific crosses of this group were the most difficult to obtain, with low embryo formation and fewer F_1 plants. BC_1F_1 embryo formation was low, 1.31 and 0.62%, respectively producing only a small number of plants having 2n=35 or 36 chromosomes. However, 2n=34 BC_1F_1 plants could be selected providing an adequate number of families for selection, with low, but acceptable backcross seed set of 7 to 10%. Alternatively, BC_1F_1s resulting from NMS HA89 x F_1s of this group resulted in all 2n=34 plants, with an average seed set of 66.2 and 68.3%, respectively. Therefore, the BC_1F_3 families can be equally utilized without the complication of having to select for 2n=34 plants in BC_1F_1.

Crosses involving tetraploid Amps were represented by Amps *H. grosseserratus*/P 21 and *H. nuttallii*/P 21 crossed with HA 410. Plants with 2n=34 were first observed in the BC_2F_1 generation, and the majority of families with 2n=34 observed in the BC_2F_2 and BC_3F_1 generations. Useful families adequate for breeding would require one or two more generations of self-pollination when reaching BC_2F_3 or BC_3F_3. Seed set of this group does not present any problem from either further backcrosses or selfing.

Crosses involving hexaploid Amps were represented by Amp *H. strumosus*/P 21 x HA 410. The speed of advancement of this group is similar to that of the tetraploid Amps, with usable materials available at BC_2F_3 or BC_3F_3. As for the tetraploid Amps, plants of this group are expected to have adequate backcross and selfing seed set after identification of 2n=34 plants.

DISCUSSION

Even though the Or_5 gene was effective against broomrape race E for up to 20 years, the fast pace of new and more virulent races being identified since the mid-1990s and the lack of sufficient resistance in sunflower hybrids presents a major challenge to the international sunflower community threatening its survival as a major global oil seed crop. Wild *Helianthus* species possess valuable genes to increase the genetic diversity of cultivated sunflower by continually providing genes resistant to diseases and pests. In our ultimate war against broomrape, the high resistance in perennial wild *Helianthus* species has been established, with occasional discovery of resistance in cultivated germplasm and in wild annual species. The main question today is how to transfer the resistance genes into cultivated lines for easy access and utilization by sunflower breeders.

A special project "Interspecific gene transfer for resistance to sunflower Sclerotinia utilizing wild *Helianthus* species" funded by the National Sclerotinia Initiatives has demonstrated good progress utilizing the various perennial *Helianthus* species and interspecific Amps since 2005. Since the nature of Sclerotinia resistance involves quantitative genes with mostly additive effects, and screening for resistance in the F_1 and BC_1F_1 generations is impossible, early backcross progeny families were produced and the screening conducted in the field trials with artificial inoculation. For a proactive breeding program for broomrape resistance, similar progeny families could be produced and screened against the new broomrape races, after resistance confirmation of the contributing wild species. In addition, early generation families produced from the Sclerotinia project could also be used for the broomrape resistance, as well as for resistance genes for numerous sunflower diseases, and other agronomic characteristics.

However, interspecific hybridization between perennial *Helianthus* species and cultivated sunflower has always been difficult or nearly impossible. This problem was solved with the development of embryo rescue techniques in the 1980s. Embryo rescue also can be used for the backcrosses whenever needed. For the crosses involving the tetraploid and hexaploid *Helianthus* species, and the interspecific Amps, the 2n=51 generation has been the most problematic for backcross seed set with additional backcrosses required to reduce the chromosome number to 2n=34, the same as cultivated sunflower. Valuable genetic variability from the wild species parents will be further reduced after each additional backcross. Efforts to shorten this process by using the 2n=51 plants as the pollen parents was shown to be effective, which resulted in all 2n=34 progeny plants in BC_1F_1 for diploids *H. salicifolius* and *H. occidentalis* and tetraploid *H. hirsutus*. Similar rapid reduction of extra chromosomes is expected for other wild tetraploids and hexaploids by utilizing 2n=51 generation plants as the pollinator in further backcrosses. The same rule should apply when using tetraploid interspecific amphiploids and the hexaploid interspecific Amps. This is a time savings of two backcross generations when using the polyploid wild species parents and the interspecific amphiploids. This rapid reduction of chromosome number when using 2n=51 plants as the pollen parents in backcrossing is expected to have a much stronger selection pressure against male gametes with extra chromosomes other than the normal n=17, since this selection pressure is generally known to be less for the female gametes.

The recent spread of broomrape in China is seen as a good example of broomrape race evolution from continuous planting of sunflower without rotation, the importation of contaminated hybrid seeds, and the gradual introduction of resistant hybrids. Highly virulent broomrape biotypes have started to appear and rapidly spread over areas of the main sunflower production regions. The migration of highly virulent broomrape biotypes to areas with less virulent biotypes is of great concern and needs to be closely monitored, and breeding strategies need to be considered. The appearance of the virulent broomrape races in China could be the result of selection or from the imported seeds from broomrape-infected areas. The breeding strategy is critical for areas like China where a mixture of all the races exists. A single gene resistance to all races is likely to lead to even more virulent races, and the competition between the broomrape race shift and resistance hybrids will never end. We were lucky that the Or_5 gene was able to keep broomrape under control for over 20 years, but looking at the rapid race shifts in recent years, this may not be the case in the future.

In conclusion, optimistically we consider broomrape is just one of the major sunflower pests or diseases that can be controlled by major genes. The resistance genes exist in abundance in wild species, especially the perennials. Utilization of valuable perennial *Helianthus* species for resistance genes has been greatly improved with the development of embryo rescue and the subsequent improvement in backcross seed set. The breeding cycle for producing selectable progeny families has been shortened by reciprocal backcrossing using 2n=51 plants as the pollen parents when starting from polyploid *Helianthus* species. Interspecific Amps have been produced for immediate selection and use in the following generations while monitoring broomrape resistance. Progeny families from the Sclerotinia project could also be used for immediate selection for resistance after the resistance confirmation of their wild species parents. With our new approach, in most cases when starting with resistant wild *Helianthus* species, the time required to identify resistant backcross progeny with reasonable self-compatibility will be at BC_1F_1 for the diploid wild and the tetraploid wild and interspecific Amps, and BC_2F_1 for the wild hexaploids and the interspecific Amps. Breeding for broomrape resistance is a continual challenge. Since we have a large pool of resistance genes and good tools to engage the battle against broomrape, the only other tool needed will be a good breeding strategy and international cooperation.

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Broomrape occurrence in natural populations of annual *Helianthus sp.*

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ABSTRACT

Wild annual *Helianthus* species native to North America have demonstrated to be a valuable genetic resource for sunflower crop improvement. The search of genes for broomrape (*Orobanche cumana* Wallr.) resistance demonstrated a natural resistance to this parasitic weed in a wild sunflower species, *H. petiolaris*. This species along with the wild *H. annuus* form natural populations in extended areas of Argentina. In Europe, mostly the wild *H. annuus* is found across the main sunflower crop production areas. This species seems to be susceptible to the specialized parasitic weed, native from Europe. The environment and plant phenotypes of five wild *H. annuus* populations in their natural habitats in Argentina were compared to agrestal populations of *H. annuus* attacked by broomrape, located in Fernan Nuñez, Spain, and Kovilovo, Serbia. The study comprised four susceptible *H. annuus* and five resistant *H. petiolaris* populations. The goal was to realize if wild populations in Argentina could be in risk to be invaded by the parasitic weed. The plant morphology was not affected by the broomrape attack in Spain. Cluster analysis based on morphology showed differences among the two annual species but grouped *H. annuus* natural populations from Argentina and Europe. Clustering of ecological variables did not separate species and geographical localization. No differences in the environment for broomrape attacked and susceptible populations were found. Natural *H. petiolaris* populations could be considered a genetic resource for resistance against broomrape but natural *H. annuus* populations should be considered as a potential reservoir of the parasitic weed.

Key words: Argentina – ecology – Europe – morphology – *Orobanche* – wild sunflower

INTRODUCTION

The sunflower/broomrape (*Helianthus annuus/Orobanche cumana*) complex is one of the most intriguing biological relationships in the modern crop production systems. Broomrape is native to the Caucasus region, where it develops on natural vegetation. After the discovery of the New World, the introduction of sunflower crop and gradually migration to Eastern Europe brought together these two taxa in the present Russian territory (Acimovic, 1998). In Krasnodar, Russia, Dr. Pustovoit devoted much of his breeding activity to create sunflower varieties with genetic resistance to broomrape (Pustovoit, 1967).

In the century following to Pustovoit's work, there is still continued effort to search and generate new sources of genetic resistance to broomrape. In Southern and Eastern Europe broomrape causes considerable yield losses and reduces sunflower seed quality. Genetic resistance had proved the most efficient method for avoiding severe broomrape attacks in the field, but natural selection promotes the emergence of new and more aggressive races of the parasite (Kaya et al., 2012).

Annual and perennial wild *Helianthus* species have been used as a source of resistance genes against broomrape (Terzic et al., 2010; Petcu and Pacureanu, 2011). The wild *H. annuus* is a successful plant invader all over the world (Cantamutto and Poverene, 2010). Extended populations of these taxa, growing in ruderal and agrestal habits, are present in South America and Europe (Stankovic-Kalezic et al., 2007; Muller et al., 2009; Cantamutto et al., 2010). Natural populations of the wild *H. annuus* met the parasitic weed only in Europe, because broomrape is absent in the sunflower cultivation region from America (Cantamutto et al., 2012).

The *H. annuus* populations of Argentina are susceptible to broomrape attack (Miladinovic et al., 2013), but the wild species *H. petiolaris*, also present in Argentina (Poverene et al., 2008), is resistant to the parasitic weed. The resistance profile of the *H. annuus* populations of Europe has not been described. Poverene and Cantamutto (2010) observed the presence of broomrape in an agrestal *H. annuus* population growing within a sunflower crop in Spain. Broomrape infection in wild *H. annuus* has also been detected in a ruderal population of Serbia (Dr. Dragana Miladinovic, personal communication).

Broomrape infection in *H. annuus* natural populations has not been reported in the modern literature. The objectives of this work were to communicate the observations on *H. annuus* natural populations attacked by broomrape and to answer the following questions: 1- Are Argentinian populations susceptible to broomrape morphologically different from the infected populations of Europe? 2- Do these European populations develop in a different environment than Argentinian populations? 3- Could *H. petiolaris* resistance be related to the environmental conditions?

The absence of morphological and environmental differences between infected and not infected populations entails a high risk for wild populations to be reservoir of broomrape populations for sunflower attack. The goal of this study was to compare morphological traits and environmental factors of wild populations infected and not infected by the parasitic weed in Europe and America.

MATERIALS AND METHODS

Materials comprised four wild *Helianthus annuus* and five *H. petiolaris* populations sampled in Argentina, one *H. annuus* population from Spain, and one from Serbia. These latter two accessions were broomrape infected (Poverene and Cantamutto, 2010, Miladinovic personal communication), with more than 30% incidence. Population data included botanical name, collection site (district, province, latitude, longitude, and altitude), estimated population area and size, and morphological variation registered according to Poverene et al. (2008). Morphological data consisted of plant height, head number, head angle, leaf shape, leaf margin, petiole/leaf rate, phyllary (bract) length, disc color, branching, and the presence of a main head. These traits were registered in the natural site. Broomrape presence was registered for each plant if tassels were visible in the stem base.

The average annual rainfall, and mean temperature of the hottest and coolest month were obtained from the nearest locality (web data). The population site was classified as roadside, riparian or crop when plants were found growing adjacent to cultivated lands (sunflower, maize, or wheat), within crop if plants were growing in the same row and/or between sunflower plants, and sunflower volunteers were recorded if present.

Morphological and ecological data were subjected to ANOVA and cluster analysis with the Infostat program (Di Rienzo et al., 2013).

RESULTS

Table 1 presents information about the surveyed populations. Site data corresponded to temperate lowlands. Population data were variable but similar in both hemispheres. The *H. annuus* (ANN) populations from Spain and from Serbia were broomrape infected at the moment of data collection. The *H. annuus* and *H. petiolaris* (PET) population from Argentina were free from the parasite.

Table 1. Spontaneous *Helianthus* populations studied in Argentina and Europe.

Population	Species	Locality	Province*	Altitude	Area m^2	Plants (n)
AAL	ANN	Puan	B. Aires	240	6600	6000
DIA	ANN	Diamante	Entre Rios	14	35000	12000
LMA	ANN	Malvinas	Mendoza	609	1680	5000
RCU	ANN	Rio Cuarto	Córdoba	366	30000	20000
FNU	ANN	Fernan Nuñez	Andalusia	125	1200	200
FK	ANN	Kovilovo	Vojvodina	77	50000	60000
CAT	PET	Q. Quemú	La Pampa	117	12000	10500
HLA	PET	H. Lagos	B. Aires	124	560	200
NGA	PET	N. Galia	San Luis	306	2750	2000
PEL	PET	La Zanja	B. Aires	101	1320	4300
SRO	PET	S. Rosa	La Pampa	183	1350	4050

*Except for Andalusia (Spain) and Vojvodina (Serbia) the remaining are provinces of Argentina

All morphological traits but three were polymorphic and were included in the analyses. All surveyed plants had red discs, total branching from the bottom, and none of them had a main head. Cluster analysis based on morphological traits comparing *H. annuus* and *H. petiolaris* populations is shown in Fig. 1 (Gower distance, cophenetic correlation 0.967). There were two main clusters (A and B), one for each

species. Plant position, branching type, anthocyanin pigment, head diameter and red disk of Spanish and Serbian populations corresponded to the wild or weedy *H. annuus* taxonomic descriptors. In Spain, plant height, stem diameter, leaf large, petiole length, phylary size, and head diameter of parasitized plants (n=4) did not differ from the healthy plants (n=6).

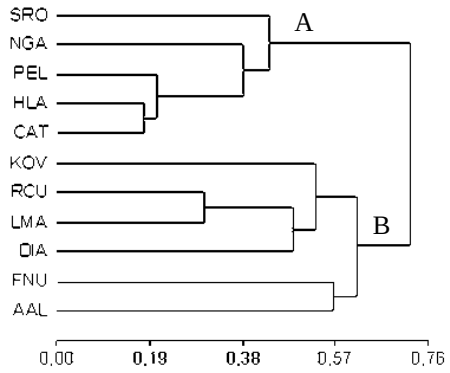

Fig 1. Cluster analysis of *Helianthus annuus* and *H. petiolaris* populations based on morphological traits.

When ecological variables were taken in consideration both species and origins failed to separate broomrape resistant from susceptible populations (Fig. 2, Gower distance, cophenetic correlation 0.882).

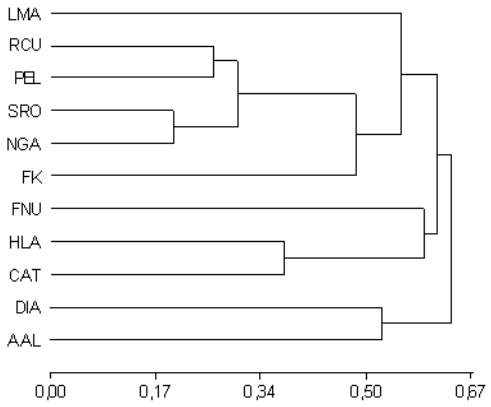

Fig. 2. Cluster analysis of *H. annuus* and *H. petiolaris* populations based on ecological variables.

DISCUSSION

Wild *Helianthus annuus* and *H. petiolaris* are important germplasm sources for sunflower breeding. These species have been established in central Argentina as feral populations since at least 60 years ago, when they were unintentionally introduced from the centre of origin (Cantamutto et al., 2010). Nine populations were chosen so as to cover the whole territory. None of them were invaded by broomrape, but artificial inoculations in Serbia proved that *H. annuus* accessions were susceptible whereas *H. petiolaris* accessions were resistant to broomrape (Miladinovic et al., 2013). Both *H. annuus* populations from Spain and Serbia were infected by broomrape at the moment of sampling. A former morphological characterization showed that Argentinian *H. petiolaris* populations were similar to those of the central part of the US, Texas, New Mexico and Nebraska (Salomón et al., 2008). Three among five *H. petiolaris* accessions with resistance to the parasite found by Terzic et al. (2010) come from the same US states (http://www.ars-grin.gov).

Cluster analysis based on morphological traits clearly differentiated between both species, showing differences among populations. Diversity level was higher among *H. annuus* populations than among *H. petiolaris* populations. There was no clear cut separation between susceptible *H. annuus* populations and those attacked form broomrape. The Argentinian population AAL, free from the parasite, was more similar to Spanish population than to the remaining populations from Argentina. Clustering did not reveal correspondence with geographical distribution within the species.

Cluster analysis based on ecological variables failed to discriminate *Helianthus* species from Argentina. RCU, PEL SRO, and NGA populations clustered close to Serbian *H. annuus* population (FK). Spanish FNU population was close to *H. petiolaris* populations HLA and CAT. As the European FK and FNU populations were invaded by the parasite, it could be concluded that there were no differences in the environment for broomrape resistant and susceptible materials. DIA and AAL *H. annuus* populations formed another cluster and LMA was the most dissimilar accession, probably because rainfall in Malvinas location is very low and there is a need for irrigation.

The wild *H. annuus* populations from Europe infected by broomrape and the susceptible populations from South America where the weed is absent showed a high morphological similarity and developed in similar environmental conditions. The ruderal and agrestal *H. annuus* populations should be considered a potential reservoir of the parasitic weed, and thus very vulnerable to broomrape invasion, while natural populations of *H. petiolaris* could be of a high value as genetic resource for resistance against this parasitic weed.

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Pathogenic comparison of highly virulent *O. cumana* affecting sunflower in Moldova, the South of Russian Federation, Serbia and Spain

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ABSTRACT

The most effective method for controlling the parasitic weed *Orobanche cumana* (sunflower broomrape) is the incorporation of genetic resistance into cultivated sunflower. According to different genes of resistance that are overcome, several races of the parasite are pathogenically identified. Race G is the most virulent one because it infects the universal resistant inbred line P96. Besides, populations of *O. cumana* are molecularly clustered according to geographical origin, and pathogenic traits are secondary criteria for genetic differentiation. In this work highly virulent parasite accessions collected in Moldova, the South of Russian Federation, Serbia and Spain were pathogenically compared. Six accessions were inoculated onto differentials of highly virulent races of the parasite: NR5, L86 and P96. Plants were grown under shadehouse and glasshouse for ten weeks following a factorial on a completely randomised design. At the end of the experiment none of the accessions infected P96. According to the reactions of the inbred lines NR5 and L86, accessions from Moldova and Serbia were identified as race E, and accessions from Spain were identified as race F. Accessions from the Russian Federation showed intermediate pathogenic behaviour onto NR5 and L86. Since a highly virulent biotype of *O. cumana* is present in Rostov oblast and seed used in this work comes from individual broomrape plants, the presence of race F in this area might be confirmed through the study of genetically heterogeneous parasite accessions from the area. Our results show that, in order to efficiently manage the genetic resistance into cultivated sunflower, there is a need for a worldwide study of highly virulent populations of *O. cumana*.

Key words: Broomrape – pathogenic characterization – Europe – *Helianthus annuus* L. – pathogenic race – virulence.

INTRODUCTION

The parasitic weed *Orobanche cumana* Wallr. (broomrape) is regarded as one of the most important constraints for sunflower production in most countries of the Middle East and eastern and southern Europe but not in the Americas, were it has not yet been described. At present genetic resistance of sunflower is the most effective, feasible and reliable control method against the parasite. *Orobanche cumana* is the only species of *Orobanche* that exhibits a clear race structure with respect to sunflower genotypes. The occurrence of six races, A to F, of increasing virulence on sunflower has been described (Vrânceanu et al., 1980; Saavedra Del Río et al., 1994).

The race F of *O. cumana* is widely spread in sunflower growing countries in Europe including Spain (Molinero-Ruiz et al., 2009). Effective sources of resistance against race F have been identified and released (Fernández-Martínez et al., 2004; Pérez-Vich et al., 2006; Velasco et al., 2007). In the last years, the occurrence of broomrape into commercial sunflower carrying genetic resistance to race F has raised concern about the existence of a new race, termed race G, in some growing areas. Race G has been confirmed in Romania (Dicu et al., 2011) as well as in the Russian Federation (Antonova et al., 2013) and Turkey (Molinero-Ruiz et al., 2014).

According to molecular data, clustering of populations of *O. cumana* depends first on geographical location (Pineda-Martos et al., 2013; Molinero-Ruiz et al., 2014). Pathogenic traits, i.e. race, are secondary criteria for genetic differentiation (Molinero-Ruiz et al., 2014). Moreover, molecular analyses did not identify race F populations from Hungary, Spain and Turkey as a genetically differentiated group (Molinero-Ruiz et al., 2014). Because strategies of control based on the introduction of effective genetic resistance into sunflower are globally developed, having an accurate knowledge of the racial structure of parasite accessions infecting sunflower worldwide arises as a critical issue for a sustainable control of the parasite. Therefore the objective of this work was the pathogenic characterization of parasite accessions identified as highly virulent and collected in Moldova, the South of Russian Federation, Serbia and Spain.

MATERIALS AND METHODS

The amount of seed of five accessions of *O. cumana* collected in Moldova, the South of Russian Federation and Serbia (Table 1) was increased in 2013 by infection of the confectionary susceptible sunflower cultivar B117. In March, a minimum of ten individual sunflower seedlings were transplanted into pots with 50 g of soil uniformly infested with each of the *O. cumana* accessions. Inoculum densities ranged from 0.1 (accessions 2501R12, 2520R12 and 2528R12) to 0.4 (accession 15S11) mg of parasite seeds/g soil. Plants were grown in shadehouse for three weeks, when they were transplanted into 5 L pots and kept under the same growth conditions until July 2013. This initial increase would also allow obtaining homogeneously vigorous seed for the following pathogenic characterization which was conducted in the autumn of 2013. *Orobanche cumana* plants parasitizing B117 cultivar were covered with two bags of microholed transparent plastic from before flowering and until full maturity of sunflower. Stems of the parasite at full maturity were uprooted and air-dried for 1 week prior to collection and cleaning of seed. *Orobanche cumana* from Spain (two accessions) were collected in May 2013; parasite seeds were recovered and kept until used, as previously described (Molinero-Ruiz et al., 2009).

Table 1. Geographical origin and year of collection of seven accessions of *Orobanche cumana* used in this work

Accession of *O. cumana*	Geographical origin (country, region, location)	Year
8M11	Moldova, Chisinau	2011
15S11	Serbia, Negotin	2011
2501R12	Russia, Krasnodar, Botanica	2012
2520R12	Russia, Rostov, Kirovskaya	2012
2528R12	Russia, Rostov, Glinki	2012
LPA13	Spain, Seville, La Palmera	2013
PER13	Spain, Seville, Pernía	2013

The pathogenic characterization of four viable accessions of *O. cumana* collected outside Spain together with two parasite accessions from Seville (Spain) (Table 1) was performed in the autumn of 2013. The experiment was conducted under shadehouse from Sept. 17 to Oct. 30 and in greenhouse at 15-25 ºC and natural photoperiod from Oct. 30 until Nov. 25.

Accessions were inoculated onto the differentials NR5 (Or_5), L86 and P96 (Or_5, or_6, or_7) (Fernández-Martínez et al., 2010; Molinero-Ruiz et al., 2014). Reactions of these sunflower inbred lines to different races of *O. cumana* are presented in Table 2. Eight sunflower seedlings (replications) of each differential were germinated and thereafter inoculated with each of the accessions of *O. cumana* according to previous methodology (Molinero-Ruiz et al., 2009, 2014). The inoculum density was 0.06 mg of parasite seeds/g soil. After inoculation by transplant into infested soil, plants were grown first under shadehouse and later in glasshouse, until physiological maturity. Plants were watered as needed and, after finishing the experiment, all the material was carefully autoclaved and discarded. The experiment was set up as a factorial on a completely randomised design.

Table 2. Reaction of differential sunflower inbred lines to races of *Orobanche cumana*[1]

Differential line	Race of *O. cumana*			
		F		
	E	Central Spain	South Spain	G
NR5	R[2]	S	S	S
L86[3]	S	S	R	S
P96	R	R	R	S

[1] Adapted from Molinero-Ruiz et al., 2014.

[2] R, resistant; S, susceptible.

[3] The inbred line L86 is susceptible to race F populations from Central Spain and resistant to populations of race F from the South of the country (Molinero-Ruiz et al., 2008).

The degree of attack in sunflower genotypes (DA, i.e. the number of emerged *O. cumana* stems per sunflower plant) was assessed weekly, from the emergence of the first parasite stem until the end of the experiment. Final DA was transformed [log (FDA+1)] prior to analysis of variance. When significant effects were obtained, Fisher's protected LSD tests (P = 0.05) were used for comparisons of inbred lines, accessions and their interaction.

RESULTS

Only five out of the six accessions of *O. cumana* from abroad Spain infected the universal susceptible B117. Accession 2501R12 did not attack B117, and therefore it could not be subsequently characterized for pathogenic traits or race.

In the pathogenic characterizations of parasite accessions, first emergence of *O. cumana* stems occurred seven weeks after inoculation (data not shown). As expected, the highest infections occurred in the universal susceptible confectionary cultivar B117. Average values of FDA ranged between 8.3 and 0.0 stems per sunflower plant in B117 and P96 respectively. Mean values of FDA for the accessions of *O. cumana* in each of the differentials are shown in Table 3.

Table 3. Final degree of attack (stems per sunflower plant) by *Orobanche cumana* in sunflower inbred lines differentials of races of the parasite. The experiment was conducted under shadehouse and glasshouse conditions in the autumn of 2013 in Córdoba (Spain)

	Differential sunflower line			
Accession of *O. cumana*	B117	NR5	L86	P96
8M11	11.1 ± 1.1	0.0 ± 0.0	5.6 ± 1.2	0.0 ± 0.0
15S11	13.2 ± 2.6	0.0 ± 0.0	4.7 ± 1.6	0.0 ± 0.0
2520R12	6.5 ± 0.8	1.0 ± 0.5	2.0 ± 0.6	0.0 ± 0.0
2528R12	7.0 ± 1.8	1.0 ± 0.6	2.0 ± 0.8	0.0 ± 0.0
LPA13	4.6 ± 1.1	3.0 ± 1.4	0.0 ± 0.0	0.0 ± 0.0
PER13	7.5 ± 1.2	4.7 ± 1.3	0.1 ± 0.1	0.0 ± 0.0
Average	8.3 ± 0.7	1.6 ± 0.4	2.4 ± 0.5	0.0 ± 0.0

The attack of accessions of *O. cumana* in sunflower at the end of the experiment was highly dependent on the parasite accession (p=0.0304), the host genotype (p<0.0001) and the interaction between both factors (p<0.0001). Mean values of FDA in B117 ranged from 13.2 stems per sunflower plant for 15S11 to 4.6 for LPA13. The inbred line P96 was fully resistant to all the accessions of *O. cumana*. Inbred lines NR5 and L86 showed intermediate reactions. The differential NR5 showed mean FDA that ranged between 0.0 (accessions 8M11 and 15S11) and 4.7 stems per sunflower plant (accession PER13). Significantly high values of FDA in L86 were obtained as compared to those in NR5. Mean FDA in L86 varied between 0.0 stems of LPA13 per sunflower plant and 5.6 stems of 8M11 per sunflower plant.

DISCUSSION

In spite of the favourable outdoor conditions for *O. cumana* infection between March and June 2013, accession 2501R12 was unable of infecting B117. The lack of infection of susceptible sunflower by 2501R12 might be related to a loss of seed viability or to the use of a too small amount of inoculum for soil infestation. However, seed of 2501R12 was collected in 2012 and *O. cumana* seeds can hold their viability for up to 17 years (Molinero-Ruiz et al., 2008). Besides, in autumn 2013, under suboptimal conditions for infection, lower inoculum densities than the one of 2501R12 used in spring resulted in good infection of sunflower by *O. cumana*. Whether other experimental or biological circumstances prevented 2501R12 from infecting B117, is unknown.

Conditions of temperature and photoperiod in southern Spain from Sept. to Nov. 2013, both in shadehouse and in glasshouse with no supplementary illumination, were suboptimal for infection of sunflower by *O. cumana*, but still they allowed a statistically valid autumn pathogenic characterization of accessions. Concerning this characterization, three different patterns of resistant/susceptible reactions were observed in the differentials. First, the one of 8M11 and 15S11 accessions: they did not overcome the resistance into NR5 neither the one into P96 but caused significant infections on L86. Both accessions were identified as race E. The results of this work show therefore that, additionally to Hungary, Romania

and Central Spain (Molinero-Ruiz et al., 2014) race E is also present in Moldova and Serbia. Secondly, LPA13 and PER13 accessions behaved as typical race F from Southern Spain: they were effectively controlled by L86 and P96 but not by NR5. Finally, 2520R12 and 2528R12 accessions showed intermediate pathogenic traits: NR5 and L86 were statistically resistant to them, but they consistently showed very few broomrape stems per sunflower plant. A highly virulent biotype of *O. cumana* is present in Rostov oblast (Antonova et al., 2013), the region in the South of Russian Federation where 2520R12 and 2528R12 accessions were collected; therefore, a significant infection of line P96 by 2520R12 and 2528R12 was expected. But seed of accessions 2520R12 and 2528R12 used in this work comes from individual broomrape plants (Antonova, pers. comm.) and therefore the resistance of P96 might be related to a reduced pool of genotypes into 2520R12 and 2528R12, because progenies of individual *O. cumana* plants are not representative of the pathogenic traits of whole populations.

In spite of the high virulence initially suspected for the accessions from Moldova, the South of Russian Federation and Serbia included in this work, none of them was able of overcoming the genetic resistance into P96 which remains, so far, as the universally resistant differential. On the other hand, resistant/susceptible reactions displayed by differential sunflower lines when inoculated with accessions of *O. cumana* from the South of Russian Federation differed from previously observed patterns. This work reveals the need of a worldwide study of pathogenic traits of *O. cumana* populations in order to efficiently manage the genetic resistance into cultivated sunflower.

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Toward a better understanding of the genetic architecture of sunflower (*Helianthus annuus*) resistance to the parasitic plant *Orobanche cumana*

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ABSTRACT

The plant parasite *Orobanche cumana* is a major threat for the sunflower crop. The emergence of new, virulent "races" during the ten past years reinforced the need to develop new approaches, knowledge and tools in order to control this pest efficiently. Breeding is the most sustainable approach to control broomrape in the field. A RIL population derived from a cross between HA89 and LR1, an inbred line bred from an interspecific cross with *Helianthus debilis* had been previously characterized for resistance to *O. cumana* race E, but no data is available for new races. The aim of this study was to characterize the HA89xLR1 RIL population for resistance to race F and to identify QTLs associated with this resistance. The population was phenotyped by counting the number of healthy broomrape tubercles and the rate of tubercle necrosis on young sunflower plants raised in a growth chamber with four biological replications. Differences in l resistance were observed among the RIL population, with some resistant genotypes and some highly susceptible genotypes. The polymorphism of 111 SNP markers previously mapped on a consensus genetic map was used for the QTL detection. Four QTLs were detected on four linkage groups (LG01, LG07, LG15 and LG17), with two QTLs controlling the number of tubercles per plant and two others controlling necrosis. This study suggests that the resistance to *O. cumana* race F is controlled by several QTLs affecting differently the number of tubercle and the induction of tubercle necrosis.

Key words: *Orobanche cumana* – race F – resistance – QTL mapping – *Helianthus annuus*

INTRODUCTION

O.cumana is a root parasite of sunflower and is an important threat around the Mediterranean Sea, especially in the Black Sea region, and south-west Europe (Molinero-Ruiz et al., 2013). Several methods of control are available: soil solarization (Mauromicale et al., 2005), biological control (Thomas et al., 1998 ; Louarn et al., 2012), Imidazolinone-tolerant crops (Tan et al., 2005) and breeding, which remains an efficient and sustainable method, particularly in using genes introgressed from interspecific crosses (Akhtouch et al., 2002 ; Velasco et al., 2007). Single dominant genes (Or1 to Or5) control resistance to *O. cumana* races A to E (Vrânceanu et al., 1980). New highly virulent isolates, threatening genotypes previously known as resistant have been reported. (Fernández-Martínez et al., 2000). Resistance to these new races appears to be more complex, with, in some cases, more than one gene controlling resistance. Akhtouch et al. (2002) showed that resistance to race F is recessive and controlled by two different interacting loci. These results provide evidence that there are traits other that those involving monogenic dominant inheritance which may be involved in resistance to new races of *O. cumana*. Quantitative Trait Loci (QTL) for the resistance to *O. cumana* race F were mapped in the population P96xP21 (Pérez-Vich et al., 2004). Six QTLs were detected, on five linkage groups (LG), with small effects associated with the number of broomrape shoots per sunflower plant. However, the molecular mechanisms involved in these QTL are not known and no gene has been cloned up to now. The population HA89xLR1 has been characterized for its interaction with the race E (Labrousse et al., 2004) and different phenotypes for the

expression of resistance have been observed (level of infection, necrosis and differential germination of *O.cumana* seeds).

The aim of this study was to phenotype the population HA89xLR1 using an ecotype of the *O. cumana* race F (from Spain) by counting the number of healthy and necrotic tubercles and to associate these phenotype to genetic markers.

MATERIALS AND METHODS

Sunflower and *O.cumana* material

107 RIL in F8 generation derived from the cross between HA89 and LR1, an inbred line derived from an interspecific cross involving the wild species *H. debilis* (ecotype 215 from the INRA collection) were analyzed for their resistance to *O. cumana* race F. The resistance of the same RIL population had previously been analyzed using the *O. cumana* race E (Labrousse et al., 2004). *O. cumana* seeds, race F, were collected in Spain in 2012 and kindly provided by BIOGEMMA (France).

Phenotyping experiment

In order to prevent any phytosanitary risk regarding the manipulation of this parasite for which no regulatory procedure has been implemented up to now, the experiments were conducted within a laboratory space where the manipulation of another sunflower parasite (*Plasmopara halstedii*) has been approved. Broomrape resistance test was performed according to Louarn et al. (2012). The substrate (charred clay, Oil Dri, Klasmann, France) was inoculated with 5mg per pot (9cm x 9cm x 8cm) of *O. cumana* seeds, After 7 days preconditioning, two pre-germinated sunflower seeds were sown in the pot and kept in a growth chamber under a 16 h photoperiod (20°C day, 20°C night). One of these two plants was thereafter discarded to obtain the best homogeneity for seedling vigour across the experiment. Plants were watered daily with a low-phosphate half strength Long Ashton Nutrient Solution (LANS, Hewitt, 1966) containing a final concentration of sodium dihydrogen phosphate of 7.5 mM. After 5 weeks, infection by *O. cumana* was determined by counting the number of healthy and necrotic tubercles.
Four successive biological replicates (one sunflower plant for each RIL in each replicate) were made.

Data analysis and QTL Mapping

Initial phenotypic data were transformed to stabilize error variance (X' = square-root(X)), assuming that the number of events (healthy or necrotic tubercles) s followed a Poisson curve. The RIL were genotyped for 111 polymorphic SNP markers previously mapped on a consensus genetic map (https://www.heliagen.org/Web/public/consensus_INEDI_FUxPAZ2/mapping_INEDI_FUxPAZ2_2012-07.html) according to Vincourt et al. (2012).The resulting genotyping data were used to detect QTL with the MCQTL software (Jourjon et al., 2005). Due to the poor density of this map (around 6markers per linkage group), we used 10% as genome wide threshold for the first type error rate. QTL detection was performed on each replicate and on the average of the four replicates.

RESULTS AND DISCUSSION

Resistance to *O. cumana* race F was tested on 107 different RILs HA89xLR1 (Fig. 1, 2). The two parents HA89 and LR1 were moderately resistant to *O.cumana* race F, as less than 4 healthy tubercle per sunflower plant were counted on these two parental lines. As shown in Fig. 1, transgressions were observed within the RIL population. Several highly resistant plants (less than 1 tubercle) and many susceptible plants (more than 10 tubercles) were observed. Labrousse et al. (2004) showed that LR1 was resistant to the race E and that HA89 was more susceptible, and that the RILs HA89xLR1 showed a large t number of resistant plants (34 RILs showing no tubercles). This indicates that the genetic background of the RIL population is more susceptible to *O. cumana* race F than to race E. Nevertheless, resistant plants were observed, indicating that this material contains a resistance mechanism against *O. cumana* race F.

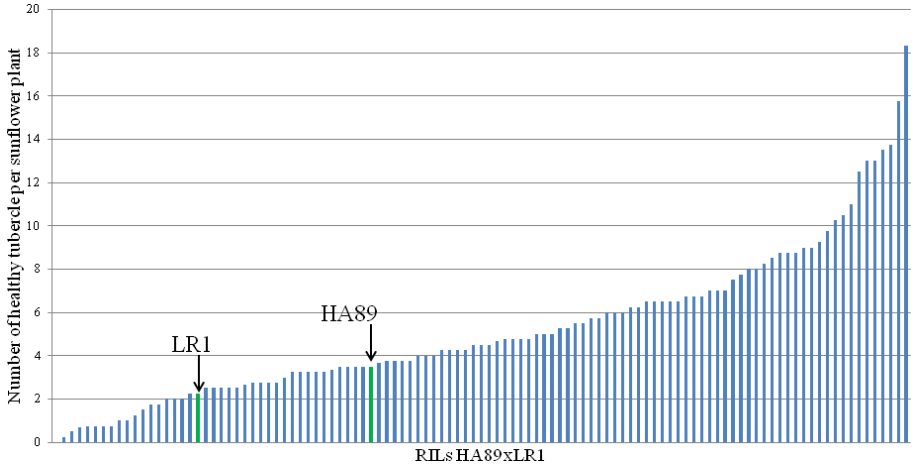

Fig. 1. Average over the four biological replications of the number of healthy tubercles per sunflower plant in RILs population HA89xLR1.

Different cytological observations have been previously described regarding the induction of necrosis in tubercles as a mechanism of resistance, especially with the interaction between the sunflower genotype LR1 and the *Orobanche* race E (Labrousse, 2001). The rate of tubercle necrosis was evaluated for the RIL population. Whereas HA89 and LR1 did not exhibit tubercle necrosis after 5 weeks, necrotic tubercles were observed in some RILs, with a rate varying from 0% to 75%, with an average of 8%. These results appear to indicate that the induction of tubercle necrosis for t race F is a complex character controlled by several loci, presumably interacting through epistasis, inherited from both LR1 and HA89. More investigations are necessary to understand the different resistance mechanisms controlling the induction of necrosis.

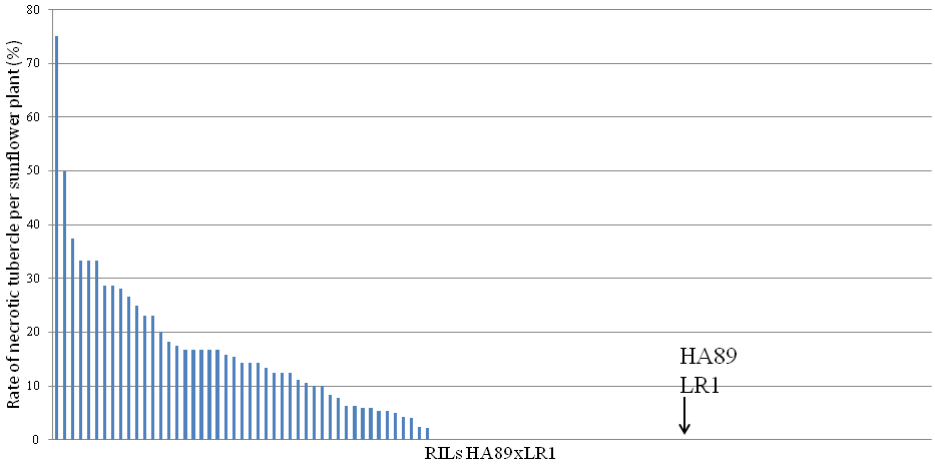

Fig. 2. Average on the four biological replicates of the rate of necrotic tubercle per sunflower plant in RILs population HA89xLR1.

111 genetic markers (SNP) mapped on the seventeen LG of sunflower were used to identify QTL associated with broomrape resistance (Fig. 3). With a relaxed Type I genome wide error rate (10%), four QTL were identified in this study. One QTL identified on LG01 is associated with the number of healthy tubercles. Another QTL, influencing the total number of tubercles per plant, was mapped on LG07. In addition, two QTLs involved in the control of necrosis were detected on LG15 and LG17. Pérez-Vich et al. (2004) identified a QTL on LG1 involved in resistance to race F, as evaluated by the number of broomrape stems in a field infected by this race. This QTL could be important for the control of *O.*

cumana, as it has an effect on race E and F for both the P96xP21 population and the HA89xLR1 RIL population, respectively.

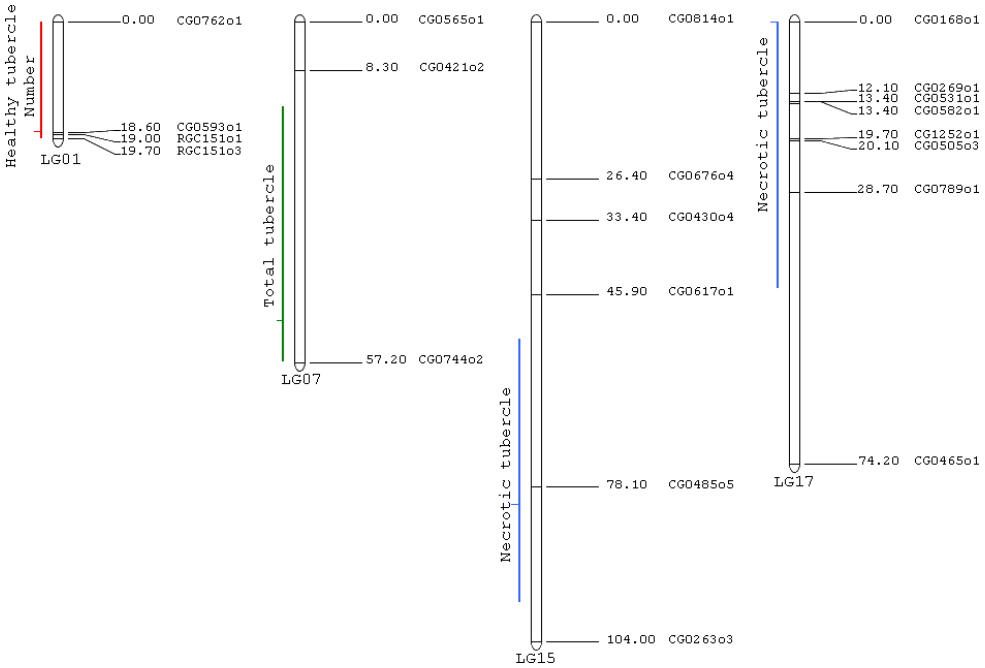

Fig. 3. QTL mapping in the HA89xLR1 RIL population. showing mapped QTL for healthy, total and necrotic tubercles.

PERSPECTIVES AND CONCLUSION

The preliminary QTL mapping described in this study needs to be improved, with better coverage (in progress) of genetic markers across the different LG. In this study, the phenotyping data were obtained in a growth chamber under a controlled environment. They will be compared in 2014 with data from field trials carried out in Spain, where the occurrence of race F has been established. Furthermore, we have developed a medium/high throughput phenotyping approach in order to characterize the level of induction of *O. cumana* seeds by sunflower root exudates (Fig. 4) and will apply this to the HA89xLR1 RIL population, l to try to decipher one of the components of the sunflower * orobanche interaction. This study will also give us the opportunity to apply a rhyzotron approach to describe more precisely the response of differential sunflower differentia inbred lines to several *O.cumana* races.

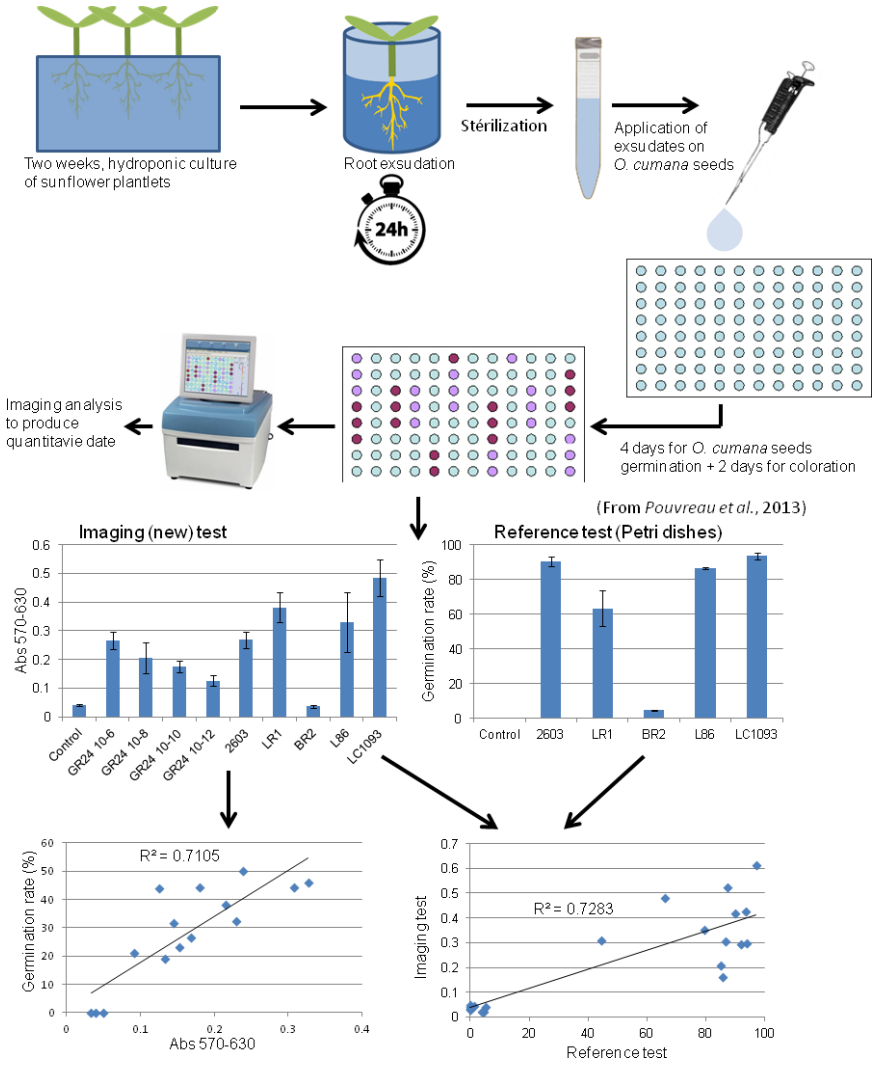

Fig. 4. Medium/high phenotyping method to quantify the level of induction of sunflower root exudates toward the germination of *O.cumana* seeds. The reference test was implemented according to Louarn et al. (2012).

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Determination of resistance to broomrape in newly developed sunflower inbred lines

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ABSTRACT

Breeding for broomrape resistance requires continuous work, since broomrape responds as soon as resistance to the latest race is found by evolving a more virulent race. Sources of broomrape resistance were mostly found in certain wild species and incorporated into cultivated sunflower genotypes by interspecific hybridization. Current work is focused on screening newly developed inbred lines in order to find resistance to highly virulent races of broomrape present in Southern and Eastern Europe. In the breeding material of the Institute of Field and Vegetable Crops, Novi Sad, Serbia, twelve inbred lines were selected as potentially resistant or highly tolerant to virulent races of broomrape, overcoming race F. The preliminary results of the F_1 hybrids, from crosses between four newly developed CMS lines that are resistant to highly virulent races F and G, and three restorer lines that are resistant to race E, indicate recessive gene action in the resistance to races overcoming F. Their recessive nature requires the incorporation of genes of resistance to the two parental lines for the development of resistant hybrids.

Key words: Broomrape – sunflower – inbred line – F_1 hybrids – resistance – screening

INTRODUCTION

Broomrape (*Orobanche cumana* Wallr.) is considered one of the most severe constraints in sunflower (*Helianthus annuus* L.) production, as it infects the roots of sunflower plants causing severe yield losses. Broomrape seed is powdery small and spreads easily and the appearance of new races requires continuous searching for new sources of resistance in sunflower germplasm. Vrânceanu et al. (1980) identified five races of *O. cumana* in Romania, designated as A, B, C, D and E. Over the past 20 years, new virulent races overcoming race E have been identified in many regions of Romania, Bulgaria, Turkey, Russia, Spain and other countries. They have been named F, G and H (Fernández-Martínez et al., 2008; Păcureanu-Joita et al., 2009; Škorić et al., 2010). The current racial situation of broomrape in the main infested areas remains unclear, i.e. there is a lack of information on whether races under the same classification reported in different countries are the same or differ in terms of virulence (Fernández-Martínez et al., 2012).

Extensive research on broomrape resistance is focused on finding resistant genotypes by screening the existing material to new virulent races of pathogen. Cultivated sunflower has limited genetic variability and lacks genes for broomrape resistance, though wild species constitute a valuable source of resistance genes (Miladinovic et al., 2012). Vrânceanu et al. (1980) set five sunflower differential lines carrying the dominant resistance genesOr_1, Or_2, Or_3, Or_4 and Or_5, respectively. The gene Or_5, which provides resistance to all five races (A-E), was successfully introduced into inbred lines with high combining ability, which were used as parents of existing or prospective hybrids (Škorić and Jocic, 2005). The appearance of race F in Romania led to the identification of resistant line LC-1093, controlled by a single dominant gene Or_6 (Pacureanu-Joita et al., 1998) which was added to a set of differentials. Resistance to race F was found in germplasm of both cultivated and wild sunflower and resistant material has been developed by Jan et al. (2002) and Fernández-Martínez et al. (2004). The latest research is focused on finding sources of resistance to the newest population of *Orobanche* (races G and H) found in Romania, Turkey and Spain. Resistant genes have been present in certain inbred lines (Škorić et al., 2010; Velasco et al., 2012; Cvejic et al., 2012).

The objective of this research was to screen new sunflower inbred lines, from the gene pool of the Institute of Field and Vegetable Crops from Novi Sad, Serbia, for broomrape resistance to races overcoming race F. Since some lines were previously tested and found to be resistant, the aim is to determine the inheritance of their resistance to this new race of broomrape.

MATERIALS AND METHODS

A hundred new sunflower inbred lines, developed at the Institute of Field and Vegetable Crops in Novi Sad (NS lines), were used for screening: 20 lines from each of following gene-pool LIV, AB-VL, NS-KOD, RUB, HA. LIV originated from the population developed from interspecific hybridization with *Helianthus tuberosus*, AB-VL were selected from the population developed from interspecific hybridization with *Helianthus divaricatus*, NS-KOD and RUB originated from populations derived from old Russian varieties, while HA was selected from the Novi Sad gene-pool. Three of these lines (LIV-10, LIV-17 and AB-VL-8) were previously tested and determined as being resistant to new races in Spain and Romania (Cvejic et al. 2012). These three lines, plus one new HA-267, were crossed with three restorer lines, carryingOr_5. Restores were chosen in case to provide screening to races higher then race E. Twelve F_1 hybrids were tested in four locations and different levels of resistance to broomrape were evaluated. The set of differential lines were used as in to Imerovski et al. (2013).

The screening procedure for resistance to broomrape was carried out in field trials during the 2012-2013 periods. Inbred lines were planted in two locations in Serbia (Kula and Vrbas), two locations in Romania (Constanta and Tulcea), one in Spain (Seville) and one in Turkey (Edirne). Each inbred line was planted in two rows (30 plants) in two replications. In Serbia, infested locations are situated in the northern part of the country. In Spain, inbred lines were planted in a location near Seville, in a naturally infested field. Both locations in Romania are situated in areas where highly virulent races are present, in the Black Sea region, where race F is widely spread and race G is identified (Pacureanu-Joita et al., 2008). The trial in Turkey was in the Trakya region, close to Edirne, where the broomrape population is very virulent and changes frequently (Kaya et al. 2004).

Reactions of the tested sunflower lines to broomrape were evaluated by calculating incidences, i.e. ratio of infected sunflower plants per total number of plants. Inbred lines were considered resistant (R) when no broomrape stalk was found within the complete entry, moderately resistant (MR) when 1-50% of plants had at least one broomrape stalk and susceptible (S) when more than 50% of plants were infested.

RESULTS AND DISCUSSION

Newly developed inbred lines were planted in six locations in four countries (Serbia, Spain, Turkey and Romania) in 2012 and 2013. The performed experiment showed different reactions of selected sunflower inbred lines to broomrape, which depended on location i.e. race composition of the broomrape. Selected inbred lines which showed resistance to broomrape of races existing in the six examined regions, during this two-year period, are listed in Table 1. Completely new lines HA-267 and RUB-1-4 were introduced to the screening procedure in 2013.

All selected inbred lines were completely resistant to broomrape attack in two locations in Serbia, as expected. This indicates that examined inbred lines were resistant to race E of broomrape present in Northern Serbia, which was confirmed by differential lines carrying Or_5 and Or_6 genes (LC-1003, LC-1093). In Serbia race E has been dominant for over ten years, especially in northern parts of the country that represent the main sunflower-growing regions. Lately, broomrape has spread to new regions in Eastern Serbia (Dedic et al., 2009). In the infested location in Spain most of the selected inbred lines showed resistance, with the exception of lines from the NS-KOD gene pool, incidence range from 1 to 5%, but with only one broomrape stalk per infected sunflower plant. The differential line LC-1093, carrying Or_6, had 10% of plants infested, aiming at the presence of races which overcome race F (race G). Similar results were reported by Molinero-Ruiz et al. (2008) suggesting that a broomrape race higher than F is possibly present in the Seville area (SE295), since sunflower lines L86 and P96, resistant to race F, showed low values of broomrape incidence.

Selected inbred lines showed different level of resistance in two infested locations in Romania. In the Constanta region complete resistant to broomrape attack was identified in the lines HA-267, AB-VL-8 and AB-VL-7. The inbred line LIV-17 was moderately resistant, with 10% being attacked but with only one broomrape stalk per infected sunflower plant. In the second location, Tulcea, only line HA-267 showed complete resistance in 2013. The differential line LC-1093 showed a range from moderate resistance to susceptible. Results suggest that race G is present in locations in Romania and it is possible that a more aggressive race is present in the Tulcea region. Pacureanu-Joita et al. (2008) tested genotypes in different location in Romania and their results showed that the differential line for the race F (LC-1093) lost resistance in the Black Sea area. Our results indicate the presence of a new race in this area, but the severity of attacks is higher in the Tulcea region, as plants from the differential line LC 1093 were highly infested. Pricop et al. (2011) identified some more aggressive populations than race G were present in lower frequency in the Constanta area.

Table 1. Broomrape incidence (%) and reaction of sunflower inbred lines in six different locations in 2012 and 2013

Genotype	Serbia			Spain		Romania				Turkey	
	Kula-2013 (race E)	Vrbas-2012 (race E)	Vrbas-2013 (race E)	Seville-2012 (race F, G)	Seville-2013 (race F, G)	Constanta-2012 (race F, G)	Constanta-2013 (race F, G)	Tulcea-2012 (races F, G, maybe new)	Tulcea-2013 (races F, G, maybe new)	Edirne-2012 (races F, G, maybe new)	Edirne-2013 (races F, G, maybe new)
NS-KOD-2	0 (R)	0 (R)	0 (R)	0 (R)	2 (MR)	10 (MR)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)
NS-KOD-4	0 (R)	0 (R)	0 (R)	2 (MR)	1 (MR)	10 (MR)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)
NS-KOD-10	0 (R)	0 (R)	0 (R)	5 (MR)	5 (MR)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)
HA 267	0 (R)	0 (R)	0 (R)	-	0 (R)	-	0 (R)	-	0 (R)	-	0 (R)
RUB-1	0 (R)	0 (R)	0 (R)	-	0 (R)	-	100 (S)	-	100 (S)	-	100 (S)
RUB-2	0 (R)	0 (R)	0 (R)	-	0 (R)	-	100 (S)	-	100 (S)	-	100 (S)
RUB-3	0 (R)	0 (R)	0 (R)	-	0 (R)	-	30 (MR)	-	100 (S)	-	0 (R)
RUB-4	0 (R)	0 (R)	0 (R)	-	0 (R)	-	25 (MR)	-	100 (S)	-	0 (R)
LIV-10	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	20 (MR)	15 (MR)	10 (MR)	100 (S)	20 (MR)	0 (R)
LIV-17	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	10 (MR)	0 (R)	40 (MR)	100 (S)	0 (R)	0 (R)
AB-VL-8	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	5 (MR)	10 (MR)	0 (R)	0 (R)
AB-VL-7	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	25 (MR)	15 (MR)	0 (R)	0 (R)
Differentials											
LC-1093 (*Or*$_6$)	0 (R)	0 (R)	0 (R)	10 (MR)	10 (MR)	10 (MR)	20 (MR)	50 (MR)	100 (S)	100 (S)	100 (S)
LC-1003 (*Or*$_5$)	0 (R)	0 (R)	0 (R)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)
LC-1002 (*Or*$_4$)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)
LC-231 (*Or*$_3$)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)
LC-215 (*Or*$_2$)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)
AD-66	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)

No data

In the infested location in Turkey several lines (HA-267, RUB-3, RUB-4, LIV-17, AB-VL-8 and AB-VL-7) were fully resistant to broomrape attack, although the differential line, carrying *Or*$_6$, was susceptible. This result indicates different racial composition in Turkey than in Romania and Spain. Race F was more virulent in Turkey than in other countries, such as Spain, and at least one more race other than F was observed in the Trakya region (Kaya et al. 2004).

Newly developed sunflower inbred lines were selected as potentially resistant to highly virulent races of broomrape. Inbred lines HA-267, AB-VL-8, AB-VL-7, LIV-10 and LIV-17 lines are considered as being resistant to broomrape attack in most infested areas in Spain, Romania and Turkey. Only line HA-267 was fully resistant to races which overcome race F in all tested areas in 2013. This line is characterized as good general combiner and with good agronomic traits and could be used directly for developing commercial hybrids. A new source of resistance to the new races of the broomrape, overcoming F, was reported by Pacureanu-Joita et al. (2009) in genotypes LC 009 and AO-548.

The results of Cvejic et al. (2012) indicated that some inbred lines (AB-VL-8, LIV-10 and LIV-17) were resistant to new races in Spain and Romania and their hybrids were tested in four locations and evaluated as having a different level of resistance to broomrape (Table 2). F_1 plants from the cross with line AB-VL-8 showed moderate resistance in the Constanta region, while the line was resistant. In Tulcea all F_1 hybrids were susceptible compared to the moderately resistant line, indicating recessive inheritance. Similarly, the F_1 plants from crosses with line LIV-10 and LIV-17 were susceptible and moderately resistant in the Seville and Constanta regions. Line HA-267 and its F_1 hybrids were showed different level of resistance in four locations. Although, line HA-267 was resistant in Romania, its F_1 hybrids were completely susceptible. These results present a recessive gene action in resistance to races overcoming F (race G). Previous reports indicated that resistance to race G has been dominant (Pacureanu-Joita et al. 2008; Velasco et al. 2012). Velasco et al. (2012) found resistance to race G in a wild *Helianthus debilis* subsp. *tardiflorus* and the F_1 plants from the cross with cultivated sunflower were resistant, indicating the dominance of resistance gene(s). Moreover, the resistance of the line AO-548 to the latest race of broomrape in Romania is controlled by two independent dominant genes (Pacureanu-Joita et al., 2008). The different results obtained with resistance to broomrape race G indicate that lines are derived from different sources and suggests the importance of the wild sunflower species as a source of unique resistant genes. Line AB-VL-8 was selected from the population developed from interspecific hybridization with *Helianthus divaricatus* and another two new inbred lines (LIV-10 and LIV-17) were selected from the population developed from the source of *H. tuberosus* (Cvejic et al., 2012). Because of their recessive nature, resistance has to be incorporated into both parental lines in order to develop resistant hybrids.

Table 2. Broomrape incidence (%) and reaction of F_1 hybrids in four locations in 2013

Genotype	Vrbas	Seville	Constanta	Tulcea
AB-VL-8 x R1	0 (R)	0 (R)	30 (MR)	100 (S)
AB-VL-8 x R2	0 (R)	0 (R)	50 (MR)	100 (S)
AB-VL-8 x R3	0 (R)	0 (R)	0 (R)	100 (S)
AB-VL-8	0 (R)	0 (R)	0 (R)	10 (MR)
LIV-17 x R1	0 (R)	25 (MR)	100 (S)	100 (S)
LIV-17 x R2	0 (R)	0 (R)	100 (S)	100 (S)
LIV-17 x R3	0 (R)	0 (R)	100 (S)	100 (S)
LIV-17	0 (R)	0 (R)	0 (R)	100 (S)
LIV-10 x R1	0 (R)	30 (MR)	100 (S)	100 (S)
LIV-10 x R2	0 (R)	5 (MR)	100 (S)	100 (S)
LIV-10 x R3	0 (R)	0 (R)	100 (S)	100 (S)
LIV-10	0 (R)	0 (R)	15 (MR)	100 (S)
HA-267 x R1	0 (R)	10 (MR)	100 (S)	100 (S)
HA-267 x R2	0 (R)	10 (MR)	100 (S)	100 (S)
HA-267 x R3	0 (R)	0 (R)	100 (S)	100 (S)
HA-267	0 (R)	0 (R)	0 (R)	0 (R)

Broomrape is becoming a serious problem for sunflower production in Southern and Eastern Europe. Searching for new sources of resistance is extremely important. Resistance was found in the new NS lines HA-267, AB-VL-8, AB-VL-7, LIV-10 and LIV-17 against the new populations, which overcome race F

but their recessive nature requires the incorporation of genes of resistance to the two parental lines for the development of resistant hybrids.

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Evaluation of resistance to parasite broomrape (*Orobanche cumana* Wallroth) of new inbreed sunflower lines

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ABSTRACT

Broomrape (*Orobanche cumana* Wallr.) is well known parasitic plant which attack sunflower and cause economic loses to farmers in many parts of the world. Among the strategies to control this parasite is the breeding of cultivars with resistance to the parasite. Sources for such a resistance have been found in wild *Helianthus* species and transferred to sunflower cultivars through interspecies hybridization. In the present study an evaluation of the resistance to highly virulent race H of *O. cumana* was made of 5 inbreed lines with known resistance to less virulent broomrape race (F), one F_1 hybrid and 7 lines with American parent. One *R* line and the F_1 hybrid have demonstrated 100% resistance while the rest of the lines were partially or completely susceptible to the attack of the parasite. Thus the resistant lines could be used in the practice where the new highly virulent broomrape race is present or as a source for resistance in the breeding programs.

Key words: Broomrape – inbreed lines – resistance – sunflower

INTRODUCTION

Orobanche cumana Wallroth is highly specialized obligate, high color parasite that is completely lacking chlorophyll and the capacity for self photosynthesis. Broomrape contacts the host in soil (through haustorium) and grows along with plant feeding with ready-assimilates. The first appearance of the broomrape over the surface of the soil is about 50 days after germination of sunflower. Flowering parasite coincides with the completion of flowering of sunflower (Batchvarova, 1978). According to Batchvarova (2004) attack of the parasite in sunflower affects to reduce plant height by 6.4%, the inflorescence diameter with 27.8%, while yields at high attack are reduced to 7 times. Broomrape has high self-fertility, but can be pollinated and crossed. Broomrape seeds moved with sunflower seeds. The weight of 1000 seeds is around 0.001 grams. The seeds can survive in the soil for over 30 years. Broomrape was observed for the first time in Russia in 1890. In Bulgaria it was widely propagated in the 40s.

According to the physiology and proliferation of the parasite, relatively high level of variability is found - the presence of the eight world races (A, B, C, D, E, F, G, and H). The last three of these are the most virulent and were discovered in the last ten years in Romania, Spain, Turkey and Russia (Kaya et al., 2004; Fernández-Escobar et al., 2008; Păcureanu-Joita, 2008; Goncharov, 2009). Researches of Shindrova (2006) point to the spread of three races in Bulgaria - D, E and F. Antonova et al. (2012) identified the available parasite races in Russia and assessed the degree of virulence of populations in different regions of the southern part of the country. The authors observed the mixed nature of the virulence of the parasite in different areas. The authors concluded that in the southern part of the Russian Federation have spread higher virulent races *O. cumana*, overcoming the action of genes *Or5*, *Or6*, *Or7*, *or6or7*, coding sunflower resistance.

Sunflower has natural sources of resistance to broomrape. Sources of resistance to the parasite lines were cultivated sunflower, wild species and forms received after interspecies crossing (Pustovoit and Gubin, 1974; Pustovoit, 1975; Burlov and Kostyuk, 1976; Škorić, 1988, 1992; Burlov and Artemenko, 1983; Christov, 1990; Christov et al., 1998, 2009; Gulya et al., 1997; Fernández-Martínez and Ruso, 1997; Fernández-Martínez et al., 2000; Melero-Vara et al., 2000; Batchvarova et al., 2001; Pérez-Vich et al., 2002, 2004; Velasco et al., 2007; Kaya et al., 2009, etc.). The first variety of sunflower resistant to race A of broomrape, Saratovskij 169, was developed by Plachek at 1918. The most important varieties resistant to race B were Peredovik and VNIIMK 8931 (Pustovoit, 1966).

MATERIALS AND METHODS

Object of this study are 5 new Bulgarian lines (Hristova-Cherbadzhi, 2007, 2012), one random choice hybrid and 7 lines with the participation of American parental (Dr. Hulke) from *Helianthus annuus* L. (Table1). The five Bulgarian lines were obtained after interspecific cross with line 6116A. American material was obtained after intraspecific (interline) hybridization with line 6116A.

Line 6116 is resistant to common Bulgarian broomrape races. Line 6116 was the result of irradiation of seeds of variety VNIIMK 8931 by gamma rays (mutant) and has specific morphological characteristics (highly serrated leaves, petiole as a "broken knee" and wherefore leaves occupy a specific position in relation to the stem), controlled by recessive genes (Christov, 2000).

Used lines were selected under field conditions infectious plot infected with *O. cumana* race F.

Table 1. Origin of inbreed *R* lines.

Bulgarian materials		American materials (F_6)	
OR-7R	*H. annuus* - 6116A x *H. nuttallii* ssp. *rydbergii*	12-517-1US	*H. annuus* - 6116A x RHA 462
CK9	*H. annuus* - 6116A x *H. puaciflorus* ssp. *subrhomboideus*	12-529-1US	*H. annuus* - 6116A x RHA 462
CK10	*H. annuus* - 6116A x *H. puaciflorus* ssp. *subrhomboideus*	12-540-4US	*H. annuus* - 6116A x 07-500 R
CK29-2	*H. annuus* - 6116A x *H. puaciflorus* ssp. *subrhomboideus*	12-547-3US	*H. annuus* - 6116A x 07-500 R
XYM-27-2	*H. annuus* - 6116A x *H. bolanderi*	12-554-5US	*H. annuus* - 6116A x 07-500 R
A78xOR-7R	F_1 hybrid - A-78 (*sensitive*) x OR-7R	12-555-2US	*H. annuus* - 6116A x 07-500 R
Line 364A	*Sensitive control*	12-558-1US	*H. annuus* - 6116A x 07-500 R

Evaluation of resistance to broomrape (*O. cumana*) of selected new inbreed lines was carried out through artificial infection in controlled conditions by standard method (Panchenko, 1975). Infection was done with broomrape race H (with origin from Edirne, Turkey). This race is not spread in Bulgaria. Reporting of the number and degree of infested plants was done after 45 days.

RESULTS

From 14 sunflower lines and one F_1 hybrid that have been tested. two have shown complete resistance to *O. cumana* with zero incidents of infection in all of the plants (Fig 1). These are line OR-7R and the F_1 hybrid line A78xOR-7R, which have as father the same OR-7R line and line A78, a line sensitive to *O. cumana* as mother. This result suggests dominant control in first hybrid generation.

Lines CK10, XYM-27-2 and CK-29-2 which are inbreed lines obtained from crosses with wild *Helianthus* species have been infected at rate of 75% and had very similar attack severity with average number of *O. cumana* tubercles between 2 and 2.6. Distinctively from these three inbreed lines the susceptibility to broomrape of inbred line CK9 was relatively smaller as 50% of the tested plants were attacked. This line also had the lowest number (0.86) of attached tubercles per plant from all susceptible lines.

The infection incidents for all the lines with American parent were 100% as the severity varied from 2 tubercles per plant in line 12-554-5 to 5.8 in line 12-588-1.

Line A364 was used as control since this line is known by its high level of susceptibility and in the experiment it was attacked at 100% with over 3 broomrapes per plant.

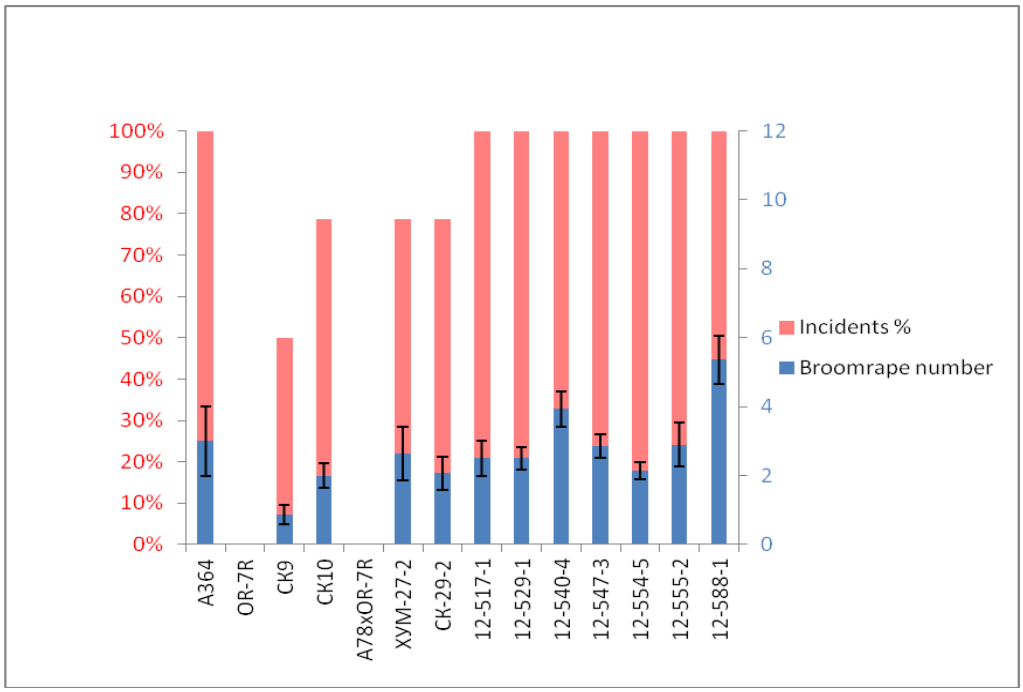

Fig. 1. Reaction of sunflower plants to O. *cumana*. Incidents - number of infected plants/total number of plants; Broomrape number - average number of broomrape tubercles per plant. Bars represent standard error of the mean.

DISCUSSION

In the present study the resistance of 14 sunflower inbred lines and one F_1 hybrid to *O. cumana* has been evaluated. One of the inbred lines has shown complete resistance to the parasite as well as the F_1 hybrid of the same line as a father and a susceptible line as a mother, suggesting dominant control. These results are in unison with previous reports for dominant control of broomrape resistance. 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. According to Antonova (1978), in the resistant forms there is a layer of lignin between parenchyma and cambium which does not allow the penetration of broomrape's haustorium. Mechanism of sunflower resistance is at the same time physiological, biochemical and mechanical. The genetic mechanism is characterized by the presence of genes responsible for the control of resistance that can refer to the full, racially-specific vertical resistance controlled by major gene. Our results have shown complete inheritance of broomrape resistance in F_1 hybrid (A-78 x OR-7R). Further research is needed in other to investigate the nature of this resistance by analysis of the segregation of the F_1 progeny.

Imidazolinones (IMI) herbicides control broomrape by suppressing its development. However, sunflower must be IMI-resistant. Lines ХУМ-27 and СК29 are resistant of herbicides Pulsar 40 + Stomp 330 ЕК, but based on different sources (first from BASF and second from line HA425 of Dr. Miller). Breeding of sunflower for broomrape resistance is an important alternative to the IMI treatment saving costs for herbicide application. *H. annuus* L. (wild) resistant to imidazolinones herbicides were first identified in Kansas, U.S.A. in 1996 in a soybean field that had been treated with the herbicide (Al-Khatil et al., 1998). USDA-ARS (NDSU) research group quickly transferred sunflower resistance to imidazolinone herbicides into cultivated sunflower genotypes and released the public populations IMISUN-1 and IMISUN-2. However, the emergence of new *O. cumana* races that overcome the developed resistance is a serious challenge for this approach.

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Broomrape resistance breeding in sunflower: a case study in Turkey

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ABSTRACT

Broomrape is the main limiting factor in sunflower area in Turkey. The competence between broomrape and sunflower breeders has continued since more than 50 years. Broomrape struggles with sunflower creating of new races. Even with the support from chemical control with IMI (Imidazolinone) herbicide recently as post application, the overcoming of broomrape problem has been possible only with genetical resistance until today. Broomrape was appeared firstly in sunflower areas in 60-70s years as known 5 races respectively. Then the most aggressive race, as called F, appeared firstly in some parts of Spain and Turkey and spread to neighbor production areas rapidly then observed in many countries later. Even first resistant hybrids developed by private seed companies in Turkey, resistant hybrids and lines also were developed lately in National Sunflower Project conducting by Trakya Agriculture Research Institute (TARI). In last three years, four broomrape resistant sunflower hybrids were registered by TARI in Turkey. Respectively, many resistant inbred male and female lines also developed and registered too. On the other hand, three joint hybrids developed with collaboration Szeged GKI from Hungary and one with Agroplazma Seed Co from Russia too.

Key words: Sunflower – broomrape – resistance breeding – Turkey – research

INTRODUCTION

Broomrape (*Orobanche cumana* Wallr.) is a plant parasite that causes reductions in the sunflower yield up to 100% in Turkey, Europe and the Balkan countries. This parasite could create many races containing very small seeds and then could spread more easily (Kaya et al., 2004). To develop effective and sustainable resistance against broomrape which is a major limiting factor in sunflower is one of the most important goals of breeding programs (Fernández et al., 2012; Kaya et al., 2012). Broomrape resistance genes in wild sunflower species such as *H. tuberosus*, *H. maximiliani* and *H. debilis* are determined and successfully transferred in the cultural types of sunflower (Christov et al., 2009; Fernández et al., 2012; Kaya, 2014).

There are many races in broomrape in sunflower and resistance is determined by a single dominant *Or* gene. However, although a single dominant gene controls broomrape resistance against known five resistance race, it was different from these as new races of broomrape, broomrape resistance have been determined by the dominant additive allelic or very closely genes which were not allelic acting together in the recent studies in Spain (Fernández et al., 2012).

First sunflower varieties resistant to race A has been developed in Saratov Research station in Russia at the beginning of the 20th century. Following this invent, a new B race was determined in Rostov station and then a large number of broomrape resistant sunflower varieties against B race have been obtained. Additionally, a new different race other than A and B strains was determined in Moldova (Antonova et al. 2011). Vrânceanu et al. (1980) were able to identify of existence of the known five races and resistance genes against these races in Romania. While F race which is new determined race other than know races that resistance single dominant gene or genes is controlled by two recessive locus in (Pérez-Vich et al. 2002; Pacureanu et al. 2009), resistant genes against to A, B, C, D and E races are determined by single dominant genes (Vrânceanu et al., 1980). On the other hand, Velasco et al. (2012), indicated recently in their study in Spain that the resistance to the newest races G was controlled by dominant alleles in a single locus obtained from wild sunflower *H. debilis* ssp. *tardiflorus*.

To facilitate the identification of broomrape race; Vrânceanu et al. (1980) set up a differentiation set with Kruglik A-41, Jdanovsky 8281, Record, S-1358 and P-1380 lines having *Or1*, *Or*2, *Or3*, or *Or4* and *Or5*. This set was completed LC1093 line carrying the gene *Or6*. In the last thirty years, new broomrape races was appeared and observed in Spain (Fernández-Martínez et al., 2012), Romania (Pacureanu et al., 2009), Russia (Antonova et al., 2011), Bulgaria (Christov et al., 2009) and in Turkey (Kaya et al., 2004).

The racial composition of broomrape in the Trakya region of Turkey is changing frequently; Evci et al. (2009) reported that E race has been the dominant race in the period of 1983-1990 until F race came along. However, Kaya et al. (2004) determined at least one new race which cannot be controlled by current genes in Turkey, also in the other conducted studies, they have reported new resistant genes

against that virulent races in a large number of lines and hybrids (Evci et al., 2011; Kaya et al., 2012). New broomrape races covered in almost all of production areas in Trakya-Marmara region which has 50% of the sunflower planting areas of in Turkey. Currently sunflower production could be possible in the region, only with genetic resistant hybrids to broomrape and Imidazolinone (IMI) herbicide through with chemical control with planting IMI herbicide resistant hybrids (Kaya et al., 2004, 2012).

Broomrape reduces seed yield in sunflower production as well as affects many other important yield traits. Decreasing of 1000 seed weight in sunflower oil and protein content, plant height, head diameter, and the seed yield by broomrape, but not affecting grain quality and fatty acids have been indicated in many studies (Kaya, 2004, Pacureanu et al., 2009).

Broomrape resistance was the primary goal in both public and also private sunflower breeding program in Turkey. Due to that main production areas are being in Trakya region which is European part of Turkey, TARI which is regional institute played key role on both broomrape resistance breeding and also dissemination of infested areas in Turkey. Similarly, this region also is center and also testing areas for many local and international private seed companies and breeding programs too. Since 1970, many broomrape resistant inbred lines and hybrids have been developed in the National program conducted by TARI but the broomrape resistant breeding studies were accelerated especially after discovering resistant genes obtained by mutation (Evci et al., 2009). Therefore, the successful results mainly have been obtained in last 5 years term in the project both in broomrape resistance and also Clearfield types too (Demirci and Kaya, 2009; Evci et al., 2011and 2012; Kaya et al., 2004; 2009; 2012, 2013).

The goal of the study is indicating recent results on broomrape resistance breeding in TARI, Edirne, Turkey and determining broomrape resistance and the performance of some yield traits of sunflower parental lines and hybrids developed in National Sunflower project conducted in TARI in Trakya conditions.

MATERIALS AND METHODS

The candidate hybrids derived from and female and restorer lines resistant against to new broomrape races developed in the National project conducted in TARI and resistant commercial varieties existed as controls were used as material in the study between 2009 and 2013. Regional yield trials were conducted in dry conditions in Lüleburgaz and Edirne in this period with randomized complete block design with 4 rows and 4 replications and the parcel size was 7.5 m based on registration office rules. Yield trials were planted by hand in April and two mid-rows were harvested and threshed by hand in September generally. Seed yield (kg ha^{-1}), oil yield (kg ha^{-1}), oil content (%), thousand seed weight (TSW) (g), head diameter (HD) (cm), plant height (PH) (cm), broomrape resistance, flowering and physiological maturity duration (days) were observed and measured in the experiments.

Broomrape Tests

In Naturally Infested Field Conditions: broomrape tests of new breeding material and candidate hybrids were conducted in the field which was infested new broomrape races in Malkara County, Tekirdag in Trakya Region. Each material in the study was planted in a single row with two replications and then its broomrape infestation was observed before physiological maturity period. Susceptible Sanbro and resistant P- 4223 sunflower varieties has been planted in every 40 rows as controls. Parcel size was 6 m, row spacing distance was one m distance.

Laboratory Conditions: the resistance of genetic material against to broomrape were tested in soil artificially infested with using mixed broomrape seeds collected from Trakya region. In the growth chamber tests, each plastic cup filled up 1-2 g broomrape seeds (as half a teaspoon in a glass, putting and mixing in the lower half of the glass) and mixed soil. Plants were removed from the glasses after 35 days from planting, then its roots washed and attached broomrape tubers were counted. Additionally, all breeding materials in the summer nursery were planted artificially infested with a mixture of broomrape seeds then their infestation was observed.

In the project, in many female (CMS), male (restorer) lines and hybrid in the growth chamber, breeding genotypes in the summer nursery were observed of broomrape resistance then selection process were performed based on these results between 2009 and 2013. These lines and hybrids were tested in that period also against broomrape in naturally infested conditions in Malkara except 2013.

RESULTS

National Sunflower breeding program conducted by in Turkey consist developing initial material utilizing wild species, interspecific hybrids, etc. The inbred A (female) and B (maintainer) lines, restorer (male) (R) lines, developing F1 hybrid and seed multiplication finished inbred lines, constituting test hybrids other parts of the program. Moreover, broomrape (both natural infested areas in Trakya region with new races and artificial conditions at the research field and in the pots at lab) and herbicide resistance tests, preliminary and regional yield trials, quality analysis (oil and oleic acid content, 1000 seed weight, etc.) are other issues completed this breeding program. However, the national program is designed only based on classical breeding processes and molecular tools is missed part of it. Due to that sunflower is a summer crop and need in a breeding cycle at least 110-120 days to be mature, it is possible easily to obtain only two generations per year in sunflower with green house breeding cycle at limited scale in the winter season in Turkey conditions. With support of green house and growth chamber process in last 5 years, national sunflower breeding program in TARI accelerated as much as rapidly and then many inbred lines and hybrids are developed recently.

In the project, almost 15000 genetic materials were tested at growth chamber in winter season in artificial conditions against broomrape between 2009 and 2013 (Table 1). The selection of that genetic materials was performed based on that test results against new races of broomrape which their seeds were collected from different part of Trakya region in previous years and then selected material which had broomrape tolerance continued in further generations. After determining resistance of them at initial and segregating phase, these materials were observed of their broomrape tolerance only in summer nurseries in the field which infested artificially also. For double check and confirmation of the artificial tests, some of these materials, especially hybrids and inbred lines at further generations, were tested also in naturally infested fields in Tekirdag, Malkara. On the other hand, the broomrape infestations of candidate hybrids were also observed in preliminary and regional yield trials too.

Table 1. The number of Broomrape tests conducted by TARI between 2009 and 2013.

Years	Broomrape tests at growth chamber	Broomrape tests at natural infested fields
2009	3237	347
2010	2631	628
2011	2260	529
2012	3230	374
2013	3444	-
Total	14702	1878

Yield trials of candidate broomrape tolerant hybrids were tested and compared with the most selling commercial hybrids every year firstly in preliminary trials conducted in Edirne TARI fields. After evaluation of their performances, promising ones were tested again in regional yield trials to adapt different environmental conditions mainly in Edirne and Luleburgaz locations in this period. Both locations were also infested new races of broomrape.

In the project, trying efforts to discover new resistant broomrape sources were performed also continuously every year both utilizing from mutation to current inbred lines and genetic materials and also using with wild types and interspecific crosses in this period. First test hybrids were also obtained from those sources from wild types and new mutations. On the other hand, segregating process still continues in that new initial materials and selections were performed for having talented characters. These candidate hybrids will be tested next years in the project to obtain promising hybrids that could compete with commercial ones currently in the market.

On the other hand, exchanging CMS lines were also performed largely to produce joint hybrids resistant to broomrape with both local seed companies in Turkey, international seed companies and also other institutions from Russia, France, Hungary Serbia, Ukraine, Bulgaria, Spain, South Africa, US, Iran, etc. The successful and promising results were also obtained recently in this way of project and now some joint hybrids were already testing currently to in the registration trials both on broomrape resistant and also Clearfield types in that countries.

One regional yield trial conducted in the project results consisting broomrape tolerant candidate hybrids for Edirne and Luleburgaz locations in 2013 were given in Table 1 and 2. Based on the results of that trial, 13 TR 009, 13 TR 013, 13 TR 001 and 13 TR 002 candidate hybrids were performed over standard average for seed yield and exhibited also other desired characters. These hybrids and also other candidate hybrids were downy mildew resistant also because downy mildew (*Plasmopora halstedii* (Farl.) Berl. et Toni) is the most limiting factor for sunflower production after broomrape in Turkey in recent years. Among these promising sunflower hybrids, 13 TR 009 candidate hybrid was selected and sent for registration process in 2014. For this candidate hybrid, production permission were also obtained and so

both hybrids and also its inbred lines could be produced officially and certified then its seeds will be ready to distribute for farmers in next years, if it exhibits good performance in 2014 and 2015 then it will be registered. Other three promising candidate in this trials will be tested one year again and if they continue that higher performances in 2014 regional trials, they will be send also to registration process.

Table 2: Broomrape tolerant sunflower candidate hybrids in Yield Trial-1 at Edirne in 2013

Hybrids	S Yield (kg ha^{-1})	Rk	Rate to Std (%)	Oil Yield (kg ha^{-1})	Rk	Oil Ct (%)	TSW (g)	Flowr (Day)	PM (day)	PH (cm)	HD (cm)
13 TR 009	2700	1	110,5	1300	1	48,0	61,52	59	98	170	15
LG 5580 (C)	2590	2	106,1	1160	3	44,9	55,20	62	99	155	14
BOSFORA (C)	2490	3	101,8	1140	4	45,7	60,96	59	99	161	17
13 TR 002	2480	4	101,6	1190	2	47,8	45,88	59	97	175	15
13 TR 001	2470	5	101,1	1120	6	45,3	51,68	59	97	167	15
13 TR 013	2450	6	100,3	1120	7	45,6	53,28	61	100	165	15
P64G46 (C)	2350	7	96,2	1120	8	47,5	53,24	61	100	150	16
COLOMBI (C)	2350	8	96,1	1030	13	43,7	55,28	62	101	181	16
13 TR 016	2310	9	94,4	1050	10	45,4	56,72	59	100	148	14
13 TR 015	2290	10	93,8	1140	5	49,5	57,52	57	98	159	16
13 TR 010	2250	11	91,9	1050	11	46,7	57,12	55	93	172	15
13 TR 004	2240	12	91,7	1070	9	47,9	48,56	63	100	173	15
13 TR 008	2240	13	91,7	970	16	43,3	42,48	60	96	184	14
13 TR 017	2460	14	100,6	1150	14	46,6	50,44	62	103	147	15
13 TR 014	2130	15	87,1	920	19	43,2	55,40	61	100	191	13
13 TR 005	2070	16	84,8	930	18	44,6	48,28	63	100	163	16
13 TR 012	2070	17	84,5	910	20	43,8	42,48	61	99	166	17
13 TR 018	2050	18	83,9	940	17	45,8	50,40	62	100	163	16
13 TR 006	2000	19	82,0	1050	12	52,4	48,44	63	102	183	17

CV (%) =7,91; LSD=252,4 kg ha^{-1} for seed yield; CV (%) =7,87; LSD=116,4 kg ha^{-1} for oil yield,

Table 3: Broomrape tolerant sunflower candidate hybrids in Yield Trial-1at Lulebugaz in 2013

Hybrids	S Yield (kg ha^{-1})	Rk	Rate to Std (%)	Oil Yield (kg ha^{-1})	Rk	Oil Ct (%)	TSW (g)	Flower (Day)	PM (day)	P H (cm)	HD (cm)
13 TR 013	2030	1	112,2	930	3	46,1	43,28	60	101	159	16
LG 5580 (C)	1950	2	108,0	930	1	47,5	42,64	60	100	154	14
13 TR 009	1920	3	106,5	890	4	46,5	48,76	58	97	137	14
13 TR 002	1870	4	103,4	930	2	49,9	37,16	58	96	160	15
BOSFORA (C)	1830	5	101,6	880	5	48,0	49,52	58	98	153	16
13 TR 017	1830	6	101,4	860	8	46,8	38,48	61	101	151	14
13 TR 001	1810	7	100,2	840	9	46,5	39,72	58	97	146	15
13 TR 014	1800	8	99,7	750	17	41,8	47,07	60	99	171	16
P64G46 (C)	1780	9	98,4	870	6	48,8	38,84	58	101	139	13
13 TR 010	1740	10	96,4	780	14	44,8	39,32	54	92	141	15
13 TR 004	1710	11	95,0	870	7	50,8	42,68	62	100	149	15
13 TR 016	1700	12	94,1	790	13	46,4	47,44	57	99	148	15
13 TR 007	1670	13	92,4	820	10	49,4	37,24	61	99	179	17
COLOMBI (C)	1660	14	91,8	760	16	46,1	49,32	63	102	171	15
13 TR 003	1630	15	90,1	800	11	49,1	37,84	61	101	154	13
13 TR 018	1590	16	88,2	710	18	44,8	33,88	61	101	158	15
13 TR 015	1580	17	87,5	780	15	49,3	42,68	56	97	154	15
13 TR 012	1530	18	84,7	660	19	42,9	30,80	60	99	144	14
13 TR 006	1500	19	83,4	800	12	53,0	37,88	61	100	176	15

CV (%) =9,16; LSD=219,9 kg ha^{-1} for seed yield; CV (%) =8,98; LSD=102,2 kg ha^{-1} for oil yield,

In the last five years period of National Sunflower Breeding Project, promising results were obtained and the registered inbred lines tolerant to new races of broomrape were given in Table 4 and oil type hybrids developed both in Turkey and in other countries were given in Table 5.

Table 4: The number of inbred lines tolerant to broomrape developed by TARI between 2009 and 2014

Inbred Line	Type	Registration Year
9702 R	Male line	2011
9758 R	Male line	2011
TT 119 4 R	Male line	2012
97583 R	Male line	2013
9979 R	Male line	2014
9728 A	Female line	2014
9987 R	Male line	2014
97581 R	Male line	2014
9718 A	Female line	2014

Table 5: The number of hybrids tolerant to broomrape developed by TARI between 2009 and 2014

Cultivar Name	Type	Country	Registration date
LARISSA	Oil type joint hybrid with Szeged GKI	Romania	2010
INESSA	Oil type joint hybrid with Szeged GKI	Romania	2010
LAURA	Oil type joint hybrid with Szeged GKI	Romania	2010
08 TR 003	Oil type hybrid	Turkey	2012
DUNA	Oil type hybrid	Turkey	2013
KAAN	Oil type hybrid	Turkey	2013
SARAY	Oil type hybrid	Turkey	2013
ORACLE	Oil type joint hybrid with Agroplazma Seed Co	Russia	2014

DISCUSSION

After evaluating and indicating that successful results, it could be concluded that national sunflower breeding project conducting by TARI reached to their goals for this five year period in sunflower breeding in Turkey both developing broomrape resistant inbred lines and also obtaining promising sunflower hybrids. These new developed inbred lines and hybrids had not only broomrape tolerance both also had higher seed and oil yield performance, other desired yield traits and also downy mildew resistance too. As a result, National Project continues in productive and profitable way and also enlarges internationally both exchanging inbred lines and also sending developed hybrids directly via local and foreign seed companies to register in that countries then sell in there and other countries too.

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HELIOS Project: Search for marine bioactive compounds to prevent the growth of *Orobanchaceae* in crops

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ABSTRACT

Orobanche and *Phelipanche* spp. (broomrapes) are parasitic plants in the Mediterranean region and southeast Europe that can be responsible for devastating losses in several important crops particularly oilseed rape, sunflowers, vegetables. Indeed, broomrapes are organisms devoid of chlorophyll that develop a haustorium serving both as an attachment organ to host roots and as a bridge to divert and exploit the sap produced by the host plant. With a strong spreading potential and a high adaptability, their dissemination is difficult to control. Several control strategies are employed against broomrapes but none has shown unequivocal success. The methods are either uneconomic, hard to achieve, or result in incomplete protection. In addition, resistance to broomrape is scarce and/or complex, making resistance breeding difficult. Thus alternative or supplementary methods should be considered to prevent infection. The aim of the HELIOS (2011-2014) project is to develop new marine bioactive compounds inhibiting the growth of *Orobanchaceae* and to offer farmers effective, environmentally-friendly solutions ensuring the optimization of crop yields. We focus our research on the development of preventative treatments that should be applied before the host infection and thus the attachment and emergence of the parasite. The aim of this innovative approach is to reduce the parasite's interest for the host or even to generate a repulsion between both partners.

Key words: *Orobanchaceae* – broomrape – oilseed – marine plant

Screening of wild *Helianthus* species for resistance to *Orobanche cumana* Wallr. and *Phomopsis helianthi* Munt.-Cvet. et al.

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ABSTRACT

The phytopathological investigation of eighty-eight accessions of wild annual *Helianthus* species was carried out. The studied *H. annuus, H. argophyllus, H. debilis, H. petiolaris and H. praecox* accessions from the collection of DAI were tested for resistance to broomrape and to grey (*Phomopsis helianthi*) spots on sunflower. The obtained testing results showed that some of the accessions were immune to the gray spots on sunflower and possessed full resistance to the parasite broomrape. These accessions could be used as donors for resistance and as useful initial material in the breeding programs for obtaining of new resistant hybrids. The presented results confirmed that wild *Helianthus* species could be used as valuable sources for resistant genes.

Key words: *Helianthus* – wild species – resistance – *Orobanche cumana* – *Phomopsis helianthi*

INTRODUCTION

During the last years a serious enlargement of sunflower production areas was observed. That led to systematically breach of crop rotation. Actually, sunflower crop has been back to the same place after one or two years. As a result of this "intensive" crop rotation, suitable conditions for multiplication of the parasite broomrape, as of some other fungus pathogens infection – phomopsis, phoma, downy mildew, sclerotinia, macrophomina, alternaria, etc., were established especially in case of susceptible hybrids production. Broomrape attacks were determined as one of the most serious problems, connected to sunflower seed production in Bulgaria, which seriously decreased sunflower yield, seed oil and protein content (Shindrova, 1994; Shindrova et al., 1998). The gray spots on sunflower caused by the pathogen *Phomopsis/Diaporthe helianthi* Munt. Cvet.et all appeared to be one of the more serious problems nowadays. As a result of severe attacks the plant core has been destructed and the broken plants have fallen to the ground. The main measures for overcoming such problems were connected to pesticides use and applying of good agricultural technics. There is another way for effective protection against broomrape attacks. Increasing of genetic resistance nowadays is the important factor for successful breeding work. Jan and Fernández-Martínez (2002), Seiler (2010), Gulya et al. (2010), and Christov (2013) reported for successfully transferred resistance to broomrape from wild *Helianthus* species into cultivated sunflower. Studies of Christov et al. (1998), Nikolova et al. (1998), Batchvarova et al. (2001), Atanasova (2004) showed, that wild *Helianthus* species could be valuable sources for developing new sunflower forms and lines, possessing genes for resistance to the broomrape. Seiler and Marek (2011) investigated a collection of wild sunflower species and determined the accessions PI 617026 (*H. grosseserratus*), PI 617028 (*H. divaricatus*), PI 617027 (*H. maximiliani*), PI 617029 (*H. divaricatus*) as resistant to race F, and the accession PI 468691 (*H. debilis* ssp. *tardiflorus)* as resistant to race G. Jan and Fernández-Martínez (2002) reported for successful transfer of genes for resistance to race F from perennials *H. angustifolius, H. cusickii, H. divaricatus, H. grosseserratus* and *H. maximiliani,* and on this base Fernández-Martínez et al. (2004) registered new sources for resistance to it. The race F appeared also in Russia (Goncharov et al., 2004) and in Bulgaria (Shindrova, 2006). Pérez-Vich et al. (2002) studied the inheritance of resistance to race F, established in interspecific hybrids, obtained from *H. divaricatus* and *H. grosseserratus*. They presumed that the resistance was controlled by one dominant gene. Velasco et al. (2006) studied hybrid material J1, obtained from *H. grosseserratus* and supposed there were two genes connected with the inheritance and the second one was influenced by the environment. There are many researchers (Treitz, 2003; Nikolova et al., 2001; Hahn and Degener, 1999; Vear and de Labrouhe, 1997; Škorić, 1985), who work in the field of sunflower breeding for increasing the productive potential of the crop and its resistance to the gray spots. Encheva et al. (2006) studied the reaction of different annual and perennial species from DAI collection and established immune type of reaction for eight *H. annuus* accessions and high level of resistance for some perennial accessions of *H. mollis, H. nuttallii, H. resinosus, H. hirsutus, H. divaricatus.* Seiler (1992) reported for establishment of genes for high tolerance to *Phomopsis/Diaporthe helianthi* Munt. Cvet. et al. Treitz (2003) studied and used purposefully the wild species for transfer of genes for resistance to gray spots on sunflower.

MATERIALS AND METHODS

The investigations were carried out in Dobrudzha Agricultural Institute near General Toshevo. Different accessions from some annual species were used. They were chosen from the collection of wild *Helianthus* species maintained in DAI. The accessions were from the follow species:

- *Helianthus annuus* L. (w.f.) (Table 1)
- *H. argophyllus* (GT-E-006, GT-E-007, GT-E-008, GT-E-091, GT-E-130, GT-E-131, GT-E-132)
- *H. debilis (H. debilis,* ssp. *cucumerifolius* - GT-E-012, GT-E-137; and *H. debilis,* ssp. *silvestris* - GT-E-138, GT-E-139)
- *H. petiolaris* (*H. petiolaris,* ssp. *petiolaris* - GT-E-022, GT-E-142;)
- *H. praecox (H. praecox,* ssp. *praecox* - GT-E-145, GT-E-146)

Table 1. *Helianthus annuus* accessions included in the investigation.

Species	Accessions										
Helianthus annuus (w.f.)	E-002	E-003	E-004	E-005	E-035	E-040	E-042	E-043	E-044	E-045	E-046
	E-049	E-053	E-055	E-056	E-057	E-058	E-059	E-060	E-061	E-062	E-063
	E-064	E-066	E-077	E-078	E-079	E-081	E-088	E-092	E-093	E-103	E-104
	E-105	E-106	E-109	E-110	E-111	E-112	E-113	E-114	E-115	E-116	E-117
	E-118	E-119	E-120	E-121	E-122	E-123	E-124	E-125	E-126	E-127	E-128
	E-129	E-153	E-154	E-155	E-170	E-171	E-172	E-173	E-174	E-175	E-176
	E-177	E-178	E-179	E-180	E-182	E-183	E-184				

The evaluation for resistance to *Orobanche cumana* Wallr., race E, was made in greenhouse conditions. The standard methodology /Panchenko, A., 1975/, adapted for the conditions of DAI was used. The evaluation for resistance to gray spots on sunflower (*Phomopsis helianthi* Munt.-Cvet. et al.) was carried out by the method of Encheva, V and I. Kiryakov, 2002 on an artificial infection plot. The type of attacks was reported a week after full flowering on 5 degree scale / 0 - 4/.

RESULTS AND DISCUSSION

In Bulgaria the problem with broomrape was always solved by developing resistant cultivars and hybrids (Encheva and Shindrova, 1994). This led to decreasing the multiplication and dissemination of the parasite and guaranteed obtaining of good results.

The variation of resistance to broomrape was studied for 73 accessions of the wild *H. annuus* (Table 2). It was established that the reaction varied in wide ranges. Full resistance (100 %) showed 7 accessions or 9, 6% from all, included in the investigation. Resistance from 76% to 99% was specified for 14 accessions or 19,3 % from all tested materials. The rest ones were susceptible.

Table 2. Evaluation of *H. annuus* accessions to the parasite *Orobanche cumana* Wallr.

Resistance %	Accessions from the wild *H. annuus*	Total number
0	E -042 E -043 E -046 E -055 E -056 E -059 E -061 E -062 E -103 E -104 E -105 E -106 E -116 E -125 E -155	15
1 - 50	E -005 E -044 E -045 E -049 E -058 E -064 E -066 E -117 E -119 E -120 E -124 E -126 E -129 E -172 E -175 E -176 E -178 E -179 E -180	19
51 - 75	E -003 E -057 E -060 E -063 E -081 E -093 E -109 E -110 E -111 E -112 E -114 E -115 E -118 E -127 E -173 E -177 E -182 E -184	18
76 - 99	E -002 E -004 E -040 E -053 E -077 E -079 E -113 E -123 E -153 E -154 E -170 E -171 E -174 E -183	14
100	E -035 E -078 E -088 E -092 E -121 E -122 E -128	7

The impressive were the results of other annual accessions testing. The evaluation of *H. argophyllus, H. debilis, H. petiolaris* and *H. praecox* accessions were shown on table 3. The resistance to broomrape here varied in wide ranges too.

Table 3. Evaluation of resistance of wild annual sunflower accessions to *Orobanche cumana* Wallr.

Species Accession	Resistance, %				
	0	1 - 50	51 - 75	76 - 99	100
H. argophyllus Torrey & Gray		E-008	E-006 E-007	E-091 E-132	E-130 E-131
H. debilis ssp. *cucumerifolius* (T. & G.) Heiser			E-082	E-012	E-137
H. debilis ssp. *silvestris* Heiser		E-089	E-138	E-140	E-139
H. petiolaris ssp. *petiolaris* Nuttall		E-024	E-022		E-142
H. praecox ssp. praecox Engleman & Gray		E-147	E-145		E-146

Full resistance (100%) was established for the accessions E-130, E-131, E-137, E-139, E-142 and E-146. Resistance from 76.0% to 99.0 % was reported for the accessions E-091, E-132, E-012 and E-140. They could be used as donors for resistance in interspecific hybridization as well as for enrichment of geneplasm of cultivated sunflower.

The same materials were inoculated with the pathogen *Phomopsis helianthi* . It was established that the reaction of 73 accessions of wild *H. annuus* to the fungus pathogen varied in wide ranges – from immune to susceptible. The different categories, in accordance with accessions' reaction, were determined and shown on table 4. Immune type of reaction was determined for 14 accessions, which was 19,2% of all studied *H. annuus* accessions. Resistance to the pathogen was established for 37% of the materials.

Table 4. Evaluation of *H. annuus* accessions to *Phomopsis helianthi* Munt.-Cvet. et al.

Type of reaction	*H. annuus* accessions	Number of accessions per groups according to the type of reaction
Immune	E -004 E -035 E -078 E -088 E -092 E -117 E -121 E -122 E -124 E -128 E -171 E -174 E -175 E -178	14
Resistant	E -003 E -060 E -063 E -077 E -081 E -093 E -109 E -110 E -111 E -112 E -113 E -114 E -115 E -116 E -118 E -119 E -120 E -123 E -125 E -126 E -127 E -129 E -153 E -154 E -155 E -180 E -183	27
Medium Resistant	E -002 E -005 E -040 E -042 E -043 E -045 E -049 E -053 E -057 E -058 E -064 E -066 E -079 E -170 E -172 E -173 E -176 E -177 E -179 E -182 E -184	21
Medium Susceptible	E -046 E -056 E -059 E -062 E -103 E -104 E -105 E -106	8
Susceptible	E -044 E -055 E -061	3

Immune type of infection was observed for 14 accessions, which was 19, 2% of all studied *H. annuus* accessions. They are E-004, E-035, E-078, E-088, E - 117, E-092, E-121, E-122, E-124, E-128, E-171, E-174, E -175 and E -178. Resistant were 27 *H. annuus* accessions or 37.0 % of all tested materials. The rest tested materials varied from medium resistant (28, 8%) to susceptible (15%). These accessions are of great importance, because they could be used as donors of resistance for improving cultivated sunflower. Using purposeful interspecific hybridization a new and valuable genetic material could be obtained.

The results of testing of some wild annual sunflower accessions were presented on table 5. Six accessions from *H. argophyllus* (E-130, E-131, E-132); *H. debilis* ssp. *silvestris* (E-139) and *H. petiolaris* (E-142, E-146) were with immune type of reaction. Another eight accessions were distinguished with resistance to the pathogen.

Table 5. Evaluation of resistance of wild annual sunflower species to *Phomopsis helianthi* Munt.-Cvet. et al.

Species Accession	Type of reaction		
	Immune	Resistant	Med. resistant
H. argophyllus Torrey & Gray	E-130 E-131 E-132	E-006 E-091	E-007 E-008
H. debilis ssp. *cucumerifolius* (T. & G.) Heiser		E-137	E-012 E-082
H. debilis ssp. *silvestris* Heiser	E-139	E-138 E-140	E-089
H. petiolaris ssp. *petiolaris* Nuttall	E-142	E-022	E-024
H. praecox ssp. praecox Engleman & Gray	E-146	E-145 E-147	

The results of this investigation gave us the reason to suppose, that the tested accessions carry genes for resistance to the cause agent of gray spots on sunflower and could be used as donors for resistance in future purposeful sunflower breeding.

CONCLUSIONS

Dobrudzha Agricultural Institute possesses a large collection of wild sunflower species. Greater part of the studied accessions could be used as donors for resistance to *Phomopsis helianthi Munt.-Cvet.* et al., and to the parasite broomrape. Full resistance (100 %) to *Orobanche cumana* Wallr. showed 7 accessions of *H. annuus* species, 2 accessions of *H. argophyllus,* 2 accessions of *H. debilis,* 1 accession of *H. petiolaris* and 1 accession of *H. praecox.* They could be successfully included in the future breeding programs for developing resistant hybrids. Accessions E-035, E-078, E-088 E-092, E-121, E-122, E-128 from *H. annuus* species were characterized with full resistance to broomrape and immune type of reaction to *Phomopsis helianthi.*

There are also accessions of great interest among the wild annual *H. argophyllus, H. debilis, H. petiolaris* and *H. praecox* species. Accessions E-130, E-131(*H. argophyllus),* E-139 (*H. debilis),* E-142 (*H. petiolaris)* and E-146 (*H. praecox*) were characterized with full resistance to broomrape and immune type of reaction to *Phomopsis helianthi.*

The parasite broomrape and the caused agent of gray spots on sunflower are problems not only in our country, but for all countries, sunflower producers in the Mediterranean region and whole East Europe (Höniges et al. 2008). Most of wide spread commercial cultivars and hybrids are tolerant or resistant to broomrape as well as to pathogens, but only for a certain period of time. Shortly a new race(s) is appeared and the hybrids have been fallen away from the production lists.

The presented results showed that the collection of wild *Helianthus* species, located on the territory of DAI possesses wild annual sunflower species, which could be included in the breeding programs as initial material for developing new hybrids.

The results of this investigation give the possibility to suppose that the studied accessions carry genes for resistance to *Orobanche cumana* Wallr. and *Phomopsis/Diaporthe helianthi* Munt. Cvet.et all., and could be used as donors for resistance.

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Screening resistance of new NS sunflower hybrids to broomrape

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ABSTRACT

Continued work in sunflower breeding program at the Institute of Field and Vegetable Crops (IFVC) on creating new sunflower hybrids resistant to broomrape demands the screening of breeding materials for resistance in both field and conditions of a greenhouse. New NS hybrids were produced by crossing *Rf* lines RHA-D-2, RHA-D-6, RHA-D-9 developed from interspecific population DES-1474-1 resistant to broomrape race E and possibly race F and G with *cms* female lines resistant to broomrape race E. NS hybrids were tested in Serbia have shown complete resistance to race E in artificial infestation in greenhouse and natural infestation 2008 and 2009. The testing continued with the resistant hybrids in Serbia, Spain, Romania in natural infestation and in Turkey artificial infestation where is present races F and higher in 2012 and 2013. In Northern Serbia NS hybrids was completely resistant to broomrape race E present, in Spain, Romania, Turkey showed different level of resistance to broomrape depending on the country where they were tested. The highest number of resistant hybrids was in Spain (Andalusia), that number was smaller in Turkey (Trakya), and the smallest number of resistant hybrids was in Romania (Constanta). In Spain the highest number of resistant hybrids in both years of testing was presented by combinations RHA-D-6 restorer. The identified hybrids BT-VL18xRHA-D-6, Cms-1-90xRHA-D-6, As92xRHA-D-6 possess resistance/moderate resistance to highly virulent races of broomrape in Spain, Romania and Turkey.

Key words: Broomrape – hybrids – resistance – screening – sunflower

INTRODUCTION

Broomrape (*Orobanche cumana* Wallr.) has been the most serious problem in sunflower production in Southern and Eastern Europe and in the Middle East, leading to considerable yield losses up to 100% and reducing sunflower seed quality (Kaya et al., 2012). Although genetic resistance is the most effective control method, new parasite races evolve overcoming sunflower resistance (Molinero-Ruiz et al., 2013). Since broomrape is a highly variable parasite, the breakdown of resistance is a frequent phenomenon, and multiple sources of resistance are needed (Seiler, 2012). Achieving sustainable sunflower resistance to broomrape is one of the most important goals in sunflower breeding (Pacureanu-Joita et al., 2009; Fernández-Martínez et al., 2010; Kaya et al., 2012). Cultivated sunflower is genetically narrow and deficient in many desirable genes. Its genetic variability can be increased by the use of wild sunflower species and interspecific hybridization (Hladni et al., 2009). The resistance to races E, F, G have been found in certain wild species of the genus *Helianthus* and incorporated into cultivated sunflower genotypes by interspecific hybridization Jan et al. (2002), especially in species *Helianthus tuberosus* L., *Helianthus maximiliani* Schrad, and *Helianthus debilis* Nutt (Fernández-Martínez et al., 2008). Cvejić et al. (2012) found the source of resistance to race G and more virulent races in an inbred line derived from interspecific hybridization with *Helianthus divaricatus*. The population of broomrape has been stable in Serbia for a long period of time, but the racial composition has changed in recent years, with race E being predominant in the regions of North Bačka and Banat (Hladni et al., 2010). Continual monitoring of the broomrape population in Serbia is very important due to changes in race composition and evolution of new more virulent races in neighboring countries and also due to the fact that climate changes are favorable for expansion of *Orobanche* species to large areas (Maširević et al., 2009). A new race, called race F, has occurred in Romania, Bulgaria, Spain and Turkey (Pacureanu-Joita et al., 1998; Christov et al., 2009; Alonso et al., 1996; Molinero-Ruiz et al., 2008; Kaya et al., 2004). The changes in the broomrape population and the occurrence of the new race are new challenges for sunflower breeding, forcing the breeders to continually test the breeding material against new broomrape races while creating differential lines (Hladni et al., 2010).

Testing races F from different geographic areas, it has been detected that broomrape race seed from different countries differs in virulence. Most virulent races are from Turkey, followed by the ones from Romania and Spain Pacureanu-Joita et al. (2003). In research Molinero-Ruiz et al., (2013) race F populations not be identified as a genetically differentiated group but aspolyphyletic origin for race F, arising independently at different geographical origins. The more virulent race G that affects cultivars resistant to race F, was identified (Škorić et al., 2010; Dicu et al. 2011). Thus, different authors reported different models of inheritance of resistance to race F: controlled by single dominant gene *Or6* (Pacureanu-Joita et al., 1998; Pérez-Vich et al., 2002) or two recessive genes (Akhtouch et al., 2002) or two partially dominant genes (Velasco et al., 2007). The genetic control of the resistance to race F into sunflower germplasm has been described as both qualitative and quantitative (Fernández-Martínez et al., 2004; Pérez-Vich et al., 2006). Preliminary results of resistance to race G indicate that it is controlled by dominant alleles at a single locus (Velasco et al., 2011). Continued work on creating new sunflower lines and hybrids resistant to broomrape in sunflower breeding program at IFVC demands the screening of breeding materials for resistance in both field conditions and in controlled conditions of a greenhouse (Hladni et al., 2012). Hybrids produced by crossing *Rf* lines developed from interspecific population (DES-1474-1) and *cms* female lines susceptible to broomrape race E was test in Serbia 2006-2008 and in Romania (Baragan, Braila) 2008. This result indicates that some inbred lines developed from interspecific populations originating *H. deserticola* DES-1474-1, are resistant to broomrape race E and possibly race F, and indicated that this population can be used for the production of new resistant sunflower hybrids (Hladni et al., 2009; Hladni et al., 2010; Hladni et al., 2012).

The objective of this research was to screen new NS sunflower hybrids originating from different *cms* line resistant to race E and *Rf* lines RHA-D-2, RHA-D-6, RHA-D-9 derived from interspecific population *H. deserticola* DES-1474-1, resistant to broomrape race E and possibly race F and G for broomrape resistance using different races of parasite.

MATERIALS AND METHODS

New NS hybrids were produced by crossing *Rf* lines developed from interspecific population (DES-1474-1) resistant to broomrape race E and possibly race F and G with *cms* female lines resistant to broomrape race E. *Rf* inbred lines (RHA-D-2, RHA-D-6, RHA-D-9) were produced from an interspecific population originating from *H. deserticola* in Institute of Field and Vegetable Crops (IFVC). Initially, the plants were selected from the interspecific population DES-1474-1 provided by Dr. Gerald Seiler (USDA-ARS, Fargo ND, USA) originating from *H. deserticola.*

NS experimental hybrids were tested for resistance to broomrape race E in two locations (Lipar and Feketić) in Serbia 2008-2011. In year 2012 and 2013, hybrids known to be resistant to broomrape race E, tested against the new broomrape races under natural and artificial conditions in Romania, Spain and Turkey. In the trials the following differential lines were used: Romanian differential lines (Vrânceanu, 1980) were used as checks in evaluation of hybrids in all locations (instead of line P-1380-2, another Romanian differential line LC-1003(*Or5*), was used, as well as lines LC-1093(*Or6*) (Pacureanu-Joita et al., 2009; Fernández-Martínez et al., 2004). Spanish differential line P-96 (*Or6*) is resistant to races E and F but susceptible to race G (Molinero-Ruiz et al., 2008). NS commercial line Ha98 was used as tested due to its susceptibility to all broomrape races.

Hybrids were planted in two locations in Serbia (Lipar and Feketić), in Romania in region Constanta location (Cogealac) and in Spain in region Andalusia on two locations (Cordoba, Molino Pavia). Each hybrid was planted in two rows (24 plants) in two replications. Reaction of the tested sunflower lines to broomrape was evaluated by calculating incidence, i.e. ratio of the infected sunflower plants per total number of plants. Inbred lines were considered resistant (R) when no broomrape stalk was found within the complete entry, moderately resistant (MR) when 1-50% plants had at least one broomrape stalk and susceptible (S) when more than 50% plants were infested. In Turkey broomrape tests test was done in pots under conditions of artificial infestation, mainly in winter time. Under the conditions of artificial infestation, broomrape seeds were cleaned threshing the plants collected from different infested fields of Trakya region. Before planting, about 1-2 g broomrape seeds were placed at the bottom of the pots. Broomrape seeds were placed under sunflower seeds so that's sunflower root must penetrate into the zone of broomrape seeds. *Orobanche* plants were counted on each root after 35 days from planting, after washing the rots. Broomrape attack was assessed by uprooting and careful observation of root system for broomrape nodules and stalks. Nodules on the sunflower root were counted. If the number of nodule was higher than 10 the marking was S susceptible, from 1-5 number of nodule MR moderately resistant and without nodule R resistant to broomrape.

RESULTS AND DISCUSSION

New NS hybrids were tested in Serbia have shown complete resistance to race E in artificial infestation in greenhouse and natural infestation 2008 and 2009. The testing continued with the resistant hybrids in Serbia and in countries Spain, Romania, Turkey where is present races F and higher in 2012 and 2013. NS sunflower hybrids reacted differently to broomrape, which depended of location and race composition of the broomrape (Table 1).

Table 1. Broomrape tests under the conditions of natural infestation conducted in summer and in artificial infestation mainly in winter time.

Country	Serbia		Spain		Romania		Turkey	
Year	2012	2013	2012	2013	2012	2013	2012	2013
Races	E	E	F,G	F,G	F,G	F,G	F,G	F,G
Number of tested hybrids	111	98	100	100	97	97	95	95
Number of resistant mat.	111	98	26	30	4	10	13	9

In Northern Serbia where is broomrape race E NS hybrids was completely resistant but in Spain, Romania and Turkey test hybrids showed different level of resistance to present highly virulent races of broomrape which is in accordance with the findings of Pacureanu-Joita et al. (2003) who concluded that the same race from different countries differs in virulence, the most virulent races were from Turkey, followed by the ones from Romania, Spain, and Serbia. The existence of different aggressiveness of populations of *O. cumana* race F have been reported (Molinero-Ruiz et al., 2009) and genetic heterogeneity within some of these populations that has been suggested (Molinero-Ruiz et al., 2008) has also been confirmed in this research. The analysis NS hybrids of broomrape resistance showed different reaction of selected sunflower hybrids depending on the restorers and the country where it was tested (Table 2).

Table 2. Resistance of hybrid combinations to broomrape analyzed by restorers (RHA-D-2, RHA-D-6, RHA-D-9)

Spain	2012				2013			
Hybrids	TM	S	MR	R	TM	S	MR	R
RHA-D-2	42	23	14	5	39	13	18	8
RHA-D-6	20	2	6	12	23	6	5	12
RHA-D-9	38	11	18	9	38	12	16	10
Turkey	2012				2013			
Hybrids	TM	S	MR	R	TM	S	MR	R
RHA-D-2	40	29	7	4	42	25	13	4
RHA-D-6	20	4	9	7	20	9	10	1
RHA-D-9	35	27	6	2	33	16	13	4
Romania	2012				2013			
Hybrids	TM	S	MR	R	TM	S	MR	R
RHA-D-2	39	13	26	-	39	11	23	5
RHA-D-6	20	1	16	3	20	14	6	-
RHA-D-9	38	14	23	1	38	13	20	5
Serbia	2012				2013			
Hybrids	TM	S	MR	R	TM	S	MR	R
RHA-D-2	38			38	28			28
RHA-D-6	26			26	29			29
RHA-D-9	47			47	41			41

TM-tested material, R-resistant, MR-moderately resistant, S-susceptible

It has been determined that the highest number of resistant hybrids was in Spain (Andalusia), that number was smaller in Turkey (Trakya), and the smallest number of resistant hybrids was in Romania (Constanta). In Spain the highest number of resistant hybrids in both years of testing was presented by combinations RHA-D-6 restorer. In Romania the hybrids were tested during two years on one location, the resistance of hybrids was different, a high number of hybrids with all three restorers exhibited moderate resistance (MR) while a significantly smaller number of hybrids exhibited resistance (R), table 2. Which is confirmed by the results on broomrape resistance under natural conditions depend on weather conditions during sunflower growing season and inconsistency in broomrape distribution in all parts of

the experiment field. Chosen were female lines resistant to race E which have demonstrated moderate resistance/resistance to broomrape in all hybrid combinations with all three restorers (Tab. 3).

Table 3. The chosen female lines which have demonstrated moderate resistance/resistance to broomrape with all three restorers

	Country	Serbia	Spain		Romania		Turkey	
CMS	RF	2012/ 2013	2012	2013	2012	2013	2012	2013
		Lipar/ Feketić	Cordoba	Molino Pavia	Cogealac	Cogealac	Trakya	Trakya
	Races	E	F,G	F,G	F,G	F,G	F,G	F,G
BT-VL18	RHA-D-2	R	R	R	24MR	S	S	S
	RHA-D-6	R	R	3MR	11MR	33MR	R	MR
	RHA-D-9	R	R	R	3MR	S	MR	MR
BT-VL24	RHA-D-2	R	R	R	8MR	S	R	MR
	RHA-D-6	R	R	3MR	12MR	S	R	S
	RHA-D-9	R	1MR	3MR	3MR	S	S	MR
OD4A	RHA-D-2	R	R	3MR	28MR	20MR	S	MR
	RHA-D-6	R	R	R	R	S	R	S
	RHA-D-9	R	R	R	4MR	5	S	S
Cms-1-223	RHA-D-2	R	3MR	9MR	13MR	14MR	S	S
	RHA-D-6	R	R	3MR	17MR	S	S	S
	RHA-D-9	R	R	R	8MR	S	S	S
Cms-1-90	RHA-D-2	R	R	6MR	6MR	S	MR	S
	RHA-D-6	R	R	R	5MR	14MR	R	MR
	RHA-D-9	R	4MR	3MR	13MR	S	MR	R
As92	RHA-D-2	R	R	R	12MR	S	S	S
	RHA-D-6	R	R	R	R	15MR	S	MR
	RHA-D-9	R	R	R	12MR	S	S	R
Ha98	RHA-D-2	R	10MR	14MR	12MR	35MR	MR	S
	RHA-D-6	R	R	R	4MR	S	MR	MR
	RHA-D-9	R	R	3MR	4MR	S	S	MR
P-96	(*Or6*)	R	R	R	R	50MR	S	S
LC-1093	(*Or6*)	R	S	S	50MR	50MR	S	S
LC- 1003	(*Or5*)	R	S	S	S	S	S	S
S-1358	(*Or4*)	S	S	S	S	S	S	S
Record	(*Or3*)	S	S	S	S	S	S	S
Jdanov	(*Or2*)	S	S	S	S	S	S	S
Kruglik A-41	(*Or1*)	S	S	S	S	S	S	S
AD-66		S	S	S	S	S	S	S

Hybrid combinations with all three restorers with female line Ha98 sensitive to all broomrape races and with female lines resistant to broomrape race E demonstrated different resistance to broomrape by years and by countries where it was tested. All hybrids were completely resistant to broomrape in two location in Serbia in two years, this indicates that examined hybrids were resistant to race E (Tab. 1, 2). In Spain all hybrids combinations demonstrated resistance and moderately resistance towards broomrape in both years of testing, in Romania on the same location the results differed by years, while in Turkey a smaller number of hybrids demonstrated resistance, tab. 3.

In Serbia differential lines LC-1003, LC-1093 and P-96 resistant to races E and F were resistant while other differential lines are susceptible. In Constanta region (Cogealac) differential lines which have *Or6* (P-96 and LC-1093) was resistant or had half of plants infected indicating the presence of races which overcome race F (race G) this results are in agreement with Pacureanu-Joita et al. (2008) which results showed that the different lines LC-1093 lost resistance in Black Sea area. Both these lines were susceptible in region Andalusia, this results are agree with results Cvejić et al., (2012) and Molinero-Ruiz et al., (2009) whose reported the low infection levels found in P-96 in Spain are not due to genetic heterogeneity within parasite populations, but to the quantitative resistance of the inbred lines. In Turkey P-96 and LC-1093 lines were susceptible, these results confirm the findings that highly virulent race F was identified in Spain and Turkey also suggest presence of race G (Tab.3). Test in natural infestation and inartificial infestation in greenhouse hybrids showed different level of resistance to present highly virulent races of broomrape race F and G in Spain, Romania and Turkey which is in accordance with the research

done by Pacureanu-Joita et al. (2004). In a breeding material were identified hybrids BT-VL18xRHA-D-6, Cms-1-90xRHA-D-6 and As92xRHA-D-6 resistance/moderately resistant to highly virulent races of broomrape present in Spain, Romania and Turkey. Our results indicate that *Rf* lines RHA-D-2, RHA-D-6, RHA-D-9 developed from interspecific population DES-1474-1 can be used for the production of new sunflower hybrids resistant to highly virulent races of broomrape.

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Morphological characterization of broomrape resistant sunflower lines

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ABSTRACT

The objective of the study was to select new Bulgarian inbreed lines that demonstrated resistance to broomrape. Characterization of morphological traits was made in field conditions in infested plot with O. cumana race F. From all 200 forms (88 B lines and 112 R lines), 78 lines were not attacked by the parasite. Single plants from 93 lines showed broomrape attack. These genotypes are suitable for inclusion in future breeding programs as a source for resistance to broomrape.

Key words: Broomrape – inbreed lines – resistance – sunflower

INTRODUCTION

Orobanche cumana Wallroth is highly specialized obligate parasite. Broomrape has become one of the most important threats to the cultivated sunflower, with high level of variability. Researches of Shindrova (2006) point to the spread of three races in Bulgaria - D, E and F. Sources of resistance to the parasite broomrape were cultivated sunflower, wild species and forms obtained after interspecies crossing (Christov, 1990, 2013; Christov et al., 1998, 2009; Hristova-Cherbadzhi et al., 2007; Hristova-Cherbadzhi, 2007, 2012; Hristova-Cherbadzhi and Christov, 2008, etc.). Resistant lines were also obtained after mutagenesis (Christov, 2000).

MATERIALS AND METHODS

The objective of the study was to select new Bulgarian inbreed lines obtained after interspecific or intergeneric crosses with cultivated sunflower. Materials were growing in infected plot with *O. cumana* race F. Lines were screened and selected. Characterization of morphological traits made in field conditions. There were made crosses between sensitive and resistant lines, too.

RESULTS AND DISCUSSION

New selected Bulgarian inbreed lines were growing in not-treated chemical and infected plot with *O. cumana* race F. From all screened 200 forms (88 B lines and 112 R lines), there were attacks in 93 numbers on single plants (Fig.1). These genotypes are suitable for inclusion in future breeding programs as a source for resistance to broomrape.

Fig. 1. Attacked single plants from different numbers

From all, 78 lines are not attack from the parasite. Five of these lines were obtained after interspecific cross with line 6116A. Line 6116 is resistant to broomrape races to F. Line 6116 was the result of irradiation of seeds of variety VNIIMK 8931 by gamma rays (mutant) and has specific morphological characteristics (highly serrated leaves, petiole as a "broken knee" and wherefore leaves occupy a specific position in relation to the stem), controlled by recessive genes (Christov, 2000). Some from screened of these materials were typical for line 6116 characteristics - highly serrated leaves and extended ray flowers (Fig.2).

Fig. 2. XYM-29.

More interesting not-attack lines were OR-7R, CK8, CK9, CK10, CK13, CK29, XYM-7, XYM-26, XYM-27, XYM-29, and others.

After the observation of the presence of the parasite *O. cumana*, some broomrape resistant sunflower lines were characterized for duration of the growing period, plant height, unbranched or branched stem, diameter of the stem, 1000 seed weight, and presence of specific morphological markers (Table 1).

Table 1. Biomorphological characterization of resistant and sensitive lines.

Line Characteristics	A-78	A-364	OR-7R	XYM-27	CK-29	CK10
Phenological characteristics						
Vegetation period, days	112	118	110	112	107	114
Duration of flowering, days:						
- of the central inflorescence	8	9	9	8	9	8
- of full plant	8	9	43	46	42	40
Morphological characteristics						
Plant height, cm	120	130	130	120	135	110
Number of branches	0	0	5	7	3	5
Length of branches, cm	-	-	60	40	45	50
Leaf length, cm	31	26	21	19	22	25
Leaf width, cm	29	24	20	17	22	23
Length of leaf petiole, cm	13	11	10	10	10	12
Head diameter, cm	17	19	25	22	26	23
Received seeds after open-pollination						
N	1173	1169	1139	838	1645	1260
%	80.0	76.4	76.2	57.6	63.7	74.4
Technological characteristics						
1000 seed weight, *g*	62.2	61.4	57.3	40.4	35.2	50.8

Lines A-78 and A-364 were sensitive to the pest. The two forms are suitable for *B* line. Stem of the plant is unbranched. Plant height was 120 cm for A-78 and 130 cm for A-364. Stems and leaves are green. The inflorescence was a few curved and convexes, with measure 17 cm for A-78 and 19 cm for A-364. Weight of 1000 seeds was about 62 g. There are sterile analogues based on CMS PET-1, after being checked for the absence of the *Rf* genes.

Line OR-7R was resistant to the parasite race E (laboratory controlled study). The form has regenerative capacity for CMS PET-1 because there had restoration (*Rf*) gene and was suitable for *R* line. Plants are branched. All inflorescence's branches were in lower position. The position of the central inflorescence was half-inverted. Form on the side of the seed was slightly convex. Ray flowers were colored in yellow. Stem and leaves are dark green. The seeds are rounded and colored in gray- black.

Two lines XYM-27 and CK-29 were based on different sources of resistance to herbicides Pulsar 40 + Stomp 330 EC. One source was from the material of company BASF, the second – from the first resistant to herbicide American HA425 line of Dr Miller. In the field condition these two lines were not attacked. These were fertility restorer lines (*R* lines) and are suitable for the paternal parent in the future hybrids. Lines are selected because of their good architectonic, i.e. due to their morphological phenotype. At the last summer environmental conditions were very harsh - drought. We separated these materials for including in an experience to infect in the greenhouse. This is important, because the resistance to the herbicide does not determine the resistance of the plant material to the parasite. If the line was not resistant to broomrape, the pathogen attaches to the plant and after treatment with herbicide broomrape can stop their development.

The *vegetation period* was an important characteristic because the received forms can participate in the creation of different types of hybrids - early mature (110-125 days), medium (125-135 days) or late mature (> 140 days) (Fick, 1978; Skoric, 1988; Ivanov et al., 1988).

Plant height of *B* and *R* lines was better is up to 150 *cm*.

Selection of characteristic *branching* was lead in two directions - creating unbranched forms suitable for *B* line and branched forms suitable for *R* lines.

The *head diameter* was link with yield. Aim of selection was to increase the head diameter in the lines and in the hybrids. Bulgarian model provides head diameter in hybrids 24-25 *cm*.

The *weight of 1000 seeds* in Bulgarian model selection for inbred lines can to be above 60 *g* (Stoyanova, 1978). This characteristic for *R* lines was usually with lower value. The weight of 1000 seeds for paternal line of hybrid Albena - line 147R was 47.6 *g*, and hybrid Musala - 29.9 *g*.

According to Fick (1978) size of the seeds, their color and the high ratio of kernel/seed hull were important parameters in the selection of high-fat sunflower forms. For many years, these objectives are achieved by selection of sunflower varieties (such Peredovik) but reached the limit, which is very difficult to overcome. This has had the transition to the selection of hybrids in which the heterosis effect is used in the first generation.

Selection of resistant to the new races of the parasite materials was very important from an economic point of view. For this reason, it was decided to evaluate resistance of the lines.

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Behavior of some sunflower cultivars at the broomrape attack in south-eastern area of Romania

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SUMMARY

In Romania, one of the most dangerous parasites on the plants is the broomrape (*Orobanche* spp). Within the sunflower fields there was identified the population of weedy broomrapes of *Orobanche cumana* Wallr. This specie has shown a significant dissemination, especially in the south and south-eastern area of the country. On sunflower cultures in the areas heavily infested with the broomrape, especially in the south and south-eastern area of the country, such as are those in Dobrogea, the losses reach 30-70% of the harvest. The behavior of some sunflower cultivars against *O. cumana* were studied under natural contamination conditions. At SC Sport Agra srl, Amzacea-Constanta county, the following hybrids showed the best results: Neoma, Alego, Sunay, Festiv, and Turbo. Due to their biological properties (good resistance to broomrape attack), Festiv and Sanay hybrids were cultivated in 2013, at Sun Agro Organic – Carasuhatu, Tulcea county within the Danube Delta Natural Reservation, part of the Natura 2000 Ecosystem.

Preliminary SSR analysis of a novel broomrape resistance source

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ABSTRACT

Broomrape (*Orobanche cumana* Wallr.) is a parasitic plant that can cause significant yield losses in sunflower. Race composition in broomrape populations changes constantly, and novel resistance genes need to be discovered and introduced into sunflower. According to results of trials in fields where broomrape races E, F and G were detected, inbred line HA-267 has resistance gene higher than Or_6. This study showed that resistance in HA-267 is under the control of a single recessive gene. An attempt to map the resistance gene using bulk segregant analyses (BSA) did not give positive results, presumably due to a low level of polymorphism between the parental lines used for mapping population development. Screening of lines HA-98-PR, OD-DI-100 and OD-DI-82 was performed with SSR molecular markers, in order to select the most suitable one for mapping population development.

Key words: BSA – *Orobanche cumana* – resistance genes – SSR – sunflower

INTRODUCTION

Broomrape is a parasitic weed that causes significant problems in sunflower production. Since the parasite lacks any photosynthetic capacity and is completely reliant on the host for all nutritional needs, seed yield losses from broomrape in susceptible sunflower genotypes can be substantial.

Compared with the other weeds, broomrape is difficult to control by conventional means because parasite has so much metabolic overlaps with the host (Aly, 2007). Breeding for genetic resistance appears to be the most appropriate and cost-effective control practice. Genetic resistance to broomrape has been introduced into sunflower by Pustovoit in 1912 at VNIIMK Institute in Krasnodar, Russia (Sackston, 1992). Sources of resistance to *Orobanche* were found within landraces of cultivated sunflowers, but was also introduced into susceptible sunflower from wild *Helianthus* species (Fernández-Martínez et al., 2010). Genes conferring resistance to races A-E are dominant (Vrânceanu et al., 1980), whereas resistance to race F was found to be under the control of recessive genes at two loci (Rodríguez-Ojeda et al., 2001) or one single dominant gene (Pacureanu-Joita et al., 1998; Pérez-Vich et al., 2002). The only mapped *Orobanche* resistance gene in sunflower is Or_5, which is located on LG3 of the public SSR map (Lu et al., 1999, 2000; Tang et al., 2003; Letousey et al., 2007). Imerovski et al. (2013). found a high associations between the markers located on LG3 (ORS1036, ORS1114 and ORS665) and three different resistance genes (Or_2, Or_4 and Or_6). Additionally, preliminary studies of the resistance source AB-VL 8 indicated that the resistance gene(s) in that line could also be on LG3. These results imply that *Orobanche* resistance genes could be closely linked or allelic, as it was also suggested by Tang et al. (2003).

Vrânceanu et al. (1980) formed a set of lines constituting genes Or_1, Or_2, Or_3, Or_4 and Or_5 which are used as differentials for broomrape races A-E. Line LC1093 carrying Or_6 was later added to the set (Pacureanu-Joita et al., 1998). The widespread use of resistant cultivars has led to the appearance of new races of the parasite (Škorić, 1988). Races overcoming race F were reported in Spain (Alonso et al., 1996), Romania (Pacureanu-Joita et al., 2008; Škorić and Pacureanu-Joita, 2010) and Turkey (Kaya et al., 2004). In Serbia, race E is present in the main sunflower-growing regions and is spreading to the new regions (Dedic et al., 2009).

The constant changes in broomrape race composition have forced sunflower breeders to continuously search for resistance genes for the new races of *Orobanche*. In order to attain their breeding goals and identify sources of broomrape resistance, sunflower breeders have to develop a breeding strategy, secure the necessary germplasm and differential lines for broomrape race identification, and choose the appropriate inoculation method and molecular marker technique (Škorić et al., 2010). Finding new sources of resistance and developing molecular markers for detecting *Or* genes is one of the most important challenges in breeding of sunflower. In the present study, we analyzed the inheritance pattern of a newly found resistance in the line HA-267. Preliminary molecular analyses with use of bulked segregant analyses (BSA) were conducted with the aim of identifying the region of the genome which could potentially carry the resistance locus. Additionally, three susceptible lines were tested in order to select the most suitable one for mapping population development.

MATERIALS AND METHODS

Plant material

Inbred line HA-267 was developed at the Institute of Field and Vegetable Crops, Novi Sad. Broomrape resistance screening trials that were conducted in Serbia, Spain, Romania and Turkey in year 2013 showed that HA-267 was resistant even in the fields where broomrape races E, F and G were detected (Cvejic et al., unpublished data), suggesting that HA-267 has resistance gene higher than Or_6. In order to establish the mode of inheritance of the resistance, HA-267 was crossed with HA 26-PR, a sunflower line susceptible to all broomrape races chosen from the genetic bank of the Institute. The F_1 generation was grown in the field, and tested in natural infestation conditions in Serbia, Spain, Romania and Turkey. The F_2 generation was grown in the greenhouse and tested in pots as described by Miladinovic et al. (2012). Based on the phenotypes of F_2 individuals, goodness-of-fit of the observed segregation ratio was calculated.

As very low polymorphism was observed between HA-267 and HA 26-PR, additional genotypes were screened in order to find a more suitable susceptible parental line for a new mapping population. For this purpose, inbred lines HA-98-PR, OD-DI-100 and OD-DI-82 were chosen.

Molecular analyses

DNA was extracted from young leaf tissue of the examined lines using a modified CTAB method described by Permingeat et al. (1998). An attempt to identify molecular markers linked to new resistance gene was carried out using BSA as described in Michelmore et al. (1991). Two contrasting bulks were prepared, each containing DNA from extremely resistant or susceptible F_2 plants. A total of 209 SSR markers was selected from 17 linkage groups from the public genetic map of sunflower (Yu et al., 2003). Additionally, lines HA-98-PR, OD-DI-100 and OD-DI-82 were tested with 30 SSR markers. PCR was performed in Biometra Thermocycler, by using 15 µl of reaction mixture containing 1xPCR buffer, 3 mM $MgCl_2$, 0.2 mM of dNTPs, 0.3 µM each of 3'- and 5'-end primers, 1 U of DNA polymerase, 25 µg BSA and 40 ng of genomic DNA. The best quality of amplification product was obtained on program with an initial denaturation at 95º C for 2 min, followed by 29 cycles at 95º C for 30 s, than 30 s at 63º C, and then 1 min at 72º C. The final extension was carried out for 10 min at 72º C. All of the products were evaluated by electrophoresis on 2% metaphor agarose gels using 1xTBE buffer. The gels were stained with ethidium bromide 10 mg/ml and visualized with the BIO-Print system (Vilber Lourmat, Marne la Velee, France). Fragment size was evaluated with BIO-CAPT V.97 program (Vilber Lourmat).

RESULTS AND DISCUSSION

The F_1 generation obtained from the cross HA-267/HA 26-PR was tested in 4 regions infested with broomrape (Serbia, Spain, Romani and Turkey), and was heavily attacked in all fields. This result suggests that the resistance in HA-267 is recessively inherited. The observed ratio of resistant and susceptible plants in F_2 generation are shown in Table 1. Segregation fitted a 1:3 ratio characteristic for a single recessive gene (χ^2 =1.6; P=0.21). Testing of the F_3 generation will give a more precise evaluation, and will also enable to discriminate between homozygous and heterozygous susceptible plants.

Table 1. Reaction of the parental lines and F_2 progeny

	Plants		Ratio
	Resistant	Susceptible	
HA-267	10	0	-
HA 26-PR	0	10	-
F_2	16	64	1:3

Thirty one (14.83%) out of 209 microsatellite primer pairs amplified clear bands, and gave polymorphisms between HA-267 and HA 26-PR. However, none of them amplified identical bands in the resistant F_2 bulk as in resistant genotype HA-267, and bands of different size in the susceptible F_2 bulk and susceptible genotype HA 26-PR. The parental lines showed high similarity on the molecular level thus preventing identification of markers linked to the resistance gene.

Table 2. Polymorphism between HA-267 and susceptible lines detected with molecular markers

Lines	Number of polymorphic markers	Percent of polymorphism
OD-DI-100	13	43.33%
OD-DI-82	11	36.66%
HA-98-PR	9	30%

BSA is a very good method for mapping genes from wild germplasm, however our results suggest that, when the resistance gene is originating from cultivated germplasm, the low level of polymorphism between the parental lines can result in unsuccessful BSA analyses. The method was originally developed for mapping resistance gene from a wild germplasm source (gene *Dm5/8* was introgressed in lettuce from the wild species *L. serriola*) (Michelmore et al., 1991). Examples of successful use of BSA for mapping gene from wild species are available in different crops, including wheat (Kang et al., 2012), rice (Chen et al., 2006), and sunflower (Tang et al., 2003; Bouzidi et al., 2002; Radwan et al., 2003; Dußle et al., 2004; Wieckhorst et al. 2010).

In case of mapping a gene from a cultivated germplasm source, a potential solution could be using more distant parental lines for mapping, which would secure a higher level of polymorphism. Therefore, we compared molecular profiles of three susceptible lines which are presumably very divergent from resistance source (Jocic, personal communication). Similar method was applied in Zhang et al. (1994), who used available data on molecular profiles of several *japonica* and *indica* strains of rice, and chose the lines that had the highest level of polymorphism (≈30%) for creating mapping population. In our study, the results of the screening obtained with 30 primers showed that polymorphism between HA-267 and the three chosen lines ranged from 30 to 42.3%. The highest percent of polymorphism was observed between HA-267 and OD-DI-100 (Table 2). This line is therefore most suitable for crossing with HA-267, and will be used as a susceptible genotype for new mapping population development.

Constant changes in *Orobanche* populations stress the need to identify new source of resistance that will enable sunflower to withstand attack of new broomrape races. Even though wild *Helianthus* species constitute the major gene pool of resistance genes, germplasm of cultivated sunflower is also a very valuable source. HA-267 is an outstanding broomrape resistance gene donor that can be used in breeding programs even in regions with the most virulent races of the parasite. HA-267 is an elite inbred line and is characterized by good general and specific combining abilities, a high level of grain quality and yield, without the deteriorative genetic linkage effect derived from the wild sunflower species. Given all these characteristics, this newly identified resistance source is a valuable asset to breeding programs. Combining known dominant *Or* genes with the recessive resistance gene from HA-267 could be a way for achieving durable resistance to a dynamic pathogen population.

In order to facilitate transfer of this novel resistance gene into other elite sunflower genotypes, closely linked molecular markers should be identified. Crossing with a more suitable susceptible line could enable successful application of BSA, which will eventually lead to identification of co-segregating molecular markers.

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SESSION 4

Herbicide tolerance and other control measures against sunflower broomrape

Herbicide tolerance in sunflower as a tool for *Orobanche* control

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ABSTRACT

Sunflower broomrape (*Orobanche cumana* Wallr.), is an obligate holoparasitic angiosperm causing significant decrease in sunflower (*Helianthus annuus* L.) yield. Several strategies were developed over years for its control or management. Any approach applied alone is often partially effective and the results are sometimes inconsistent due to variable environmental conditions. Genetic resistance was for many years the main control method, but because physiological races of broomrape seem to rapidly evolve it is necessary to combine this genetic control with chemical methodologies to avoid the rapid overcoming of the resistance genes in use. Launch of commercial selective herbicides for this crop permits to develop sunflower genotypes bearing an acetohydroxyacid synthase mutant gene and in this way a new way for chemical control was established. At this moment, three technologies were commercially available, and a new one, with a broad spectrum of resistance to AHAS inhibitor herbicides is under development and testing. Herbicides formulations developed for each of those traits were studied for *Orobanche* control under field and greenhouse conditions with good results. The addition of plant growth regulators to IMI herbicides enhances control when compared with the application of the AHAS inhibitor herbicide alone. The availability of next generation sequencing technologies, and its applications to produce continuous and massive information about parasitic weeds must be exploited for the creation of new herbicides specifically directed to this parasitic weed.

Keywords: AIR – broomrape – CLPlus – ExpressSun – herbicide tolerance – Imisun – Sures

INTRODUCTION

Sunflower broomrape is an obligate holoparasitic angiosperm that causes significant decrease in sunflower, yield. Broomrape lives attached to the roots of sunflower, depleting the plant of nutrients and water. This parasitic plant causes severe crop losses in many countries of southern and central-eastern Europe and the Middle East (Parker, 1994; Melero-Vara et al., 2000). Control of this parasite remains extremely difficult because its produce thousands of tiny seeds per plant that can be easily dispersed by wind, water, animals, humans, machinery or soil attached to agricultural products. The seeds may remain viable for 15–20 years and will germinate in the presence of the host plant (Škorić, 1988). With anticipated climatic changes taking form of increased temperatures and drought in many areas of the world, *Orobanche* species could pose greater threats to agriculture by expanding their ranges farther north in Europe and elsewhere (Mohamed et al., 2006).

Several strategies were developed over years for broomrape control or management in sunflower. These solutions were developed from very different perspectives. Basically these approaches were divided in at least six different categories, consisting in: preventing actions, physical methods, chemical methods, agronomic methods, biological methods and crop resistance (Habimana et al., 2013). Any approach applied alone is often partially effective and the results are sometimes inconsistent due to variable environmental conditions, and because of that, integrated methods combining different strategies into a given farm system are the most effective ones.

Development of traits for herbicide resistance in sunflower during the last decade (Sala et al., 2012) permits the combination of these technologies with *Orobanche* resistance genes for a more sustainable strategy but also to introduce new active ingredients in herbicides formulations that are specific for the parasitic weed control or to develop seed coatings using a variety of AHAS inhibitors.

GENETIC RESISTANCE AND CHEMICAL TOOLS

Genetic resistance was for many years the main control method for broomrape in sunflower. From 1976 to the present, various genetically simple and complex sources of *Orobanche* resistance have been described in sunflower (Tang et al., 2003). Horizontal resistance based on accumulation of multiple genes provides partial resistance, but this is difficult to breed into elite sunflower cultivars (Molinero-Ruiz et al., 2009). Despite the complexities underlying *Orobanche* resistance breeding in sunflower, race specific dominant genes seem to protect the crop and can now be managed with molecular marker assistance to introduce them in opposite sides of a hybrid pedigree.

Because physiological races of broomrape seem to rapidly evolve it is necessary to combine this genetic control with any available chemical methodologies to avoid the rapid overcoming of the resistance genes in use. Several non selective herbicides were used in preemergence or in seed coating treatments as a joint strategy for a more efficient management. The absorption, translocation and metabolism of these herbicides were studied in the *Orobanche*-sunflower system (Diaz-Sanchez et al., 2002). However, the efficacy of those treatments depends on the crop stage, soil type, and environmental conditions (García-Torres et al., 1994; Castejón-Muñoz et al., 1990; García-Torres et al., 1995).

In 1994, the launch of commercial selective herbicides for this crop permits to develop sunflower genotypes bearing an acetohydroxyacid synthase (AHAS, EC 4.1.3.18, also known as acetolactate synthase, ALS; Shaner et al., 1984; Ray, 1984) mutant gene creating a new way for chemical control. The development of sunflower hybrids resistant to AHAS inhibiting herbicides has made possible to successfully control broomrape regardless of the race composition of the populations of these weeds (Škorić and Pacureanu, 2010). At this moment, three technologies were commercially available, and a new one, with a broad spectrum of resistance to AHAS inhibitor herbicides is under development and testing (Sala et al., 2012).

CHEMICAL CONTROL USING SELECTIVE HERBICIDES

Imidazolinone (IMI) and sulfonylurea (SU) herbicides have been demonstrated to have a broad spectrum of weed control activity, flexibility in timing of application, low usage rates, and low mammalian toxicity (Brown, 1990; Tan et al., 2005). These herbicides inhibit the enzymatic activity of AHAS, the first enzyme in the pathway for the synthesis of the branched chain amino acids valine, leucine, and isoleucine (Singh, 1999). This same enzyme has been shown to be the site of action for the triazolopyrimidines (TZ, Subramanian and Gerwick, 1989), pyrimidyloxybenzoates (POB; Subramanian et al., 1990), and sulfonylaminocarbonyl-triazolinones (Santel et al., 1999).

Given their high effectiveness and low-toxicity, IMI and SU herbicides are favored for agricultural use. However, the ability to use both types of herbicides in a sunflower production system depends upon the availability of IMI- and SU-tolerant hybrid cultivars. To produce such tolerant cultivars, it is imperative to develop IMI- or SU-tolerant plants with altered AHAS genes and enzymes. These plants have been discovered in sunflower, which permitted the development and commercialization of at least four herbicide tolerant (HT) traits. Tolerance in these traits is due to a form of the AHAS large subunit enzyme (AHASL) that is less sensitive to herbicide inhibition and is conferred by a single, partially dominant nuclear gene (Sala et al., 2012)

HERBICIDE RESISTANT SUNFLOWER TRAITS AND THEIR APPLICATION IN BROOMRAPE CONTROL

The first commercial HT trait in sunflowers was known as ‘Imisun’ and its development started in 1996 (Al-Khatib et al., 1998). Inheritance of Imisun is additively controlled by two genes, where one of them is the partially dominant allele *Ahasl1-1*, and the other is a modifier or enhancer factor (Miller and Al-Khatib, 2002; Bruniard and Miller, 2001).

The use of Imisun sunflowers for *Orobanche* control was early advised by Alonso et al (1998). When imazethapyr was applied over a wild IMI-tolerant sunflower population artificially infested with *Orobanche*, an almost perfect control, no emerged broomrape shoots, was observed. Later, in trials conducted under field conditions in Serbia, in a natural race E infested region, different commercial IMI-resistant hybrids were used for V3 application with Pulsar ® (imazamox). Good results were obtained for broomrape control, although two split applications of this herbicide were needed to be effective (Masirevic et al., 2010). Under greenhouse conditions, using the same herbicide applied over an IMI-resistant inbred line, broomrape tubercles attached to the sunflower inbred line roots were evaluated. The observed damaged rate of the tubercles was 64% and this results support the idea of an efficient control (Demurin and Perstenyeva, 2010). Due to controlling both broomrape and key weeds together, the IMI herbicide use with IMI resistant hybrids in sunflower production reached about 50% market share in Trakya Region (European part of Turkey) having over 70% of Turkish sunflower areas in recent years (Kaya and Evci, 2009).

The second imidazolinone tolerance trait in sunflower, known as CLPlus®, is controlled by the expression of the partially dominant nuclear allele *Ahasl1-3* which was developed by seed mutagenesis and selection with imazapyr (Sala et al., 2008b). Based on a vast array of environmental conditions and in biochemical studies, it was determined that the CLPlus trait provides superior herbicide tolerance to IMI than the Imisun trait (Sala et al., 2008a&c, 2012d, Weston et al., 2012b). In fact, the CLPlus trait displays the lowest level of inhibition of the AHAS enzyme extracts by IMI, which results in a higher level of

accumulation of biomass after IMI application at the above-ground (Sala et al., 2012a) and root levels (Sala et al., 2012b). Moreover, this superior level of tolerance also provides a better stability of the tolerance to cope with the unpredictable portion of the environmental variation. Due to the high levels of tolerance, only one homozygous component, namely *Ahasl1-3*, or the combination of both *Ahasl1-1* and *Ahasl1-3* alleles in the final hybrid variety, are required to achieve commercial tolerance levels (Sala and Weston, 2010). CLPlus also permitted to develop new herbicide formulations(Pfenning et al., 2012).

There not exist published results regarding the use of new formulations over CLPlus technology hybrids and their possible use in broomrape control. However, taking into account the results obtained for the previous, well studied, Clearfield® technology herbicide formulations, it is advisable to obtain similar or better results with new and more effective formulations. The higher IMI tolerance of the Clearfield Plus trait allows the use of stronger adjuvants and a better formulation of IMI herbicides. Consequently, a more flexible and reliable weed control in sunflower without having any yield penalty is possible (Pfenning et al., 2012).

SU-tolerant and ExpressSun® sunflowers were developed separately, from wild sunflower populations discovered in USA (Al-Khatib et al., 1999) and by EMS mutagenesis over the line HA89 (Gabard and Huby, 2001), respectively. The tolerance allele *Ahasl1-2* from SU-tolerant sunflowers was introgressed into cultivated sunflower by forward crossing and selection with the herbicide tribenuron, and gave rise to the trait known as Sures (Miller and Al-Khatib, 2004). Even though the inheritance of these traits has not been reported, it is well established that the target-site-tolerance is the result of the mutation P197L at the *Ahasl1* locus (Kolkman et al., 2004), and that differences in crop injury among Sures-tolerant breeding lines are the result of the presence of modifier genes (Miller and Zollinger, 2004). These mutations were used independently by some public Institutes and by private companies to develop SU-tolerant hybrid cultivars in many countries, increasing the range of available herbicides in sunflower (Jocic et al., 2011; Streit, 2012).

The use of SU based herbicides to control *Orobanche* in SU tolerant sunflowers was tested under field conditions (Gabard and Huby, 2001). Treatments involving pre-emergence application of metsulfuron methyl and tribenuron methyl only had little effect on the parasitic weed at the rates tested, possibly because limited watering of the soil did not cause the herbicides to reach the sunflower roots. Treatments involving seed coating had substantial effect on *Orobanche*, but were considerably phytotoxic at these application rates to the sunflower varieties in this test, while treatments involving post-emergence application gave the best results. Metsulfuron-methyl was found to be both more efficacious in controlling *Orobanche* and more likely to cause sunflower phytotoxicity even at lower application rates than tribenuron-methyl. A split application of metsulfuron-methyl provided the same level of control while eliminating sunflower phytotoxicity.

Field and greenhouse dose-response experiments were conducted to quantify the tolerance of one tribenuron-tolerant and one IMI-tolerant sunflower hybrid to seven rates of imazamox and tribenuron-methyl. Although, the application of high rates of tribenuron (45 and 67.5 g a.i.ha^{-1}) resulted in 72-100% broomrape control at the time of sunflower flowering, imazamox in IMI-tolerant sunflower proved to be more effective in broomrape control than tribenuron in tribenuron-tolerant sunflowers (Malidza et al., 2012).

A novel HT trait in sunflowers is still under development and is known as AIR. It is controlled by the *Ahasl1-4* and presents a completely new pattern of cross-tolerance for sunflower, since it shows a broad range level of tolerance to different AHAS-inhibiting herbicides (IMI, SU, TZ and POB). Furthermore, this allele also presents a higher level of tolerance to IMI and SU than lines carrying the Imisun and the Sures traits, respectively (Sala and Bulos, 2011). It is known that sunflower lines developed to tolerate some AHAS-inhibiting herbicides are susceptible to foliar applications and, in many cases, to soil residues of other AHAS-inhibiting herbicides (Howatt and Endress, 2006). In these cases, the cross-tolerance of *Ahasl1-4* could allow sunflower hybrids carrying this allele to cope with the soil residues of other types of AHAS-inhibiting herbicides from the fallow or the previous crop.

All the features presented by this trait must be exploited to develop novel herbicide formulations in order to improve broomrape chemical management. Several trials are being conducted in many sunflower producing countries in order to determine the better formulations for weed and *Orobanche* control.

Also, new herbicides formulations and seed coating technologies using different AHAS inhibitor herbicides, proven to be usefully for *Striga* control in corn and sorghum (Tuinstra et al., 2009; Ransom et al., 2012), should be tested extensively for their use in sunflower.

During the last decade, our group has been working for the development (Sala et al., 2008a; Sala and Bulos, 2011) and characterization (Sala et al., 2008b) of new HT traits. With the objective of develop new HT traits other candidates genes are now under study. Using a mutagenesis approach coupled with forward and reverse genetics strategies these genes have been subject to modifications and selection. In this way, new herbicides, targeting different enzymes, could be formulated. The creation of these new formulations

will extend the life, not only of the previous HT technologies, but also of the broomrape resistance genes used in combination with all these technologies.

NEXT STEPS IN CHEMICAL CONTROL

With most of the important weeds being controlled by selective herbicides applied over HT sunflower using the previous described technologies, the addition of active ingredients that are specific for broomrape control is agronomical and economically possible. Plant growth regulators operate by various modes of action (Rademacher, 2000). Prohexadione, prohexadione- calcium, trinexapac and trinexapac-ethyl act as inhibitors of aminocyclopropane carboxylic acid oxidase and hence inhibit the biosynthesis of ethylene. Prohexadione calcium, a plant growth regulator, was studied previously for *Orobanche* control. This compound induces sunflower resistance against the infection (Fan et al., 2007). In some cases, herbicidally active ingredients have been shown to be more effective when mixed with other herbicides compared to when applied individually, and this is referred to as "synergism", since the combination demonstrates a potency or activity level exceeding that which it would be expected to have based on knowledge of the individual potencies of the components.

The addition of plant growth regulators to IMI herbicides enhances *Orobanche* control when compared with the application of the AHAS inhibitor herbicide alone (Pfenning and Bremer, 2013). It was found that, herbicidal active mixtures or compositions having one, two or three AHAS inhibitor herbicides and one, two or three plant growth regulator which act as ethylene modulators are very useful for controlling parasitic weeds.

The availability of next generation sequencing technologies, and its applications to produce continuous and massive information about parasitic weeds (Westwood et al., 2012; Pineda-Martos et al., 2014; Piednöel et al., 2012) must be exploited using different bioinformatics approaches for a rapid and effective characterization of other previously known herbicide target genes obtained from model plants for the creation of new herbicides specifically directed to this parasitic weed. The discovery of differences in metabolic pathways among this organism and its host will be critical for these developments. Also, the sequences generated will provide insights into the biology of parasitism and advance progress toward understanding parasite virulence.

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Potential of some commercial maize varieties to induce germination of Egyptian broomrape

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ABSTRACT

The devastating root parasitic weed, *Orobanche aegyptiaca*, is causing enormous crop losses in China, especially in Xinjiang region. Maize (*Zea mays* L.) has the potential to induce germination of at least three *Orobanche* species. We aimed to determine whether maize could be used as a "trap crop" for *O. aegyptiaca*. In this study, ten commercial maize varieties in China were tested for their ability to induce *O. aegyptiaca* germination, the maize variety with high germination rates of *O. aegyptiaca* seed were screened to control *O. aegyptiaca* on farmland. The results implied that maize could induce *O. aegyptiaca* germination and the germination rates changing in the cut-root experiment and hydroponic experiment were consistent. Changcheng799 and Zhengdan958 had the highest stimulation potential on *O. aegyptiaca*, while Luyu13 and Zhengyu203 had the lowest stimulation potential. Above mentioned four maize varieties were further studied in the pot experiment. Rhizosphere soil, root extracts, and shoot extracts from these four maize varieties induced *O. aegyptiaca* germination, and Changcheng799 displayed the highest germination rates. Root extracts generally induced higher germination rates than shoot extracts. It was suggested that Changcheng799 could be planted in front of the crops in the field where *O. aegyptiaca* occurred to prevent this malignant weed.

Key words: *Orobanche aegyptiaca* – maize – trap crop – germination

INTRODUCTION

Broomrapes (*Orobanche* spp.) are holoparasitic weeds that completely depend on their hosts for water and nutrients (Young et al., 1999). They can heavily infest many important crops with negative impact on crop yield and quality and leading economic losses for millions of people worldwide (Parker and Riches, 1993; Sauerborn, 1991). In China, there are about 20 *Orobanche* species, of which *O. aegyptiaca*, *O. cumana* and *O. ramosa* are the most common and have the widest host range (Zhang and Jiang, 1994). It was firstly reported that *Orobanche aegyptiaca* was infested melon and tomato field in 1964 at Xinjiang Uygur Autonomous Region under the condition of continuous cropping for 3 to 5 years, the average parasitic rate was 31-54%, even 100% parasitism was occurred (Du, 1964). In China, *O. aegyptiaca* is one of devastating weeds that mainly distributed in Xinjiang Uygur Autonomous Region and cause heavy direct damage to some important crops such as melon, watermelon, tomato, potato and tobacco (Zhang et al., 2012).

Seeds of *Orobanche* should be "pre-conditioned" (i.e. expose to water and suitable temperatures for several days) and they will start germinate in response to the reception of a chemical stimulus from host or non-host roots (Joel et al., 1995; Yokota et al., 1998). The germ-tube infects host roots by developing an historian that penetrates the host root and then develops a tubercle, with adventitious roots and a shoot. While the host doesn't exist, the seedling will die. The non-host which can induce *Orobanche* seed germination but not be parasitized also could be called "trap crop".

It is extremely difficult to control for *Orobanche* spp. because of their special biological characteristics and their direct connection with host roots. Some traditional methods such as manual weeding, plant quarantine, resistant cultivars, and biochemical methods have been used for *Orobanche* control. These control measures were with low effects and could not get to the root of weed, because majority of the damage has already been done by the parasite before it emerges above ground (Dhanapal et al., 1996; Parker and Riches, 1993). It is a promising way for *Orobanche* control by utilizing trap crops to induce seed "suicidal germination". This can reduce *Orobanche* seed bank in soil and improve the crop production. And the effects will be better when combining with other methods.

Maize could induce the germination of *O. ramosa* and *O. minor* (Sunderland, 1960a, b; Zehhar et al., 2003). Recently, we found that maize could also be as "trap crop" for *O. cumana* (Ma et al., 2013). We further studied the potential of maize varieties to induce germination of *O. aegyptiaca* based on the results

that maize could induce *O. cumana* germination. The objective of the experiment was to screen some commercial maize varieties for their ability to induce *O. aegyptiaca* germination. Through a cut-root assay, hydroponic and pot experiments, the variety induced highest germination rate of *O. aegyptiaca* could be identified and provided a basis for control of *O. aegyptiaca* in the parasitic field.

MATERIALS AND METHODS

Preparation of Seeds and Chemicals: Seeds of eleven commercial maize varieties were bought from Yangling Seed Company (Changcheng 799, Changdan 48, Luyu 13, Nongda 364, Shandan 2001, Tiancheng 288, Yuyu 22, Zhengda 12, Zhengdan 958, Zhengyu 203). Seeds of *O. aegyptiaca* were collected from infested melon fields in the Xinjiang Uygur Autonomous Region, China. The germination stimulant GR24 was provided by Professor Binne Zwanenburg of the University of Nijmegen, The Netherlands. Before use, the maize and *O. aegyptiaca* seeds were surface-sterilized by immersion for 3 min in 1% (v/v) sodium hypochlorite followed by soaking in 75% (v/v) ethanol for 3 min. The seeds were then rinsed with sterile distilled water and air-dried. *O. aegyptiaca* seeds were preconditioned for 3 days to broke dormancy.

Cut Root Assay: The cut-root assay method was used to determine the effect of maize roots on *O. aegyptiaca* germination (Botanga et al., 2003; Van Mele et al., 1992). Briefly, surface-sterilized maize seeds (10 seeds) of each maize variety were placed in sterile Petri dishes lined with moistened filter paper. The Petri dishes were wrapped in aluminum foil and incubated at 25°C for 96 h. The seedlings roots were cut into 0.5 cm segments. The segments were put in the center of Petri dishes lined with filter paper (~1g per dish). *O. aegyptiaca* seeds (20-50 seeds) were arranged in three concentric circles around each root segment. These circles were 1, 2 and 3 cm from the root segment and will be referred to as the inner, middle, and outer circles, respectively. A piece of aluminum foil encircled the sides of each root segment so that there was no direct contact between the root segment and the *O. aegyptiaca* seeds. Sterile distilled water (1.5 mL) was slowly poured over the top of the root segment. The water ran down the segment and then spread out evenly across the bottom of the Petri dish. The Petri dishes were sealed with parafilm, wrapped in aluminum foil, and incubated at 25°C for 10 days. Each treatment was replicated three times. Seeds of *O. aegyptiaca* were examined with a microscope to look for the emergence of a germ-tube, which indicated that the seeds had germinated. GR24 (synthetic strigolactone) treated disks (20 µL of 0.1 mgL^{-1} GR24 per disk) were used as positive controls and distilled water were used as negative controls.

Hydroponic Experiment: The surface-sterilized maize seeds germinated in the dark on moistened filter paper in Petri dishes for 48 h at 25°C. After germination, 150 seedlings were transferred to a strainer (33 × 26 × 8 cm) lined with a sheet of gauze moistened by placing it in a slightly larger container (42 × 30 × 11 cm) containing 4 L of tap water. The seedlings were placed in a growth chamber with a 12 h photoperiod at 120 µmol photons m^{-2} s^{-1} at 25°C. The tap water medium was replaced every 2 d. On Day 10, the tap water was replaced with half-strength Tadano and Tanaka (TT) medium (PH 6.0) (Tadano and Tanaka, 1980). The TT medium was replaced every two d. On Day 14, the TT medium was replaced with tap water containing 10^{-3} M $CaCl_2$. The tap water + $CaCl_2$ medium was circulated through an activated charcoal filter with an aquarium pump. The maize was grown on this medium for 1 wk and the charcoal filter was replaced every 2 d. Root exudates adsorbed onto the activated charcoal filters were eluted with acetone. The acetone was removed by vacuum evaporation in a rotary evaporator at 40°C. The residue was dissolved in 50 mL distilled water, and then extracted three times with 50 mL ethyl acetate (EtOAc). The EtOAc extracts were combined, dried over anhydrous Na_2SO4, and then evaporated to dryness under vacuum at 40°C using a rotary evaporator. The residues were dissolved in 5 mL acetone and then stored in sealed glass vials at 4°C.

Assay with root exudates: The stored root exudates were diluted with distilled water to final concentrations of 100, 10, and 1 mg L^{-1}. These samples referred to as the aqueous test solutions. Aliquots (20 µL) of the aqueous test solutions were applied to 7 mm disks of glass fiber filter paper in Petri dishes and allowed to dry. A 7 mm disk with *O. aegyptiaca* seeds was placed on top of each disk and then moistened with 40 µL of distilled water. Each test solution had three replications. The treated seeds were incubated in the dark at 25°C for 10 d and then examined the germination rate. The conditioned *O. aegyptiaca* seeds treated by GR24 (10^{-4} M) and distilled water were used as positive and negative control, respectively. Maize root and shoot were collected after cultured hydroponically for 25 d. The samples were freeze-dried, and then milled to pass through a 0.35 mm sieve. 1 mL methanol were added to 1.5 mL centrifuge tubes containing 100 mg of the milled samples. The samples were ultrasonic treated for 30 min and then centrifuged at 6400 rpm for 2 min by a centrifugator (Millipore Cat. No. XX42 CF0, 60 Lot No. N8JMB042A, Nihon Millipore LTD. Yonezawa, Japan). The supernatants are hereafter referred to as the undiluted extracts. These solutions were diluted 10- and 100-fold for use in *O. aegyptiaca* seed germination tests as described above.

Pot Experiment: A pot experiment was conducted at the Institute of Soil and Water Conservation, Yangling, Shaanxi, China in April 2012. Eight kg soil was put into plastic pots (25 cm high × 20 cm diam.). The soil, collected from a cultivated field near the research institute, is silly loam. The soil tests gave the following mean values: soil pH was 7.98, organic matter content was 13.97 g kg^{-1}; NO_3-N was 48.3 mg kg^{-1}; Olsen P was 24.2 mg kg^{-1}; and ammonium acetate extractable K was 166.1 mg kg^{-1}. Ten maize seeds were sown per pot and then thinned to four uniformly sized seedlings per pot after germination. Each maize line had three replications. Experiments 1 and 2 indicated that Changcheng 799 and Zhengdan 958 induced highest *O. aegyptiaca* germination, while Luyu 13 and Zhengyu 203 induced lowest germination rate. Therefore, these maize varieties were used in this experiment to study their induction stability on *O. aegyptiaca*. Samples of the rhizosphere soil and plants were collected at the four-, six-, and eight-leaf stages.

Five grams of rhizosphere soil and 1.5 mL distilled water were added to Petri dishes (3.5 cm diam.). Five disks of glass fiber filter paper (7 mm Whatman GF/A) with 20-50 *O. aegyptiaca* seeds were put on the surface of the soil and the Petri dishes were sealed and incubated at 25°C for 10 days. The germination rates of *O. aegyptiaca* were determined microscopically. Root and shoot extracts were assayed the ability of inducing *O. aegyptiaca* germination as the hydroponic experiment.

Statistical Analyses: The SPSS 10.0 software was used to perform one-way analysis of variance. Treatment means were compared using least significant difference tests at the 5% level of probability. The significant differences in all figures are marked by the small letters respectively.

RESULTS

The mean germination rate of *O. aegyptiaca* induced by GR24 was 81.9%. Distilled water induced no significant germination. So, the *O. aegyptiaca* seeds were viable in our studies.

Cut-root Assay: Maize varieties could induce *O. aegyptiaca* to germinate except Luyu13 and there were significant differences among germination rates induced by the maize varieties.

Germination rates of *O. aegyptiaca* ranged from 0 to 40% (Fig. 1), and it showed an increasing trend as the distance between the *O. aegyptiaca* seed and the maize root segment increased. Among the maize varieties, Changcheng799 generally induced the highest germination rates (12.7 to 40.6%) followed by Zhengdan958 (16.9 to 30.8%). Changdan48 and Zhengda12 also induce higher germination rates (16.5 to 27.2%, 8.2 to 29.4%). Zhengyu203 induced lower germination rates of less than 10%. Luyu 13 could not induce germination rate of *O. aegyptiaca*. Other maize varieties induce germination rates of 10 to 20%.

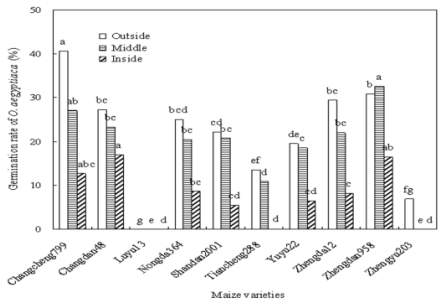

Fig. 1. Germination rates of *Orobanche aegyptiaca* seeds which placed in concentric circles 1 cm (inside), 2 cm (middle), and 3 cm (outside) from root segments of maize seedlings in the cut-root assay.

Hydroponic Experiment: The root exudates of ten maize varieties could induce *O. aegyptiaca* to germinate, and there were significant differences among the germination rates induced by the maize varieties (Table 1). At the concentration of 100 mg L^{-1}, Changcheng799 induced the highest germination rate (41.4%) followed by Zhengdan958 (29.9%), Yuyu22 also induced higher germination rate of 29.6%, and there were no significant differences between them. The germination rates of *O. aegyptiaca* induced by Zhengyu203 and Luyu13 were 11.3% and 13.3%, respectively. Germination rates induced by other maize varieties were about 20%. At the concentration of 10 mg L^{-1} and 1 mg L^{-1}, Changcheng799 induced the highest germination rates (21.8% and 5.3%). Luyu13 induced the lowest germination rates (< 10%) (Table 1). Generally speaking, the germination rates decreased as the concentration of root exudates decreased, it showed that the activity of root exudates gradually decreased.

Table 1 Germination rate of *O. aegyptiaca* induced by maize root exudates (%) in the hydroponic experiment.[1]

Maize varieties	100mg L^{-1}	10 mg L^{-1}	1mg L^{-1}
Changcheng799	41.4 b	21.8 a	5.3 a
Changdan48	22.5 cde	8.6 bc	0.0 c
Luyu13	13.3 ef	3.0 bc	1.1 bc
Nongda364	17.3 ef	4.8 bc	1.2 ab
Shandan2001	28.7 cd	21.7 a	3.5 abc
Tiancheng288	18.0 def	7.7 bc	0.0 c
Yuyu22	29.6 c	4.7 bc	0.0 c
Zhengda12	14.3 ef	8.6 bc	3.2 abc
Zhengdan958	29.9 c	10.6 b	2.9 abc
Zhengyu203	11.3 f	10.2 b	4.2 ab
Distilled water	0.0 g	0.0 c	0.0 c
GR24	81.9 a	—	—

[1]The small letters after the values indicate the significant difference at the 0.05 level，and the same letters imply no difference between them.

The methanol extracts of maize root and shoot under hydroponic condition could also induce *O. aegyptiaca* germination (Fig. 2). The 100-fold dilutions of the shoot and root extracts induced higher germination rates than the 10-fold dilutions. The methanol shoot extracts of Changcheng799 induced the highest germination rate (23.0%) followed by Zhengdan958 (20.6%). Zhengda12 and Tiancheng288 induced similar germination rate (18.2% and 18.1%). Zhengyu203 and Luyu13 induced lower germination rates (8.3% and 10%) (Fig. 2A). The methanol root extracts of Changcheng799 induced the highest germination rate (32.6%) followed by Zhengdan958 (31.3%). Zhengyu203 and Luyu13 also induced the lowest germination rates (13.6% and14.8%) (Fig. 2B).

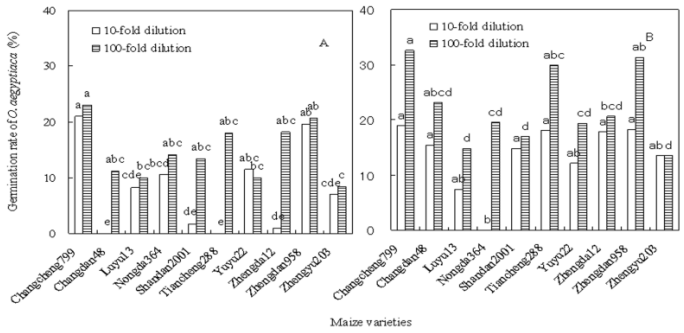

Fig. 2. Effect of root and shoot methanol extracts from ten maize varieties on *Orobanche aegyptiaca* germination in the hydroponic experiment. A and B mean methanol extracts from maize shoot and root respectively.

Pot Experiment: Results from Experiments 1 and 2, indicated that Changcheng799 and Zhengdan958 induced the highest *O. aegyptiaca* germination rates, while Zhengyu203 and Luyu13 induced the lowest germination rates among the maize varieties in this study. Therefore, they were used in the pot experiment. Rhizosphere soil collected at all three growth stages induced *O. aegyptiaca* germination. Changcheng799 and Zhengdan958 induced higher germination rate than Zhengyu203 and Luyu13 at all three growth stages, and there were significant differences (Fig. 3).

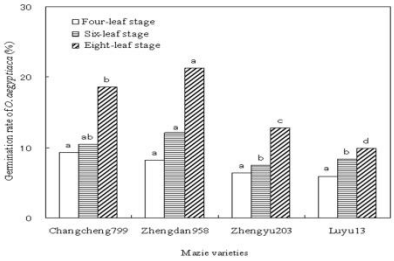

Fig. 3. Effect of rhizosphere soil from four maize varieties on *Orobanche aegyptiaca* germination in the pot experiment. The rhizosphere soil was collected at the four-, six-, and eight-leaf stages.

The ability of methanol shoot extracts to induce germination tended to increase as the plants matured (Fig. 4A). Changcheng799 induced the highest germination rate, while Zhengyu203 and Luyu13 induced the lowest germination rates (Fig. 4A).The ability of methanol root extracts to induce germination first increased and then decreased (Fig. 4B). At the six-leaf stage, root extracts of Changcheng799 induced the highest germination rate (52.6%), followed by Zhengdan958 (41.6%). Zhengyu203 and Luyu13 induced the lowest germination rates (about 20%) (Fig. 4B).

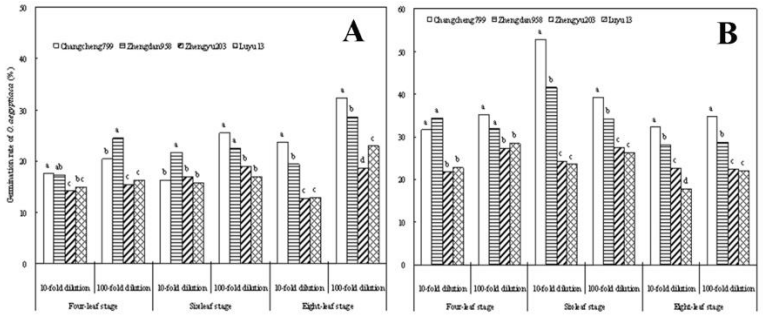

Fig. 4. Effect of shoot and root methanol extracts from four maize varieties on *O. aegyptiaca* germination in the pot experiment. A and B mean methanol extracts from maize shoot and root respectively.

DISCUSSION

Results from the three experiments indicated that maize can induce seeds of *O. aegyptiaca* to germinate, and there existed significant differences among the maize varieties. Some researchers have identified two kinds of strigolactones, strigol and 5-deoxy-strigol in the root exudates of maize, which are potent germination stimulants for *Orobanche* (Awad et al., 2006; Siame et al., 1993). So, it was reasonable that maize induced the germination not only *O. aegyptiaca*, but also *O. minor*, *O. ramosa* and *O. cumana* (Ma et al., 2013; Sunderland, 1960b; Zehhar et al., 2003). Our study displayed that two maize varieties were able to induce high germination of *O. aegyptiaca* seeds and two maize varieties that induced low germination through cut-root and hydroponic experiments, and each of the four maize varieties showed the same trend with the former experiments in the pot experiment. It showed that there existed differences among the germination rates induced by different maize verities, and the trend won't be changed as the condition varied.

All the ten maize varieties could induce the germination of *O. aegyptiaca* in the cut-root assay; this showed that maize had produced germination stimulants at the early growth stage. It has reported that maize produced more than three kinds of germination stimulants after growing for 5 days and had the allelopathic potential for cress (*Lepidium sativum* L.) (Kato-Noguchi et al., 2000).

We found that maize induced high germination rates of *O. aegyptiaca* seeds in the hydroponic experiment, and the germination rates decreased as the concentration of root exudates decreased. Yonayama et al. reported that low P concentrations in the 1/2 TT culture medium could induce red clover seedlings released more orobanchol, and they also found that low P and N concentrations in the 1/2 TT culture medium during the early growth of sorghum seedlings may promote the release of 5-deoxystrigol (Yoneyama et al., 2007a; Yoneyama et al., 2007b). So we predict that maize may release more germination stimulants through adjusting the ratio of elements or dealing with nutrient deficiency in the culture medium.

Changcheng799 and Zhengdan958 induced high germination of *O. aegyptiaca* seeds, while Luyu13 and Zhengyu203 induced low germination of *O. aegyptiaca* seeds in the cut-root and hydroponic experiments. So, these four maize varieties were selected in the pot experiment, and the results of pot experiment were consistent with that of the cut-root and hydroponic experiments, the former two maize varieties also showed high germination stimulation. This could be as a basis for further extending test in the field.

We observed that root extracts generally induced higher germination rates than shoot extracts, it coincides with the report that strigolactones were mainly synthesized in roots and transported to shoots, and we have received the same result in our resent paper (Kohlen et al., 2011; Ma et al., 2013). We got that some extracts at 100-fold dilution induced higher germination rates than 10-fold dilution; it showed that the concentration of germination stimulants in maize were high or had high activity.

In our preliminary test, *O. aegyptiaca* could not parasitize on maize. This study proved Changcheng799 had the strongest stimulation on *O. aegyptiaca*. Therefore, using it as "trap cop" is feasible.

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WORKSHOP

Sunflower broomrape research in the private sector

Towards sustainable development solution in broomrape management

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ABSTRACT

Nowadays it is not enough for seed companies to focusing only on breeding new varieties with good resistance for broomrape. The broomrape races evaluate rapidly with increasing aggressiveness, gaining surface across Europe. In this context it is necessary to be revolutionary if we want to win against this parasite. The main pillars of development durable solution:

Monitoring: Drawing the map of distribution of *Orobanche* races based on systematic collections of broomrape samples across Europe is enabling us to create customer specific recommendation. Molecular characterization of samples is a forward thinking approach what we integrated in the identification of newly emerging races. Our collection is feeding the largest private collection of Orobanche samples - "Orobanchoteque"[1] founded and preserved in Biogemma and serve the comprehension of regional evolution of races.

Breeding for resistance: Find new genetic resources is a key priority of Limagrain. This cannot be boosted without fundamentally different approach. The first innovation in this area is a high throughput screening platform[2] developed and patented by Biogemma. This permits to screen reliably 55,000 individual plants per year. The platform is serving our breeding since 2012 and is allowing particularly promising progress in exploring genetic diversities of resistance against Orobanche.

Resistance mechanism: Go beyond understanding plant-pathogen interaction is as important for us. The patented platform is used also to dissect and in-depth analysis of genetic determinism of resistance.
These activities allow us to collaborate widely with private and public partners and convert the advance of research into sustainable solution.

Limagrain launched **SUNEO®** brand in 2014. This solution integrates Limagrain's best sunflower genetics, an innovative screening platform for Orobanche resistance and the Clearfield® system.

Key words: Sustainable solution – SUNEO

Limagrain is an international agricultural co-operative group, specialized in field seeds, vegetable seeds and cereal products. Founded and managed by French farmers, the Group is the largest European seed company and 4th largest seed company in the world. For further information: www.lgseeds-europe.com

SUNEO® is a registered trademark of Limagrain Europe.

Biogemma is a leading biotech company in Europe involved in Genomics applied to Field crops. Result of the merger of the Limagrain biotech activity with two other major seed business companies, with the help of two financial growers' institutions. The company is developing R&D programs with its partners, in field crops (Corn, Wheat, Sunflower and Oilseed rape), focused on yield improvement, biotic and abiotic stresses and specialty grain compounds. For further information: www.biogemma.com

1) "Orobanchoteque": founded by Biogemma in 2011 with the support of FSRSO
2) Orobanche screening platform is a technology protected by patent application EP 13306587.0

Syngenta's integrated sunflowers broomrape management program

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ABSTRACT

Orobanche cumana Wallr. causes severe damage to sunflowers in many countries in Europe and Asia, is presently expanding and developing new virulent races. Genetic resistance has been very successful control method in sunflowers since early XX century. However, every time a new source of resistance has been introduced, the broomrape has developed new virulent races overcoming the resistance. Although genetic resistance will continue being a fundamental pillar for the sunflower broomrape control, the main obstacle in using resistant varieties is their fast break down and the appearance of new virulent races of the parasite. We recommend to keep new genetic resistances, such as resistance to race F, to be used only when the previous resistance to race E is not useful anymore, in stead of using the new genetic resistance to prevent the spread of a new race F, because it has been demonstrated, that the introduction of such resistance in a heavely broomrape infected field would select new races capable to overcome the new F resintant gene. Syngenta first introduced herbicide tolerant sunflower hybrids in Turkey and Spain in 2003. This hybrids allowed the treament with imidazolinone herbicides (The CLEARFIELD® production system). This system offered an excellent opportunity for the post-emergence herbicide control of the parasitic weed. This 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. Different crop management measures may help to the ultimate goals of: reduction of existing seed banks, prevention of further seed production and avoiding seed dissemination. Thus, an integrated control program is the best way to preserve sunflower production. Inspired on the experience gained in some eradication or control programs, such as the "Emergency plan for broomrape control in Israel" and "The branched broomrape eradication program in Australia, Syngenta, has adopted sustainable broomrape management program. Syngenta has built a broomrape excellence center in 2013, in Stein, one of the Syngenta research centers, to develop a long term sustainable solution to control broomrape in sunflowers reinforcing our leadership position in this crop. The main aims for this center are to improve our screening capacity to support our breeding efforts; to study in depth the host-parasite relationship and to develop new control solutions, including genetic, chemical or a combination of both. New control approaches will also be included.

1. Introduction

The genus *Orobanche*, recently divided into *Orobanche* and *Phelipanche*, has more than 100 species of holo-parasitic weeds that infect the root of many crops of economic importance in the Mediterranean regions, Eastern Europe, CIS and several countries around the world. Six species are regarded as either widespread or acute agricultural problems. ***O. crenata* Forsk.** including *O. speciosa* D.C., ***O. cumana* Wallr.**; ***O. cernua* Loefl.; *P. ramosa* (L.) Pomel,** (Syn. *O. ramosa* L., including *O. nana* Noe and *O. mutely* Schultz); ***P. aegyptiaca* (Pers.) Pomel**. (Syn. *O aegyptiaca* Pers.); and ***O. minor* Sm**. A few further species occur locally and many other species infect wild hosts. Yield losses are routinely about 50% and can be up to 100%. Sunflowers broomrape (*O. cumana)* is present in most sunflower production areas in Europe and Asia, creating concern due to its expansion and the development of new virulent races in all of them.

Broomrape species are difficult to diagnose and control because the parasites have a long underground development stage and by the time they emerge, much of the damage is already been produced. Source of broomrape resistance are scarce or even absent in the majority of susceptible crops and there are few selective herbicides to control it because as holoparasite, it takes water and metabolites only from the host plant. Thus, only systemic herbicides have a chance to enter in the parasitic pant. Furthermore, herbicides that interfere with photosynthesis do not affect broomrape as this parasitic plant does not have chlorophyll.

The long term impact of the broomrapes can be very serious: their seed may persist in soil for decades and their spread is facilitated by man, agricultural tools, planting seeds and animals leading to an accelerated

increase in the infested areas. In some countries there have been programs for the eradication or control broomrape such as the "Emergency plan for broomrape control in Israel" and "The branched broomrape eradication program in Australia" (Joel M., and Manor H. 2005; Warren Philip, 2005)

The broomrape problem is Israel is important due to the presence of five different species with highly diverse populations that affect to several host crops and having all country regions infested with at least one broomrape species. Broomrape is regarded as the most damaging pest in vegetables and field crops. The emergency plan was set as response to the appearance of new virulent races of several broomrape species, (*O.cumana, P. aegyptiaca* and *O. crenata*) increasing the risk for industries such as tomato and sunflowers. Thus the awareness of the farmers and the government to the problem lead to the nomination of special steering committee to develop comprehensive scheme to gradually reduce the damage caused by this parasite in Israel.

The national branched broomrape program in Australia started soon after a small area was discovered in 1992 infected by branched broomrape in South Australia. The eradication program was State funded using methyl bromide, as soil fumigant, to destroy broomrape soil seed bank in the infected area. When it was found that the area affected was more widely distributed that first thought the National program was established in 1999 and the eradication program adopted in 2001/02. Eradication remained the optimum strategy objective for years. But in 2011 the Australian Branched Broomrape National Management Group agreed that it was no longer technically feasible to eradicate the parasitic weed and the eradication program was wounded up by end 2011. Despite the failure of the Australian branched broomrape eradication program, this has provided a series of principles to manage the risks and to minimize the impact of broomrape infection applicable to sunflowers.

Syngenta, world sunflower leader, is working to provide integrated solutions for sunflower broomrape problem that is the largest threat for the crop in Europe and CIS. Recently Syngenta has created a broomrape excellence center in Stein (Switzerland) to seek for new solutions to fight against this parasitic weed. At the same time, Syngenta is setting a sustainable broomrape management program in all the countries where this broomrape represents a threat to the sunflower crop with similar goals as the emergency plan for broomrape control in Israel and adapting to sunflowers some of the leanings from Australian branched broomrape eradication program.

There are some important differences between the situation of sunflower broomrape and the above mentioned programs in Australia and Israel. These differences make us to be optimistic in forecasting reasonable good control of the problem in sunflowers if an integrated approach is applied. On one side, *O. cumana*, is very specific to sunflowers, while some of the broomrape species present in the Australian and Israel programs are capable to infect a large range of host including cultivated and wild species. On the other hand, sunflowers has been the exception among crops in regards to the development of new genetic resistant cultivars which have allowed the crop to continue expansion despite 3 temporary resistance crisis in the history. Finally, herbicide control of *O. cumana* is possible in Clearfield resistant cultivars using imidazolinone herbicides.

2. The control measures of *Orobanche cumana* in sunflowers, the pillars for integrated control

2.1. Pillar 1. Genetic resistance

The use of resistant varieties/hybrids to broomrape has been the preferred control system of the parasitic weed in sunflowers. (Alonso, 1998).

2.1.1. Russia, Ukraine and Moldova

The rapid expansion of the sunflowers in Russia by the end of the XIX century was threatened by the expansion of the broomrape. Thus the selection of resistant sunflowers to *O. cumana* has been a constant objective for breeding since Pustovoit began to lead breeding program in 1910 (Pustovoit, 1966). Selections were initially collected from farmer's fields in Saratov region, and were initially based on field trials, and since 1921, also on pot test (Pustovoit, 1973). A range of sunflower populations (Kruglik A41, Saratovskii 169, Fuxinka 3, Zelenka 10 and Tchernyanka 35) were developed with complete resistance by 1916. By 1925 95% of the crop grown in the region was based on resistant varieties. In 1925 susceptibility of these varieties to *O. cumana* was reported from Ukraine and Moldova. Field trials in Krasnodar using broomrape from these regions showed that there was a variation in the parasite virulence. Thus two races were designated to describe these pathotypes as A and B, respectively. By mid 1930s, race B had spread across all the sunflower growing areas in the former USSR and threatened to stop sunflower production (Pustovoit 1973). Intense efforts were made to locate resistance to the new race and it was found among landrace sunflowers collected from farmer's field in Ukraine. Sunflower selection in Don conducted since 1924 by academician L.I. Jdanov developed sunflower varieties tolerant to broomrape. During 1934/35 the new cultivars Jdanov 8281 and Jdanov 8885 revealed resistance to both races A and B. Short after, new resistant varieties such as Armavirsky 762; Zelenka 61; Fuxinka 62, VNIIMK 1646, VNIIMK 6540, VNIIMK 8880, VNIIMK 8931, Armavirsky 3494, Armavirsky 9343, Smena, Peredovick etc., were released and cultivated in large areas of production. After the spread of these varieties across different countries new races of *O. cumana* have been identified in different places. Breeders from different countries have followed the nomenclature started by soviet breeders. Nevertheless, the complexity of this pathogen suggest that what is known as races C, D, E, F, etc., in different countries may be different races from country to country.

In mid 1960s, a new race, named C, with virulence to all resistant varieties was identified in Moldava and Ukraine. The varieties resistant to race C, Odesski 63 and Start were developed in 1970s (Buchuchanu and Karadzhova, 1984). In 1990 a new race, D, was identified in Krasnodar region. None of the resistance available at that time was able to control this virulent new race (Antonova, 1994). This Russian race D was probably closer to other places race F than the named race D from Romania (Antonova per.com.).

With the expansion of modern hybrids in Russia and Ukraine and the reduction of the rotation period, there has been a buildup of new virulent races in several regions. In a survey made in 2013 by VNIIMK institute they collected several new races in different regions of Russia naming them F, G and H. The correspondence of these with other country races is not known

2.1.2. Romania

The breeding work carried in Fundulea institute in Romania has set a "before and after" in the fight against this parasitic weed and has served as foundation of some of "right or wrong" present ideas. The above mentioned USSR resistant cultivars to races A and B were resistant to broomrape infection in Romania until mid-1960s except in the southeastern regions near Moldava. The good broomrape control during many years was probably consequence of good resistance of the varieties and the imposed long term rotation having sunflowers only once out of six years. A total of five races were identified using a set of Romanian and former USSR sunflower varieties as differential series. Races D and E were characterized for the first time and they were predominant at the coastal Black Sea and north-eastern region. The variety Record was the source of resistance to the Romanian race C. The resistance to the Romanian race D was found in the variety S 1358 and the source of resistance to race E was found in the inbred line P 1380. This line has been has been considered the universal source of resistance until recently. Studies on the sunflower/*O. cumana* association by Vrânceanu *et al.* (1980) indicated that it fitted the gene-for-gene model. Using a set of differential lines, they found that resistance to broomrape races A to E was determined by dominant alleles at single genes named *Or1* through *Or5 r*espectively. *Or5* gene conferring

resistance to race E, but also to all previous races, has been reported to have a dominant gene plus some quantitative components. In as QTL study Peréz-Vich *et al.* (2004) determined that resistance to this race was the result of the major gene Or5 in combination with a quantitative component for which at least four QTL were identified. Such QTL had a minor effect, in some cases non-race specific and determined mainly by the number of broomrape shoots per plant. The fact that each new differential line in Vrânceanu *et al.*, (1980) study controlled all previous broomrape races created the sense that any new resistant gene controlling new races will also control all the previous races when this is not the case in most "gene for gene" relations between host and pathogens or parasites. It could be breeders were pyramid resistant genes in new cultivars by introducing new genes to already breed cultivars that carried previous genes. Inbreeding selection to create hybrids broke the pyramid breeding of broomrape resistant genes in open pollinated cultivars. Furthermore, hybrids often carry the resistant gene in heterozygous and this have shown higher infection rate than in homozygous condition (Alonso *et al*., 1996).

After the spread in 1990s of modern hybrids carrying *Or5*, new virulent races, named F, have spread across the southeastern part of Romania. Commercial hybrids carrying resistance to race F have been released during the last decade and the development of new races capable to infect these resistant to race F hybrids has followed in recent years. Presently, more than 60% of the sunflower area planted in Romania is infected with broomrape and races A, B, C, D, F, G and H have been found with different levels of presence in different parts of the country (Fierbinteanu and Dinca 2013**).** The use of a set of differential cultivars including varieties, inbred lines and hybrids is creating confusion among farmers and broomrape experts with no agreement about the name of new races, as there is no an accepted set of differentials. This situation is repeated in all the broomrape infected areas.

2.1.3 Bulgaria, Turkey and Spain

As in many areas of East Russia, these countries share a very short rotation of sunflowers only with cereals (wheat or barley). In some cases this rotation includes only wheat and sunflowers in alternate years.

In Bulgaria sunflower broomrape was first recorded in 1935 (Dovrev, 1945, in Entcheva and Shindrova, 1994) in the northern part of the country. In the mid-1940s it became a serious threat to sunflowers production. Races A and B were characterized in 1950s using differential varieties from former USSR. Varieties Jdanov 8281 and 6432 were introduced in 1945 and Peredovick in 1963. In this year a new race, C, was found (Petrov, 1970) after massive attacks on the variety Peredovick observed in North Bulgaria. A study was done in order to establish the race composition with the same differential variety set used in Romania. Thus, 5 races were also identified in Bulgaria and hybrids carrying Or5 controlled all of them and were planted in most of the sunflower area in Bulgaria (Entcheva and Shindrova, 1994).

Present situation in Bulgaria, according to a recent survey (Shindrova, and Penchev, 2012), indicate that 4 races are present in the country: E, F, G and H. Race E gradually decreased it percentage in the population but still predominant in north-east Bulgaria while becoming equal with race G in the central north Bulgaria. In the south-east the population included only race G which is expected to increase its percentage in other regions in upcoming years. Race F was sporadic occurrence and the population identified as race H was isolated only in 2007 but may be considered a primary source of infection that will be expanding in the years to come.

In Turkey, large sunflower losses were reported over the period 1956-62. This lead to a drop in the sunflower planted area until former USRR varieties Jdanov 8281 and VNIIMK 8931 were successfully introduced. In 1980 a new broomrape race o races threatened once again the crop. Different Romanian and USSR varieties were tested and it was concluded that a race E similar to the Romanian one, was present in Turkey. This was effectively controlled by hybrids carrying *Or5* (Bulbul *et al.*, 1991). In 1998, a new race (F) was found able to infect all the hybrids carrying *Or5* and this race is presently widely spread in all the

country. Resistant hybrids to this race F were released in the 2000s and new virulent races are appearing in the country.

In Spain, broomrape was first recorded in confectionary sunflowers in central part of Spain in 1958 (Diaz-Celayeta, 1974). Further records of this parasite were provided by Jimenez-Diaz and Sackston (1977). Only trace incidences were observed in the oilseed cultivar Peredovick. Surveys carried out in 1978 and 1979 (Gonzalez-Torres *et al*., 1982) showed the presence of the parasitic plant in 25,9% of the fields in Cuenca province. This was the first report of race other than A and B was present in Spain. After 1992, the very heavy infections of formerly resistant hybrids, in many areas of Andalucía and Cuenca, suggested that races similar to Romanian races D and E had spread to sunflower growing areas in Spain. The Romanian line P 1380 showed resistance to all races and several resistant hybrids carrying *Or5* were released during 1993 and 1994. In 1995 a field near Ecija showed heavy broomrape infections in a hybrid carrying *Or5*. A new race, F was confirmed in laboratory test (Alonso, *et al*., 1996). The quick spread of race F in southern Spain (Fernández-Escobar *et al.,* 2008) incentivized the competition among breeding programs to introduce new resistant hybrids and several were introduced from 2002 to 2006 and quickly adopted by farmers. In 2008, we collected a new virulent race near Carmona capable to infect all the race F resistant hybrids (Table 1).

2.1.4 Genetic resistant alone may not be enough to control of broomrape in sunflowers

Genetic resistance has been very successful broomrape control method in sunflowers thanks to the continuous discovery of new sources of genetic resistance. However, every time a new source of resistance has been introduced, the parasite has developed new virulent races. This evolution of new virulent races in *O. cumana* is probably the result of the use of sunflower hybrids that are near exclusively based on resistance determined by single race-specific dominant genes (Fernández-Martínez *et al.,* 2008; Molinero-Ruiz *et al.,* 2008). New breeding strategies such as pyramiding of major genes or combining vertical and horizontal resistance mechanisms have been suggested as the way to develop more durable resistant cultivars. But this pyramiding of genes in modern sunflower hybrids is complex and requires huge breeding efforts. The present confusing race situation may be due to different companies putting different resistance genes in the market and to some extend in pairs

The explanation of this quick buildup of new broomrape races may come from the understanding of the population dynamics of broomrape in an infected soils (Román, 2013) and the nature of broomrape seed production and dispersal (Joel, 2013). The population of broomrape seeds present in each sunflower infected field is genetically diverse in regard to virulence genes. Being an obligate parasite, broomrape cannot survive unless infect a host plant. Thus it has a range of mechanism to survive and develop new virulent races. The flowers of some broomrape species have been reported to have the ability to develop seed in three different ways: by cross-pollination, by self-pollination and by apomixis, an asexual cloning reproduction mechanism that allows the development of seed without pollen fertilization. Mating system studies in *O. cumana* (Rodríguez-Ojeda *et al.,* 2013) have demonstrated that this species produced seed by self-pollination as well as cross-pollination, having an average of 28,8% of cross pollination under field conditions. By self-pollination there is a fixation of most successful gene combinations. By cross-pollination genetic recombination may allow the selection of new gene combinations. Genetic recombination between broomrape gene polls has been suggested as an important mechanism for race evolution (Pineda-Martos *et al.,* 2013). Thanks to its reproduction mechanisms broomrape produce seeds of various genetic compositions each generation in each infected field.

The race evolution, once a new resistant hybrid is introduced in broomrape infected field, is probably determined by the constitution of the population of broomrape seed present in each field. Thus, the introduction of resistant hybrids, (i.e., resistant to race F) would screen among the many broomrape seed mutations already present in the field and some would be able to infect. These first infections would pass unnoticed to farmers, but in 4 rotation cycles would multiply to cover the field limiting the growth of

sunflower. The rotation time would determine the number of years the farmer will be able to grow the hybrid. In a 2 year rotation wheat-sunflower schema it takes period from 6 to 8 years. Thus in each broomrape infected field a new race "G" could be selected having in common among them their ability to infect the resistant to race F hybrid. (Fig. 1)

Although genetic resistance will continue being a fundamental pillar for the sunflower broomrape control, the main obstacle in using resistant varieties is their fast break down and the appearance of new virulent races of the parasite. In order to prevent the development of such populations, the use of resistant varieties should always be accompanied with other control methods, which should eliminate seed production and dispersal of individual parasites that are able to bypass the resistant mechanism of any resistant sunflower hybrid.

2.2 Pillar 2. Crop management

Broomrape control difficulties are based on the properties of their seed: Their immense number, small size, extreme longevity and ease dispersal rapid increase the parasite soil seed bank. Containment of infected areas and prevention of seed distribution should therefore be a major objective of parasitic weed management strategies. Progress report of the Australian branched broomrape eradication program reveal that human-mediated dispersal, mainly by machinery, is the most important parasite seed vector.

Broomrape seed bank rapidly increases in subsequent seasons from even a small initial infection when a suitable host plant grows in the field due to the high seed production from each successful infection (Lopez-Granados and García-Torres, 1993). Completely eliminating the parasite seed bank in the soil is practically impossible. For an eradication of *P. ramosa* in California (USA) expensive control program and strict quarantine measures have been applied. Yet, there have been several recurring outbreaks of the infestations (Goldwasser and Rodenburg, 2013).

Different crop management measures may help to the ultimate goals of: (1) reduction of existing seed banks, (2) prevention of further seed production and (3) avoiding seed dissemination. However, some of these measures may have relative little use in extensive crops such as sunflowers. For instance, hand weeding, transplanting and deep crop sowing, may have some success in vegetable crops, but are not practical in large field crops. Soil fumigation with toxic compounds such as metham sodium, methyl bromide (presently ban) and fumigants than release methyl isothiocyanate have been used in broomrape eradication programs but are expensive and in some cases extremely toxic to humans. Initial results on experimental small plots in Israel show that metham sodium, chloropicrin, dimethyl disulfide, methyl iodine and mixtures of these fumigants at reduced rates efficiently control *P. aegyptiaca* but part of the success may be due to the sandy soil conditions and a very precise application procedure. None of these conditions would be present in most commercial sunflower production fields.

Soil solarization, a method of trapping solar radiation in moist soil under a transparent plastic sheet, serve to kill many soil borne insects, fungi and weed seed including *Orobanche* seeds. On top of the high cost per hectare, the system has limited use in heavy clay soils, as the high lethal temperatures cannot be reached below the upper 15-20 cm soil layer and thus it is not useful for deep root crops such as sunflowers.

Flooding of broomrape infested fields causes a decay of parasitic weed seeds, leading to a decrease in infestation. This approach has no use for commercial sunflower production as most of sunflower crop is grown under dry land conditions.

Among the common crop management practices, long crop rotations schemas were successful in allowing sunflower production in formers USSR and East Europe countries. State imposed rotations obligations force farmers to grow sunflowers once every 6 to 9 years. This common agricultural practice together with

the use of resistant sunflower varieties allowed sustainable sunflower production during many years and many countries after the release of the varieties resistant to broomrape races A and B. But there is some inconsistency's in this approach, as only a 6 to 9 year crop rotation, by itself, would not be enough to reduce the broomrape soil seed bank as *Orobanche* seed can live more than 20 years in the soil. There may be other elements or hidden cause other than time in the reduction of broomrape seed bank in 6 to 9 years rotations practices used in USSR period.

Trap crops, (false hosts) are non-host crops than can stimulate parasite germination but further development of the parasite is impeded as no viable connection is established between host root and parasite. Important crops that were reported in reducing broomrape seed banks are sorghum (*Sorghum bicolor*) and maize (*Zea mays*) (Goldwasser and Rodenburg, 2013). Laboratory test have demonstrated that these crops induce *O. cumana* seed germination (Rodríguez-Ojeda, per. com.). Thus, the incorporation of one of these crops in the 6 to 9 year soviet rotation recommendation may be the hidden cause of the observed positive effect of the long crop rotation in broomrape management. However, long rotation only with cereals is unlikely to have the same positive impact.

Non tillage or conservation agriculture has been reported to have a positive impact on the long term prevention and reduction of the broomrape seed bank in the soil. In a long term study that started in 1986 in Cordoba, Spain, to compare rotation and tillage practices, data from 2003 and 2004 demonstrated that broomrape infections, measured as number of broomrape shoots per plant, in broad beans under non tillage practices were 10 times less (3 vs. 32 shoots/plant) than under conventional tillage (Benitez-Vega *et al.*, 2005.) A sunflower study during three consecutive years, growing sunflowers in monoculture and comparing the broomrape infection under conventional and non-tillage practices in three different sunflower cultivars was carried in Spain (García-Ruiz *et al*., 2008) it was shown that the broomrape infections, measured as % of plant infested and number of broomrape shoots per plant, were significantly lower under non tillage than under conventional tillage. The non-tillage effect is reduced if sunflower is repeated. Reanalyzing the published data, we can calculate the number of broomrape shoots per sunflower hectare in the year 2006. Broomrape shoots/Ha were about 6.000/Ha under conventional tillage, but 0/Ha under not tillage in the F resistant hybrid. If we calculate than a broomrape shoot can produce 20/40.000 seeds, under conventional tillage the "resistant" hybrids would have produced from 120-240 million broomrape seed per Ha while none would have been produced with the same hybrid under non-tillage.

No single agronomic management practice will accomplish full broomrape control as stand-alone measure. For effective and durable control, measures need to be combined. The main obstacle in the long term management of infected fields is the near indestructible seed bank. There is an urgent need to implement novel integrated parasitic weed management programs to overcome this obstacle. This should be based on new findings in different control methods and their interaction as well as the use of appropriate monitoring and decision support systems enabling precision agriculture and site-specific farming technologies (Rubiales *et al*., 2009)

2.3. Pillar 3. Chemical control

Chemical control of broomrape with herbicides has been explored since 1970s and the knowledge of broomrape biology may help to understand why most of them do not control it. Weedy holoparasites, such as broomrapes, must be treated during their underground stages of development, because they emerge during flowering, i.e., treatment has to be done before they can cause damage to the host plant. But broomrapes are non-photosynthetic plants and this exclude the use of herbicides that target photosynthesis related processes. Furthermore as broomrape takes water and nutrient only from host plant, only systemic herbicides able to translocate from host to parasitic tissues can have a chance to control *Orobanche*. In order to allow safe control of the parasite, the host plant should be selective to the applied herbicide either by metabolic or by target-site resistance.

Several systemic herbicides have shown broomrape control in vegetable and field crops. Low rates of the aromatic amino acid synthesis inhibitor herbicide glyphosate, applied up to three times, were effective for broomrape control on few hosts that are less susceptible to the herbicide in *Apiaceae* (carrot, celery and parsley) and *Fabaceae* (faba-bean, vetch and pea) (Kasasian, 1973; Jacobson and Kelman, 1980; Arjona-Berral *et. al*., 1984; Foy et al., 1989; Nadal et al., 2008). The successful *Orobanche* control in legumes during the 80s offered a technical solution for certain legume crops but not for sunflowers even at sub-lethal doses. Other herbicides that inhibit branched-chain amino acid synthesis such as imidazolinones followed. Field studies in Southern Spain from 1991 to 1994 determined that imazapyr used in both pre- and late post-emergence application could partially control broomrape. The post-emergence application at sub lethal doses in sunflowers in V10-V11 stage (10 to 11 leaves) controlled up to 85% of broomrape infection without significant damage to the sunflower plant (García Torres *et al*., 1994. García Torres, 1994; García-Torres *et al.,* 1995).The incomplete control of broomrape and the toxicity on sunflowers in case of overlapping during treating limited the use of this herbicide to control broomrape in sunflowers.

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 transferred the resistance to cultivated sunflower. This prospect was very exciting because the list of broadleaf weeds and grasses controlled by herbicides of the imidazolinone chemical family (IMI) was extensive. In 2002, USDA released "two sunflower lines (HA 425 and RHA 426) with tolerance to imazamox. A personal collection of the same wild sunflower previously found by Al-Khatib was made in Kansas in 2007 from Mr. Doug French farm. Back in Spain, we proved that this herbicide tolerance offered an excellent opportunity for the post-emergence chemical control of the parasitic weed *Orobanche cumana* (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.

The CLEARFIELD® Production System for sunflowers in Europe was introduced into different countries since 2003 and Syngenta Seeds was the first seed company marketing Clearfield sunflower hybrids. Since the introduction of the CLEARFIELD® system in several countries, broomrape control has been possible using *Imazamox* (LISTEGO®) herbicide despite the regulatory imposed by label in different countries restrict the window for treatment to one single application at a precise sunflower development stage.

The evolution of herbicide resistance to ALS-inhibiting herbicides is a common phenomenon in weeds (Corbett and Tardif, 2006). But so far, no broomrape has been found with tolerance to the imidazolinone (IMI) herbicides, despite the system has been in place for more than 10 years in several countries. This is something unique and something similar has been observed in imidazolinone-resistant (IR) maize commercialized in Africa to control *Striga*. When IR maize was beginning to be developed for Africa, modelling was performed to predict how quick herbicide resistant would evolve in *Striga* and how quick the technology would be useless. (Glessel 2013). The model suggested that five new resistant *Striga* plants should be expected per cropping season per hectare and the expanding coverage with resistant *Striga* would render the herbicide technology useless in about eight seasons. Surprisingly, no resistant individuals have been reported despite thousands of hectares have been planted with IR maize.

As for the reasons to this long period of useful control of broomrape with the CLEARFIELD® system, we should consider that most of the sunflowers hybrids to which the herbicide tolerance was incorporated already had the broomrape resistant gene OR5. Thus, genetic control of most races via genetic resistance has proven to be an effective method to prevent the loss of the chemical control due to broomrape developing resistance to IMI herbicides. Furthermore, we should consider that the driving force for water flow from host to parasite is the gradient of water potential between them. Broomrapes lower their water potential by accumulating high levels of osmotically active compounds such as mineral ions, sugars and sugar alcohols (Westwood, 2013). This higher water potential may force the concentration of the herbicide to at much higher dose in the broomrape than in the host tissue creating an extra difficulty to develop herbicide resistance.

In order to reduce the risk of the evolution of ASL resistance in the parasitic weed, which has not yet been found despite broomrape formidable mutation capacity, we should consider not using LISTEGO®, CLEARFIELD® herbicide for sunflowers, as the solo control method. Also, the timing for application should allow killing broomrape tubercles before the cause damage to sunflowers.

Sunflower plant has a slow growth during the early stages until 3 to 5 pair of leaves. As the temperature rise in the spring, the sunflower plant starts a very fast growing period. As the number of broomrape infection per host plant depend a lot on the level of broomrape seed bank in the soil, and the genetic resistance of the cultivar planted, there is not a single universal recommendation on when is best to apply the herbicide. However the possibility to customize the recommendation is often limited by regulatory labeling in each country. Thus, split application, to keep a high concentration level of the herbicide in the sunflower plant during the growing period, cannot be recommended in many countries presently using this herbicide in sunflowers.

3. Syngenta´s Sustainable broomrape management program

Sunflowers is the most profitable field crop in many countries usually call " $/cash crop" with extreme continental climate such as Russia and Ukraine or the only break crop for cereals in very dry countries such as Spain and Turkey. Either for one reason or the other, the rotation period between sunflower crops is often very short facilitating the development and spread of broomrape. In order to keep sunflower as a profitable crop a sustainable broomrape management program integrating control methods has to be established.

As in the Israel emergency plant the long term objectives of the program are: Long term reduction of the damage; Prevent the spread of sunflowers broomrape and Gradual de-infestation of the fields. In the case of sunflowers there are some distinct advantages compared with the emergency plant in Israel, or the branched eradication program in Australia. On one side, *Orobanche cumana* is restricted to *Asteraceae* mainly sunflowers, while other species of broomrape have a much larger range of alternative host including wild and cultivated species. On the other hand several genetic resistance genes, available in sunflowers, have allowed so far controlling the broomrape. Finally, the CLEARFIELD® method allows chemical control of *Orobanche* in sunflowers too.

The Syngenta's broomrape management strategy aims to secure the sunflower production on a long term in a sustainable way. Per country we are implementing activities in three main areas: combined control, agronomy practice and field activities and communication. This program is summarized in Fig. 2.

3.1. Combined control

For integrated *Orobanche* managements the use of two or more control methods for managing the parasite in each farmer field is recommended together with agronomical complementary measures. Taking into account the status of control methods available today, the most feasible combination would be the use of resistant hybrids together with the chemical control with the herbicide LISTEGO®**,** CLEARFIELD® herbicide, in herbicide tolerant hybrids.

We do not recommend, to launch new genetic resistant hybrids, such as resistant to race F, "to prevent" the spread of this race, because it has been demonstrated, that the introduction of such resistance in a heavely broomrape infected field would select new races capable to overcome the new resintant gene. Part of the confusing race situation in different countries may be caused by this approach often recommended by commercial firms. We recommend to keep the new resistance to be used when resistance to race E is not valid anymore and launch it better together with herbicide tolerance The same is advisable with any new source of resistance that may be incorporated in the future. It is probably more complex from hybrid seed prodaction management point of view but it is more efficient for longer term broomrape control.

The main obstacle in using resistant hybrids is their fast break down and the appearance of new virulent races of *Orobanche cumana.* In order to prevent the formation of such races, the use of resistant hybrids should be accompanied with other control methods, which should eliminate seed production and dispersal by individual parasites that bypass the resistant mechanism of the resistant hybrid. This can be achieved with the application of the LISTEGO® herbicide.

The use alone of the herbicide to control broomrape is not advisable either, because a non-resistant sunflower hybrid would be infected by any broomrape race seed present in the field increasing the chance for the evolution of ASL resistance in the parasitic weed. Furthermore, if a sunflower plant gets infected by several hundred of broomrape seeds, it may die before the herbicide is applied.

New solutions that may arrise from creative research in our Stein broomrape excellence centre, inagurated in 2013, or by other scientist, may be incorporated to integrated control in the future as they will be developped.

3.2. Agronomy & Field activities

Syngenta has in each country a net of demand creators and field technical experts to advise farmers on the use of different crop protection and seed technologies. This net will deliver extension services to help famers to choose the best crop management technologies to delay o reduce the broomrape seed bank. The Syngenta's CONTIVO program, launched in 2013 as a pilot in Hungary and to be extended to other countries, to promote sustainable agricultural practices such as non-tillage may play an important role in limiting and reducing the broomrape impact.

The ultimate goal is to provide farmers with a customized service to manage broomrape including creating database and mapping and developing a diagnosis service with laboratory tests. The prevention of the spread of broomrape to new areas should be a priority in most countries where the parasitic weed still do not cover the entire geography as in Ukraine, Russia, Romania, Spain, Hungary, France and other countries. Also the best control practices should be applied in infected farms. A summary of recommendations is presented in Table 2.

3.3. Communication

Educating farmers to manage properly broomrape include the develop information and education programs per country. This is a continuous process that requires printed documents, on line information, farmers meetings and personal visits as described above.

One of the most important ways for the broomrape expansion in CIS, is the recent development of a parallel market of counterfait seed. In this, F_2 sunflower harvest is bagged using the bags, tags, and even seed color of the most cultivated hybrids in the country. The counterfait seed is offered with large discount to farmers and the dimension of this fraud has reached several millons dollars in the last years, affecting all leading hybrids irrespective of the seed producer. However, the collection of sunflower F2 seed in broomrape contaminated fields, carries the parasitic weed seed attached to the coat of sunflower seed. Furthermore, in F2 hybrids the genetic resistance genes are segregating and many seedlings are not resistant, creating a new opportunty for the parasite to multiply once introduced. The reduction of this farm saved conterfeit seed is a first goal in our integrated program through education of farmers, local authorities, and stakeholders such as crushing industry, as they will be the first one to loose if sunflowers can not be grown in the area where the factory gets its supply.

4. Stein Broomrape Excellence Centre

The Syngenta's Stein broomrape excellence center was built in 2013 to develop a long term sustainable solution to control broomrape in sunflowers reinforcing our leadership position in this crop. Although the most effective way to control broomrape in sunflowers so far has been through resistant cultivars, this does not guarantee future success in finding new resistance genes. Integrated solutions must rely on different strategies which involve combined ones for durable solutions.

The main objectives for the center are:

- Screen various broomrape races under quarantine conditions to monitor apparition of new *O. cumana* races in different countries.
- Preservation of a broad broomrape bank of different races existing in the world and subsequent phenotypic and genotypic characterization.
- Perform in-depth studies of the host-parasite relationship and provide technology support to quality control
- Develop new control solutions, including genetic, chemical or a combination of both. This would include exploring new control approaches.
- Knowledge and solutions initially developed for sunflowers may be extended to other crops and species of broomrape

The broomrape excellence center will not be alone. It is integrated in our broomrape network that includes: Classical breeding activities including the search and introgression of new resistant genes from wild *Helianthus* species; Molecular Market asistant breeding, Broomrape field testing and screening and research centers activities in Stein (Switzerland) and Jealott's Hill in UK.
The Stein center is one of the major research centers in Syngenta, a world leading agribusiness which was formed in November 2000 bringing together the industry leading skills of Novartis agrobusiness and Zeneca agrochemicals. The modern facilities include custom-designed buildings, 4.200 m2 greenhouse space, 70 walk-in growth chambers and 1.800 m2 of laboratories. Greenhouses and growth chambers are dynamically controlled to ensure that conditions exactly meet the individual needs of the insect pest and fungal plant diseases. Widely recognized as a leading center for biological research, Stein is part of Syngenta international research and developmental network. Thus, the ultimate aim of Syngenta is to find novel integrated solutions which are safe, effective and form part of sustainable farming systems. Over 100 PhD scientists at Stein play an important role in developing products and solutions that fit into sustaianble integrated crop management practices. Stein is our center for crop protection research biology in fungicides, insecticides, profession product, including seed care and since 2013 broomrape control.

Syngenta chemists synthesize thousands of new chemicals every year for testing as potential fungicides, insecticides and herbicides. Starting point for synthesis is identified using a variety of state-of-the-art approaches. Using a combination of sophisticated and inventive design techniques, the aim of our scientist is to modify the structures of chemicals in order to bring about improvements in their biological, physical and environmental properties. Initially, large numbers of chemicals are prepared in small quantities in the laboratory, either by hand or by automated methods, and are subsequently tested in the glasshouse. Field evaluation is concentrated in products considered of greater interest.

Syngenta collaborates with many other private and public research institutions across the world in different research areas. The Stein broomrape excellence center has been born with the same collaborative intent. Every year, we conduct a series of internal project evaluations to choose those that deserve financial support for the development of new solutions. Broomrape, as the main threat for sunflowers, is now in our priority list and the new center offers a unique opportunity to work in a range of different solutions. We would like to invite the creative broomrape scientist around the world to let us know about their projects and actively seek.

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TABLES AND FIGURES

Table 1. Recent *O. cumana* evolution in Spain.

Race	1st Detection	Fields with Comm. Damaged	Introduction of R. hybrids
E	1988	1992	1993-1994
F	1995	1999	2002-2006
New	2008	2013	

Table 2. Agronomic Basic Principles for Sunflower Broomrape management

1º Prevent introduction into broomrape free fields	**2º Avoid dispersion & evolution, reduce soil broomrape seed bank**
I. **Prophylactic** • Use certified seeds only • Clean farm machinery, harvest first the healthy fields II. Avoid livestock moving from infected fields III. **Rotational practices** • Maintain diversified crop rotation • Include trap crops IV. **Preventive measures** • Use control solutions in nearby fields for following sunflower in these fields (genetic resistance, herbicide control or both) • Non tillage prevents new infections	**I. Diagnosis** Map parasitic weed to track race type and dispersion **II. Good practices** Adopt preventive practices for non-infested fields. Clean machinery, harvest last contaminated fields. III. **Rationalize rotational practices** • Design sustainable crop rotations • Include into rotation "Trap Crops" that induce suicide germination of the broomrape seed (i.e., corn; sorghum) IV. **Avoid broomrape seed soil incorporation** • Non tillage after sunflowers V. **Control solutions** • Use appropriate genetic resistance • Use Clearfield technology • A combination of the two above mentioned

Fig. 1. Preexisting broomrape mutations may be screened and selectively multiplied to become "new races" by the introduction of resistant hybrids.

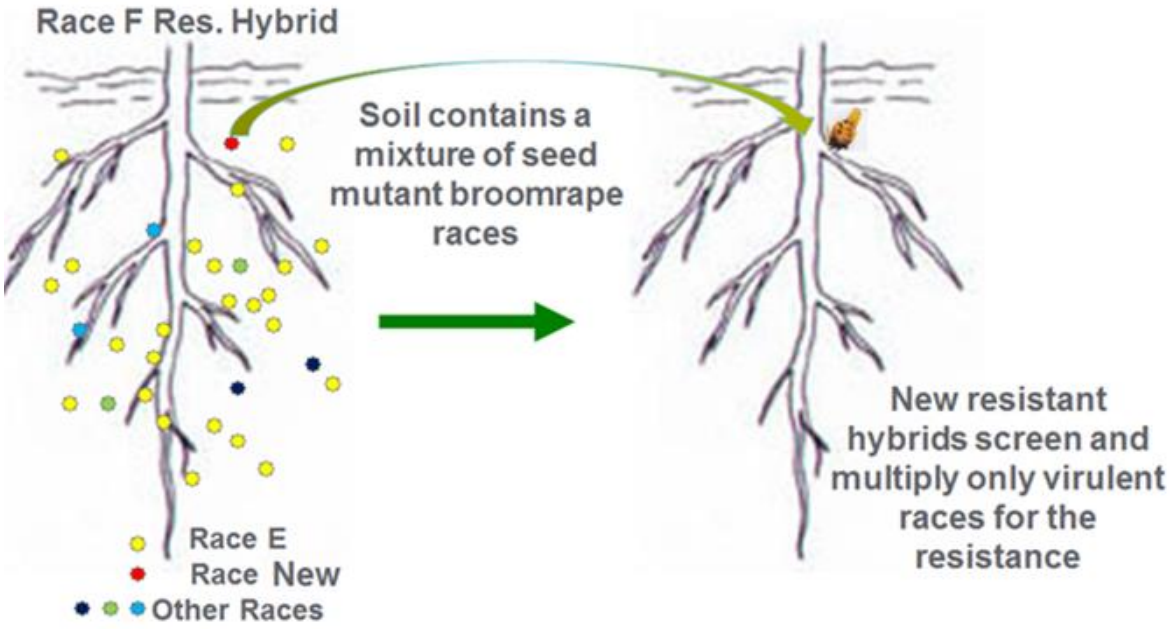
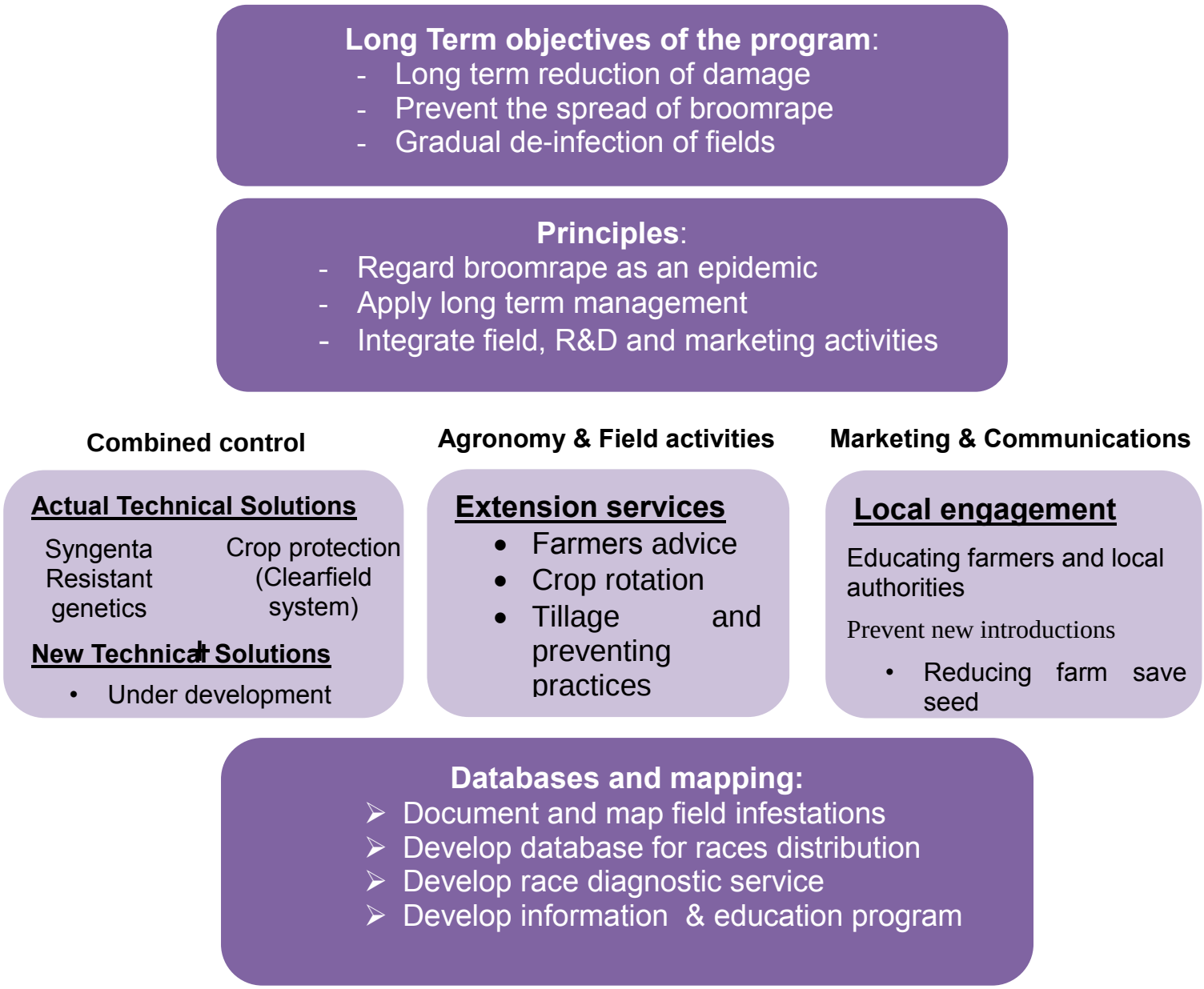


Fig. 2. Syngenta's sustainable sunflowers *Orobanche cumana* management strategy and control program



WORKSHOP

Public-private international collaboration in sunflower broomrape research

Public-private international collaboration in sunflower research on broomrape (*Orobanche cumana* Wallr.)

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ABSTRACT

The history of developing sunflower varieties resistant to broomrape began at the start of the second decade of the 20th century with the development of the first few varieties resistant to race A at several breeding stations in Russia. Since that time, the racial composition of broomrape has been changing cyclically. The appearance of new races such as B, C, D, E, F, G, H and so on has been reported in several countries of central and eastern Europe as well as in Turkey and Spain and, most recently, China. Sources of resistance to races A through E can mostly be found in Russian varieties, while those for resistance to races E through H and further are in genotypes developed by crossing cultivated sunflower genotypes with certain wild species of the genus *Helianthus*. When it comes to broomrape, the first significant germplasm exchange on an international level occurred in the late 1970s and early 1980s, when the source of CMS and Rf genes was discovered and work on developing sunflower hybrids began.

In parallel with the appearance of race E in the late 1970s and its spread across the countries that are large sunflower producers, there was a significant exchange of A lines developed at public institutions with private companies with the aim of developing joint resistant hybrids.

With the founding of the FAO-European Research Network on Sunflower in 1975, work began on studying a large number of sunflower traits including broomrape resistance. These programs involved researchers from both public and private organizations. Broomrape was studied within several Working Groups (environmental study of hybrids, disease studies, genetic research, and wild species). The best results were achieved between 1997 and 2005 within the Working Sub-Group on *Orobanche*.

In parallel with these collaborations, there was also intensive cooperation between some private companies and public institutions that involved the exchange of resistant A lines and the development of commercially based joint hybrids. This form of cooperation has produced the best results, especially in the last 10 years.

The appearance of new virulent races (F, G, H, etc) calls for the formation of a global program in which both public and private organizations would take part. The first step would be to form a list of participating organizations. Then, project tasks would have to be defined that would include precisely defined goals, time frames for their realization, division of labor, sources of financing, methods of use of the results achieved, and intelectual property rights.

Key Words: Sunflower – broomrape – sources of resistance – genetics of resistance – international cooperation

INTRODUCTION

According to Morozov (1947), broomrape was first observed in sunflower in the 1890s in the Saratov region, while according to Kukin (1970) the first report of the pathogen on sunflowers in Russia is attributed to Oldanov (1886). Broomrape causes the greatest damage to sunflower production in the countries around the Black Sea (Russia, Ukraine, Moldova, Romania, Bulgaria, Turkey). More recently, the pathogen has been causing economic damage in Spain, Greece, Serbia, Hungary, Israel, Iran, Uzbekistan, Kazakhstan, India, China, and Australia. In the last ten years, broomrape has also been reported on sunflower in southern France.

For more than a century, there is a constant tug-of-war between geneticists and breeders on the one hand and broomrape on the other, where the side having the upper hand in this struggle often changes.

According to Morozov (1947), the first sunflower varieties resistant to broomrape (race A) were developed by Plachek (Saratov Breeding Station) in 1918. The most well-known among them was Saratovskij No. 169. Afterwards, the following varieties resistant to race A were developed: Kruglik – A 41 (Kruglik Experimental Station), Zelenka (Kharkhov Experimental Station), and Fuksinska No. 10 (Voronetz Experimental Station).

According to Morozov (1947) and Pustovoit (1966), a new race of broomrape was discovered in Rostov Oblast in 1926 by Jdanov. Jdanov named the race B and soon developed varieties resistant to it

called Jdanovska 8281, 8885, 8884, 6432, and 6397, which quickly replaced varieties resistant to race A in commercial production. After that, at VNIIMK (Krasnodar) a number of varieties resistant to race B such as VNIIMK 1646 and Kruglik 1846 were developed along with a number of high-oil ones such as Peredovik, VNIIMK 8931, Smena and others (Pustovoit, 1966). Also, Jdanov (1975) developed several new high-oil varieties resistant to race B (Donskoj 309, 695, 800) (Jdanov, 1975).

After the development of varieties resistant to races A and B, there was no talk for quite a while about changes in the racial composition of broomrape, probably because the commercially grown varieties had resistance above that for races A and B.

Vranceanu et al. (1980), identified five pathogenic races of broomrape and named them A, B, C, D, and E. Based on differential lines, they determined that the resistance was controlled by a dominant cumulative genes (Or_1, Or_2, Or_3, Or_4, and Or_5) Soon after that, a new broomrape race appeared in Romania that could not be controlled by the Or_5 gene. Pacureanu et al. (1998) found resistance to this new race, named F, in the line LC-1093. The gene controlling the resistance was named Or_6.

A great contribution to developing new sunflower germplasm containing Or genes was made by Galina Pustovoit (1975) using interspecific hybridization by crossing wild hexaploid *H. tuberosus* with the variety VNIIMK 8931. This was the end result of a long and complex process (1960-1975), especially in reducing the interspecific hybrid down to a diploid form (2n=34 chromosomes). Her varieties Progres, Novinska, Oktobar, Jubilejnaja-60 and others were used by sunflower breeders worldwide to develop hybrids resistant to races E, F, and G.

More recently, a number of geneticists and breeders across the world have used wild sunflower of the genus *Helianthus* L. to search for genes for broomrape resistance. Among them, the most notable results have been achieved by Christov et al. (1992, 1998, 2009), Škorić (1988, 1989, 2005), Jan et al. (2000, 2002), Jan and Fernández-Martínez (2002), Fernández-Martínez et al. (2000, 2007), Ruso et al. (1996), Sukno et al. (1999), Melero-Vara et al. (1996), and Dozet et al. (2000). These authors have found genes for broomrape resistance in over 20 wild species of the genus *Helianthus*.

In the last five to six years, new virulent races have appeared that cannot be controlled by the Or_6 gene. These have been reported in Russia, Ukraine, Moldova, Romania, Bulgaria, Turkey, Serbia, Spain and perhaps several other countries as well (Pacureanu et al., 2004, 2008, 2009).

The objective of this paper was to make an overview of international cooperation between public institutions and private companies in studying the new races of broomrape, their identification, use of wild species to find new resistance genes, exchange of breeding materials for the purpose of developing resistant hybrids, and the possible formation of a global project for studying broomrape.

COLLABORATION BETWEEN PUBLIC INSTITUTIONS AND PRIVATE COMPANIES ON BROOMRAPE

Globally looking, there has been a long standing collaboration among sunflower breeders and geneticists in terms of germplasm exchange and other forms of cooperation. It is well known that at the time of intensive development of sunflower varieties, the work was being done predominantly at public institutions (1910-1970). Because of the bisexual nature of sunflower flowers, the use of heterosis used not to be possible in practical terms. It was not before the discovery of CMS and Rf genes that the development of sunflower inbred lines began along with the evaluation of combining abilities and actual hybrid development. At that time intensive collaboration begins between public institutions and private companies, which started their own breeding programs on sunflower. Used as the starting material were local populations and high-oil varieties developed at VNIIMK, Krasnodar, Rostov on Don, Odessa, Kharkhov, Fundulea, INTA Pergamino and other places. Concurrently with this, there was a rapid development of intensive programs on inbreeding and heterotic breeding at a number of public institutions. Lines developed at these breeding centers were available to private companies, which resulted in a rapid development of productive hybrids. It is important to note the contributions of particular institutions such as USDA-ARS-Fargo, ND, USA; INTA, Pergamino, Argentina; INRA, France; the Fundulea Institute, Romania; IFVC, Novi Sad, Serbia; IWS, General Toshevo, Bulgaria and many others. Lines developed at the above centers played a significant role in the programs of private companies as well, where the exchange was contract-based.

The rapid spread of broomrape race E in the late 1970s and early 1980s in the countries of Eastern Europe and Turkey and Spain opened up a new chapter in the collaboration between public institutions and private companies on developing sunflower hybrids resistant to broomrape. This collaboration intensified even further with the appearance of race F.

The collaboration between public institutions and private companies as well as that among public institutions from different countries has developed and become very intensive and has taken on various forms. The most extensive collaboration took place within the FAO-European Cooperative Research Network on Sunflower. Within this Network, broomrape was studied in four Working Groups:

1. Experimentation of sunflower cultivars;
2. Genetic studies of agronomic, physiological, and biochemical characters;
3. Evaluation of wild Helianthus species; and
4. Study on population dynamics of sunflower pathogens and their control.

1. <u>Working Group: Experimentation of sunflower cultivars</u>

This Group studied equally and in 2-3-year cycles hybrids from public institutions and private companies. At locations where broomrape was present, the trial executors took note of which hybrids were resistant and which were not. This was an easy way for the owners of the hybrids to find out which hybrids were resistant in which country.

2. <u>Working Group: Genetic studies of agronomic, physiological, and biochemical characters</u>

Genetic studies of broomrape within this group were done the most by the team from Spain. Over a longer period of time, they conducted extensive research and investigated the mode of inheritance of broomrape resistance and the racial composition of the pathogen. Here is a summary of their findings:

Genetics and dynamics of broomrape race composition in Spain

Before 1970, sunflower growing in Spain mostly consisted in the cultivation of confectionery sunflower susceptible to all races of broomrape, as a result of which there was an increase in the *Orobanche* population in the country.

- As oilseed sunflower genotypes began to be grown on a larger scale, a number of broomrape races were identified.
- Domínguez et al. (1996) determined that there was a low frequency of genes for race E in cultivated sunflower genotypes;
- Domínguez (1996) found that resistance to race E present in the line R-41 was controlled by two dominant genes;
- Sukno et al. (1999) determined that race E resistance found in the line NR-5 was controlled by a single dominant gene;
- Pérez-Vich et al. (2004) reported a single dominant gene controlled race E resistance found in the line P-96;
- Rodrígez-Ojeda et al. (2001) found that two recessive genes (Or_6 and Or_7) were responsible for resistance to race E present in the line KI-534;
- Rodrígez-Ojeda et al. (2001) also determined that resistance to race F was controlled by two recessive genes (Or_6 and Or_7);
- Akhtouch et al. (2002) reported that race F resistance existing in the line P-96 was under the control of two recessive genes (Or_6 and Or_7);
- According to Pérez-Vich et al. (2002), the line J1 (BR-4) has a single dominant gene for resistance to race F;
- Velasco et al. (2006), on the other hand, report that resistance to race F in the same line J1 (BR-4) is controlled by two partially dominant genes (Or_6 and Or_7).

Or7 – expression occurs under the influence of environmental factors!

When it comes to genetic research on broomrape within the FAO-European Research Network on Sunflower, particularly noteworthy is the contribution of Pacureanu et al. (1998), who were the first to report the existence of race F. Also, they determined that resistance to this race could be found in the line LC-1093 and that it was controlled by a single dominant gene (Or_6). It is very important to note that this line has become the main source of resistance to race F and that to this day it is part of a number of programs of private companies aimed at developing resistant hybrids, accompanied, of course, by obligatory contracts on the protection of intelectual property.

Also, within this Working Group work has been done on the use of marker genes in determining the race composition of broomrape. Here, too, the most has been accomplished at research institutes, CSIC and CIFA, Cordoba, Spain. Based on extensive research, a number of scientific papers have been published. Their conclusions in brief are as follows:

Marker-assisted selection (MAS) for resistance to Orobanche

- Pérez-Vich et al (2004a) and Pérez-Vich et al (2004b) have achieved significant results in using molecular markers in sunflower breeding for resistance to the various races of broomrape.
- Using segregating materials (resistant x susceptible) and QTL, RFLP and SSR markers, the above authors have found that resistance to race E is controlled by only five QTL markers and that to race F by as few as six. The markers were detected in 7 of 17 linkage groups.
- Race E (5 markers): Or1.1; Or3.1; Or7.1; Or13.1; and Or13.2;
- Race F (6 markers): Or1.1; Or4.1; Or5.1; Or13.1; Or13.2; and Or16.1.

International meetings on broomrape held between 2008 and 2011

During this short period, three important meetings were held dedicated to *Orobanche spp*. on sunflower:

1. International Symposium on Broomrape (*Orobanche spp*.) in Sunflower
   November 30-October 3, 2008
   Antalya, Turkey
   At this meeting 18 papers were presented and there were participants from both public institutions and private companies.

2. Sunflower Breeding on Resistance to Diseases
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The above three meetings illustrate just how important solving the problem of broomrape on sunflower really is.

Working Sub-Group on broomrape

The spread of race E and the appearance of the virulent race F brought about the need for more intensive collaborations on studying this parasitic plant. This Sub-Group operated as part of the working group on sunflower diseases and was established at the Technical Meeting held in Giessen, Germany in 1997. Dr Pepa Shindrova of IWS, General Toshevo, Bulgaria was elected Coordinator of the Sub-Group. After that, a concrete proposal of collaboration was made that included:

- Establishment of the diffusion areas of sunflower broomrape;
- Determination of sunflower broomrape variability;
- Exchange of breeding materials with the aim to test their reaction to the broomrape populations in different countries;
- Preparation of a bibliographic reference of publications on the problems of sunflower broomrape in each country.

The following countries (institutes and companies) took part in the program:

- Bulgaria – Institute for Wheat and Sunflower *Dobroudja* near General Toshevo
- Egypt – Field Crops Institute Agricultural Research Center, Giza (since 1998);
- France – Rustica Prograin Genetic, Mondonville
- Israel – Newe-Yar Research Center (since 1998);
- Hungary – Agroindustrial Share Corporation of Bacsalmas (since 1998);
- Romania – Research Institute for Cereals and Industrial Crops, Fundulea;
- Spain – Koipesol Semillas, Carmona
- Spain – Arlesa Semillas, Sevilla;
- Turkey – Thrace Agricultural Research Institute, Edirne;

- Ukraine – Institute of Oil Crops, Zaporozhye
- Serbia – Institute of Field and Vegetable Crops, Novi Sad.

Cooperation on *Orobanche* has developed very successfully in a short period of time. It includes researchers from Bulgaria, Egypt, France, Hungary, Israel, Romania, Spain, Turkey, and Serbia (Shindrova, 1999).

On the basis of the literature published on this subject and the information given by the participants in the program, it could be concluded that during 1996-1997 no significant changes in the geographic distribution of sunflower broomrape occurred. Countries from the Mediterranean and the Balkan peninsula and Ukraine, southern Russia and Hungary remained the main areas of the parasite's distribution.

In 1998, however, the situation with *Orobanche* changed significantly. Pacureanu-Joita et al. (1998) reported the occurrence of a new, more virulent race in Romania. The race was registered in the region of south and southeast Romania (Kostanza, Tulcha, Braila). It overcame the resistance of line P-1389 (Or_5 gene) and was identified as race F of the parasite. The same authors also reported a source of resistance to this race – line LC-1093.

It was concluded that at the time all sunflower broomrape races were established in Romania (A, B, C, D, E, and F), with races D and E having the greatest share in the population of the parasite.

According to Alonso et al. (1996, 1998) a new *Orobanche* pathotype was established in the south of Spain (Ecija-Sevilla). It was highly virulent and overcame the resistance barriers of all resistant commercial hybrids as well as the Or_5 resistance gene in homozygous lines.

This program has achieved some very significant results in several areas:

- Reaction of the breeding materials to the local broomrape populations;
- Reaction of the various genotypes to the local broomrape populations under greenhouse conditions or in an infected field;
- Reaction of the genotypes to the local broomrape populations under the conditions of an infected field and the role of the parasite in the development of genotypes and yield components (citation by Shindrova)

Bulgaria – Dobroudja Agricultural Institute

The resistance of the wild species was transferred to the cultivated sunflower through hybridization with the aim to produce resistant lines.

- By testing of mutant forms obtained via gamma irradiation and chemical mutagens treatment. As a result of the irradiation of seeds with gamma rays, lines completely or partially resistant to the parasite were produced.
- By inoculation of initial forms of cultivated sunflower obtained through selfing or cross pollination of cultivars and hybrids. This is the most commonly applied method for production of lines resistant to broomrape.

The collected populations of the parasite were used for testing the resistance of the breeding materials at different generations including: restorer lines, female lines (A, B) and hybrids. In 2004, 3,923 breeding samples were analyzed for resistance to *Orobanche*; 1,776 of them (45.2%) demonstrated complete (100%) resistance to the local populations of the parasite.

As a result new broomrape-resistant hybrids were produced at the Dobroudja Agricultural Institute. These hybrids are also resistant to downy mildew, besides broomrape. They combine resistance with high oil content and yields exceeding the standard (citation by Shindrova)

At the FAO-Consultation Meeting held in Novi Sad in June of 2005, a new program of the collaboration was made:

- Exchange of breeding materials (lines, hybrids) with the aim to test their reaction to the broomrape populations in Bulgaria, Turkey, Romania, and Serbia;
- Testing the response of breeding materials (not exceeding 100 samples) to the broomrape population in your country (local population);
- Conducting a trial with the aim to determine the effect of broomrape attack on yield elements and the development of sunflower plants;
- Carrying out observations to identify other hosts of the parasite;

- Preparing a bibliographic reference of the publications in sunflower broomrape problems in your country (for the 2004-2008 period); and
- Searching for sources of resistance among wild sunflower species and maintaining a collection of them.
-

The above program was executed as agreed and significant results were achieved.

Also, at the FAO-Consultation Meeting in 2005, Dr Pepa Shidrova presented detailed results on broomrape distribution and race composition in Bulgaria. These findings helped all the sunflower breeders in the development of new resistant hybrids.

At the FAO-Consultation Meeting held in Novi Sad, Serbia in 2005, Pacureanu reported the presence of new broomrape races in Romania. She concluded that the existence of a new race of the parasite *Orobanche cumana* in sunflower in Romania had led to many investigations for identifying new sources of resistance to the parasite; interspecific hybrids, new sunflower populations, and new sunflower lines had been investigated and some sunflower genotypes with good resistance were identified such as R-10481, R-7842, and R-5540 (inbred lines) along with three interspecific hybrids.

At the same meeting, Kaya (Edirne, Turkey) reported the appearance of new broomrape races in Turkey and concluded as follows: *Preliminary results show that there is at least three different broomrape populations based on reaction against resistant sunflower hybrids and lines. However, it is not known yet if these different broomrape populations are new races or sub populations. Infested new broomrape areas increase year by year in the region. Resistant sunflower hybrids have been developed against these new races. Results from this study would be used to establish differential lines against the new broomrape populations. The new races increase each year 50% more than in the previous year. From 1995 to 2002 the infested areas increased from 2 to 35%. It can be concluded that in 2002 in more than 60-70% of the sunflower production area in Trakya region have new Orobanche races present.*

3. Working Group: Evaluation of wild Helianthus species

Besides a number of joint programs carried out within this Group, work was also done finding sources of resistance to broomrape in the wild sunflower species.

Significant results were achieved by the team from CSIC, Córdoba, Spain and that from USDA-ARS, Fargo, ND, USA, which carried out a joint project on finding sources of resistance in certain wild sunflower species and incorporating the genes into cultivated sunflower genotypes. They presented their results in two research cycles and published a number of papers on the subject.

As the final product, they developed four broomrape-resistant populations of sunflower. Jan et al. (2002) developed new germplasm resistant to broomrape (genes x wild species):

- Four new populations
- Genetic source of resistance to race F
- BR_1 (Pl 617026):
  P21II *H. grosseserratus* – 004/P21(PD)/3/HA89
- BR_2 (Pl 617027):
  P21II *H. Maximiliani* (D) – II P21/3/HA89
- BR_3 (Pl 617028):
  H. divaricatus-830/P21/3/HA89
- BR_4 (Pl 617029):
  H. divaricatus-830/P21(D)II *H. grosseserratus*-011/P21(D)/3/P21/4/ HA89

All four of the above interspecific populations were successfully used to develop inbred lines resistant to certain broomrape races in a number of private companies.

A huge success in the use of the wild species for finding resistance genes to broomrape was achieved by Christov (1992, 1998, 2009) with his discovery of the resistance genes and their incorporation into elite sunflower lines. In his report for the FAO-Sunflower Network, he reported that resistance to broomrape was found in hybrid material originating from *H. annuus* (accesssions E-126 and E-112), *H. praecox* (E-148), *H. nuttallii* (M-088), *H. mollis* (M-082), *H. pumilus* (M-172), *H. giganthus* (M-011), *H. decapetalus* (M-043), and *H. strumosus* (M-110 and M-126). It should be noted that a certain number of new inbred lines developed by Christov and his team were also resistant to the latest, virulent races. Based on Material Transfer Agreements, these lines are used to develop resistant joint hybrids.

Also, Škorić et al. (1999) reported their findings on the presence of the Or_5 gene in some wild sunflower species, shown in Table 1.

Table 1. Škorić, Lačok, and Jocić (1998/1999). Results of testing wild sunflower species for resistance to race E of broomrape (greenhouse test with test tubes).

Wild sunflower species	Resistant populations	Susceptible populations
H. annuus	19	75
H. petiolaris	23	6
H. neglectus	3	-
H. debilis	9	-
H. praecox	10	-
H. argophyllus	4	1
H. niveus	5	-

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DIRECT COLLABORATION BETWEN PRIVATE COMPANIES AND PUBLIC INSTITUTIONS

There is long-standing successful cooperation between certain private companies and some public institutions in the form of the exchange of A-lines and the development of joint hybrids. A good example is the hybrid Albena alongside many others, all of which played or still play a significant role in sunflower production of many countries.

With the appearance of new, virulent races of broomrape collaboration in germplasm exchange has increased sharply, especially in the case of the exchange of A-lines resistant to race F. This can be seen in the use of the Romanian line LC-1093A (Or_6), which is used on contract basis by several private companies in developing joint hybrids.

In the last 5-6 years as the new virulent races of broomrape have been appearing that cannot be controlled by the Or_6 gene, collaborations between private companies and public institutions have intensified. This cooperation is based on Material Transfer Agreements at first, after which as the joint hybrids have been developed Commercial Agreements are signed on the exploitation of said hybrids. These Agreements guarantee the rights of both partners and intelectual property rights. It can be said with certainty that this model is the most widely used one in the collaborations between private companies and public institutions. There are other models of successful cooperation between private companies and public institutions. INRA, France, for example has carried out several successful projects with private companies in France.

WHAT TO DO NEXT ON AN INTERNATIONAL LEVEL

The appearance of the new virulent races in the last 5-6 years has brought about the need for greater cooperation among public institutions themselves as well as between them and private companies. The

problem dictates the set up of one or more global projects in which the above organizations would all join forces to address the problem of *Orobanche*.

The question of differential lines

Thanks to the work of Pacureanu and her team we now have a set of six differential lines for testing the race composition of broomrape, as shown in Table 2.

Table 2. Spectrum of physiological races of *Orobanche cumana* Wallr. Parasite in Constanta, Tulcea, Braila, Ialomita zones (Romania), 1997 (Pacureanu-Joita et al., 1998)

Differential lines	Resistance genes	Reaction of sunflower plants to broomrape races											
		Natural infestation (Constanta, 1997						Artificial infestation (Fundulea, 1997)					
		A	B	C	D	E	F	A	B	C	D	E	F
LC-1093	Or_6	R	R	R	R	R	R	R	R	R	R	R	R
P-1380-2	Or_5	R	R	R	R	R	S	R	R	R	R	R	S
S-1358	Or_4	R	R	R	R	S	S	R	R	R	R	S	S
Record	Or_3	R	R	R	S	S	S	R	R	R	S	S	S
Jdanov	Or_2	R	R	S	S	S	S	R	R	S	S	S	S
Krulik	Or_1	R	S	S	S	S	S	R	S	S	S	S	S
AD-66		S	S	S	S	S	S	S	S	S	S	S	S

R-resistant, S-susceptible

The question remains as to what should the official differential lines be for the new races G, H, etc. In my opinion, we should decide at this meeting which of the resistant hybrids should be adopted as the new differential lines.

Also, in the existing set of six genotypes three are varietal populations: Kruglik, Jdanov, and Record. In order to have a representative sample for them, it is necessary to determine the starting population (number of plants) so as to be sure that we have not lost a gene for resistance to broomrape.

METHODS OF ASSESSING RESISTANCE TO BROOMRAPE

In order to develop sunflower varieties or hybrids genetically resistant to broomrape, it is necessary during the selection process to use a reliable method of evaluating the breeding material. Sunflower breeders worldwide use different methods either under natural conditions or using artificial inoculation in a greenhouse or a climate chamber. Each of the methods has its drawbacks and advantages. Here are the most commonly used methods:

1. Planting breeding material on a plot naturally infested with broomrape
2. Assessing the resistance in the field by introducing broomrape seeds in parallel with planting the breeding material
3. Assessing the resistance in the greenhouse – planting in pots
4. Assessing the resistance in the greenhouse on tables
5. Testing for broomrape in the greenhouse using test tubes
6. Hydroponic co-culture system of assessing the resistance

It would be desirable to reach an agreement which of the above methods should be adopted as standard or to develop a new joint method for screening in the field and separately in the greenhouse.

THE QUESTION OF CHOICE OF OPTIMAL MOLECULAR TECHNIQUES (MARKER GENES)

The choice of optimal molecular marker methods for screening for resistance to broomrape on the molecular level

The results so far indicate that different researchers use different molecular marker methods. These include: AFLP, AP-PCR, ARMS, ASAP, ASH, ASLP, ASO, CAPS, CAS, DAF, DGGE, GBA, IRAP, ISSR, ISTR, MP-PCR, OLA, RAHM, RAMPO, RAMPs, RAMS, RAPD, RBIP, REF, REMAP, RFLP, SAMPL, SCAR, SNP, SPAR, SPLAT, S-SAP, SSCP, SSLP, SSR, STMS, STS, TGGE, and VNTR.

It would be desirable if the researchers using molecular marker methods could pick two or three so that the results they obtain are easier to compare.

POSSIBLE ESTABLISHMENT OF A GLOBAL PROJECT ON STUDYING BROOMRAPE

The dynamic changes in the racial composition of broomrape in a number of countries over the past 5-6 years are a cause of concern for all sunflower researchers, technical people and producers. It is expected that this trend of dynamic changes will continue in the years to come as well. Because of this, it would be desirable to establish a global program in which public institutions and private companies would participate on equal footing.

First, a list of participants should be formed along with finding the sources of financing and making a detailed program of work with concrete responsibilities of each party involved.

Germplasm

Globally looking, there are two big collections of cultivated sunflower – one is the national collection in Ames, Iowa, USA and the other the one at VIR, Saint Petersburgh, Russia. Also of interest is the cultivated sunflower germplasm of INTA-Pergamino, Argentina and the national collection at the Kharkhov Institute, Ukraine, as well as perhaps some other ones as well that are smaller in terms of interest regarding broomrape.

The main collection of the wild species of the genus Helianthus L. is also at Ames, Iowa. There are other collections of this type as well, but they are significantly smaller.

In order to gain full insight into which varieties, local populations and inbred lines have genes for resistance to broomrape, it would be necessary to conduct a full screening of said collections of cultivated sunflower and the wild species. This would be a very extensive and expensive job that would require qualified staff, laboratories, greenhouses, and a great deal of funding.

COLLECTION OF BROOMRAPE VARIABILITY ON A GLOBAL LEVEL AND THE ESTABLISHMENT OF A WORLD COLLECTION

Countries in which the presence of broomrape has been confirmed are well known. The fact that broomrape is a quarantine weed in most countries makes it difficult to exchange broomrape among institutions and countries. It would be necessary to determine in which country the parasite is not a quarantine weed, and such a country would also have to have all the prerequisites for establishing a world collection of broomrape seeds and their short- and long-term maintenance. This is a responsible and important task. After the establishment of a world collection, it should be determined how to manipulate it on a global level.

TESTING THE VARIABILITY OF BROOMRAPE

The variability of broomrape can be tested in the field, greenhouse or a climate chamber with the obligatory use of a set of differential lines.

Another way to test this variability is to do it on the molecular level using marker genes. In this case the exchange of broomrape could be made easier by manipulating the DNA among countries and institutions.

DEVELOPING NEW GENETIC VARIABILITY IN MATERIALS RESISTANT TO BROOMRAPE

If full screening for broomrape resistance was to be done, it would be possible to set up a project or subproject on developing new genetic variability of the cultivated sunflower. A research center would have to be chosen in which all the wild species resistant to broomrape would be crossed with, let us say, five elite lines of the cultivated sunflower. The team concerned would produce F_1, F_1BC_1, F_1BC_2, F_1BC_3 and so on. After that, the participants in this program would equally share the newly developed material for the further development of new resistant lines and hybrids.

FUNDING

If enough researchers from public institutions and private companies would be able to join forces, the next step would be to come up with the funds for the global program. The potential sources of funds could be the World Bank, EU, international financial institutions, private companies, governments of countries where broomrape is present and many others that would have an interest in the matter.

INTELECTUAL PROPERTY RIGHTS

The protection of intelectual property is a highly important segment for all the participants in the possible future program. This matter would be addressed via a special Agreement among all the parties involved.

All the above considerations about a possible global project are just a starting point for a serious discussion on research, technical and professional levels and all suggestions and proposals are more than welcome.

CONCLUSIONS

During the existence of the FAO-European Cooperative Research Network on Sunflower (1975-2005), joint research on broomrape between public institutions and private companies took place within four Working Groups.

The study of hybrids as part of environmental studies made equal use of hybrids of private companies and public institutions. In locations where broomrape was reported resistant hybrids were identified.

Extensive genetic research included the appearance of new broomrape races as well as the mode of inheritance of the resistance to the parasite. The genetics of the resistance were also studied using molecular markers.

As part of the Working Group on sunflower diseases, the appearance of races E and F and their distribution by country were monitored in detail.

As part of the study of wild sunflower species, besides other extensive researches, search was conducted to find genes for resistance to broomrape and incorporate them in cultivated sunflower genotypes.

The exchange of lines resistant to races E, F, G, H and so on took place and is still taking place between public institutions and private companies in the form of Agreements and the development of commercial hybrids.

In order to further advance cooperation between public institutions and private companies on a global level, a global program on broomrape would be required. At the start of this process, it would be necessary to choose the optimum methods of screening germplasm in the field and greenhouses and to choose the most accurate molecular marker methods so as to be able to compare the results obtained.

If a decision is made to establish a global program on studying broomrape, the participants should be known along with the modes of financing, teams responsible for particular parts of the project, and which gene banks (germplasm) to use in studying the cultivated sunflower, wild species, and interspecific hybrids. Another important point is how to use the results obtained and the protection of intelectual property.

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