EVALUATION OF GENETIC VARIABILITY FOR *Sclerotinia sclerotiorum* Lib. de Bary RESISTANCE IN A F$_2$ POPULATION FROM A CROSS BETWEEN SUSCEPTIBLE AND RESISTANT SUNFLOWER

M. Baldini[1*], M. Vischi[1], M. Turi[1], N. Di Bernardo[1], S. Raranciuc[2], M. Echeverria[3], F. Castaño[3], G.P. Vannozzi[1], A.M. Olivieri[1]

[1]*Dipartimento Produzione Vegetale e Tecnologie Agrarie, via delle Scienze, 208, 33100 Udine, Italy*
[2]*Agricultural Research and Development Institute, Titulescu street, 2, Fundulea, Romania,*
[3]*Unidad Integrada Facultad de Ciencias Agrarias-UNMdP/Estacion Experimental Agropecuaria-INTA, CC 276, RA 7620 Balcarce, Argentina*

SUMMARY

The inbred line R28, coming from Helianthus argophyllus, displays low susceptibility to both artificial and natural infection by basal stem and head rot and to artificial infection by fungus filtrate (oxalic acid). A cross was made between this line and 9304 (a susceptible inbred line). F$_2$ plants, parental plants and F$_1$ plants, were artificially infected by: i. pathogen mycelium over the basal stem (basal stem attack), ii. ascospores on the head (head rot attack).

As result of basal stem infection, 72.4% of the F$_2$ plants showed symptoms and died. Incubation period (IP), as the number of days from infection to the appearance of first symptoms, varied from 5 to 25 days, with a population mean of 12.5±6.1 days. The IPs in 9304, F$_1$ and R 28 were 7, 10 and 16 days, respectively.

The same IP character and the percentages of lesions on the capitulum, monitored at the end of flowering time (EF) and at physiological maturity (PM), were also measured after the infection with ascospores. After the ascospore test, 60.7% of the infected F$_2$ plants showed disease symptoms on the capitulum. In this case the IP ranged between 16 and 45 days, with a population mean of 27.6 ± 6.4 days. The IPs for 9304, F$_1$ and R 28 were 18, 25 and 35 days, respectively. Among all the plants with lesions at PM, 24% was completely safe at EF, thus showing different reactions of F$_2$ genotypes against *Sclerotinia*. Relationship among EF, PM and IP were investigated and the obtained results suggest two different *Sclerotinia* attack mechanisms.

This paper discusses the possibility of obtaining some results from classical selection programs utilizing these parameters. We are also considering a molecular markers assisted approach. Recently, several hundred microsatel-

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* Corresponding author, Phone: +39 0432 558663, fax +39 0432 559603, e-mail: baldini@dpvta.uniud.it

lite markers were developed for sunflower. For this purpose we have selected a first set of primer combinations on the basis of amplicon length to facilitate multiplexing. SSR markers were screened for polymorphism using three-color multiplexes.

INTRODUCTION

In many damp temperate climates of the world the fungus *Sclerotinia sclerotiorum* Lib. de Bary is one of the most dangerous crop pathogens, with an extremely large number of host plants (e.g., 225 genera) and the persistence in the soil lasting for many years (Gulya *et al.*, 1997). It is particularly aggressive in sunflower (*Helianthus annuus* L.) crops and it is responsible for seed yield reductions up to 100% under extreme circumstances. *S. sclerotiorum* is one of the most complicated pathogens to combat since the chemical control is difficult, uneconomical and very harmful to the environment. The genetic control is polygenic and often partial (Castaño *et al.*, 1993), as the pathogen can attack all parts of the plant (basal and mid-stem rot and head rot), and a genotype that is resistant to one form of attack is often susceptible to another (Tourvieille and Vear, 1990). Although several easy and rapid methods for assessing *Sclerotinia* resistance have been developed recently (Tahmasebi-Enferadi *et al.*, 1998; Castaño *et al.*, 2002), no resistant sunflower lines have been found so far. For these reasons breeding programs for *Sclerotinia* resistance nearly always involve lengthy and time-consuming traditional selection methods based mainly on morphological, biochemical and physiological traits.

The inbred line 28R (coming from the wild species *H. argophyllus*) appears to contain not only some resistance factors against both basal stem and white head rot infections (Baldini *et al.*, 2002) but also a specific resistance to oxalic acid (Tahmasebi-Enferadi *et al.*, 2000), which is a fundamental compound in white rot pathogencity (Noyes and Hancock, 1981; Tahmasebi-Enferadi *et al.*, 1998; Bazzalo *et al.*, 2000). In fact, a wheat oxalate oxidase (*OxOx*) gene has recently been used to enhance the level of resistance in the cultivated sunflower in the USA, where the transgenic form of this plant appears to significantly improve white rot resistance by degrading the oxalic acid (Burke and Rieseberg, 2003).

The advent of DNA markers has facilitated mapping of agriculturally important genes and QTLs in plants. If closely related to resistance genes, markers could be used to identify resistant sunflower lines on the basis of genotype as well as phenotype, maximizing the selection effectiveness. We report here the field evaluation of a single F_2 population after *S. sclerotiorum* infection on the head and basal stem and the preliminary results on a first set SSR markers tested on our material in the framework of a marker assisted selection program for *S. sclerotiorum* resistance in sunflower.

MATERIALS AND METHODS

Sunflower genotypes

Two inbred B lines were used, R 28 and 9304. R 28 was obtained after a selection for physiological parameters related to drought tolerance of *Helianthus annuus* × *H. argophyllus* material. The source of resistance is uncertain, but this line displayed a good level of resistance against both basal stem and white head rot infections and moreover it gave a high performance to oxalate and culture filtrate tests highlighting a specific resistance to oxalate, too. Meanwhile, the line 9304 has a low level of resistance as shown by a more rapid lesion expansion on stem and head than HA89; because of that it was utilized in previous trials as susceptible control. The 9304 × R28 cross was made and the F_2 seeds were obtained by self-pollination of F_1 plants.

Field trials

Field trials were carried out during 2002, at the University of Udine, Italy (latitude 45° 2' N, 13° 13' E, altitude 92 m) on a loamy-sandy and shallow soil (about 50 cm), with 25% of gravel.

Hand sowing was performed on 3 May and the emergence occurred 10 days later. The F_2 was sown in a uniform soil surface of about 250 m^2, while the parental lines and the hybrid were grown in four experimental units consisting of one 2-m row for each genotype randomly distributed throughout the field.

Two sprinkler irrigations (10 mm each) were applied weekly during the entire crop cycles to maintain the soil in non-limiting water condition. Weed control was performed by hand after plant emergence without any pesticide treatment.

Artificial infections and disease evaluation

Head infection. The inoculum was obtained according to Castaño and Rodriguez (1997). At flowering stage (R 5.3), with approximately three external rows of hermaphrodite flowers in pistillate stage (Schneiter and Miller, 1981), the floral surface of 159 F_2 plants as well as of 10 individuals for each parental line and hybrid, were sprayed with 10 ml (5 ml twice a week) of an aqueous suspension containing 2500 ascospores/ml. F_2 plants were infected at one of the following dates: July 19, 22, 24 and 26, 2002, according to their date of flowering. The inbred lines and the hybrid were infected at a single date. After infection, heads were immediately covered with paper bags in order to avoid drying. Two weeks later, each capitulum started to be observed twice a week until physiological maturity to detect first disease symptoms and the lesion development. At the end of flowering (EF) and physiological maturity (PM), all infected capitula were classified on the following rating scale: 0, 1-20, 21-40, 41-60, 61-80 and 81-100, according to the proportion (%) of the white rot area scored on the receptacle. The incubation period (IP), i.e.,

the number of days between the infection and the appearance of first symptoms, was also recorded.

Basal stem infection. 155 F_2 individuals and 10 plants of each parental line and hybrid were used for this resistance test. Two oat seeds, infected with *S. sclerotiorum* mycelium, were put over the basal stem of sunflower plants at R_2 stage (Schneiter and Miller, 1981); the infection site was covered with moistened cotton wool and sealed with a transparent plastic film to maintain humidity (Tahamasebi-Enferadi *et al.*, 2000). One week later, each infected plant started to be observed twice a week up to physiological maturity. The IP was estimated as in the previous resistance test.

SSR marker genotyping

DNA extraction. About 150 F_2 plants were inspected in each inoculation treatment. DNA was extracted from leaf tissue by cetyltrimethylammomium bromide (CTAB) protocol (Doyle and Doyle, 1972) and quantified by gel–electrophoresis staining comparison.

PCR amplification. The set of 20 SSR markers published by Paniego *et al.* (2002) were amplified with primers end-labeled with $[^{33}P]\gamma$-ATP and T4 polynucleotide kinase. PCR reactions were performed in a total volume of 10 μl using a Gene Amp 9700 DNA thermocycler (PE Applied Biosystems, USA). The reaction mixture contained 1.5 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM each of dNTPs; 0.25 μM each of primers, 1 × PCR buffer, 0.4U Taq DNA polymerase (Amersham Science, UK). Touchdown PCR was performed for enrichment of the template. The initial denaturation step was performed at 94°C for 5 min, followed by one cycle at 94°C for 30 s, at 59°C for 30 s and at 72°C for 30 s. The annealing temperature was decreased 1°C per cycle in the subsequent cycles until reaching 52°C. Products were subsequently amplified for 25 cycles at 94°C for 30 s, at 52°C for 30 s and at 72°C for 30 s with a final extension for 7 min. The products of PCR amplification were separated on 6% acrylamide gel under denaturing conditions and visualized by autoradiography after 1-2-day exposure.

Table 1: Primer combinations (Tang *et al.*, 2002) and fluorochorme used

SSR	Florochrome	SSR	Florochrome
ORS385	FAM	ORS152	TMR
ORS523	FAM	ORS364	TMR
ORS533	FAM	ORS423	TMR
ORS602	FAM	ORS481	TMR
ORS340	HEX	ORS499	TMR
ORS565	HEX	ORS557	TMR

We have extended the analysis on an automatic capillary sequencer (Mega-BACE, Amersham Science) using a set of 21 SSR primer pairs (Table 1), randomly selected in the range of 150-250 bp among these published by Tang *et al.* (2003). Forward primers were modified (MGW Biotech, Italy) by adding fluorophores

(6FAM, HEX, TMR) to the 5' ends. PCRs were performed using the same reaction mixture described previously for ^{33}P-labeled primers. Amplicons were desalted after ethanol precipitation and resuspended in 20 μl of H_2O; 1 μl for each sample was mixed with 3 μl of loading dye and H_2O up to a final volume of 6 μl for Mega-BACE injection. The peaks generated by the MegaBACE sequencer were analyzed by Genescan Analysis 2.0.2 software (Amersham Bioscience, UK).

Data analyses

The normal distribution of data was checked. After that, both IEF and IPM data were transformed in arc-sin √% whereas IP data followed a logarithmic transformation. The ANOVA, normality test and correlation coefficient were performed using the Cohort software. For QTL detection, analysis of variance was carried out according to Kearsey and Pooni (1996).

RESULTS AND DISCUSSION

Field trials

In our study, all plants without symptoms on heads and stems were defined as not infected and therefore were not included into the analysis because all reports about *Sclerotinia* resistance in sunflower indicate the lack of complete resistance (Gulya *et al.*, 1997; Castaño *et al.*, 2001a).

In general, the distribution of F_2 individuals was unimodal and continuous for all traits except IPM, as reported in Figures 1, 2 and 3. In fact, the D'Agostino-Pearson test revealed a deviation from the normal distribution even after transformation of IPM values.

Of 159 F_2 plants infected on the heads, 96 individuals showed white rot symptoms at physiological maturity (PM) and among these 24% did not show any lesions at the end of flowering (EF). At the end of EF period, 18% of plants had lesions from 81 to 100% (maximum range); at PM, the F_2 plants increased in the same range, up to 63% (Table 2 and Figure 1).

Table 2: Mean symptom criteria measured in F_2 progeny, parental lines and F_1 hybrid after *Sclerotinia* head artificial infection

Character	Unit	9304	9304 × R 28	R 28	Mean of the F_2 population
EF	%	30.0 a	36.7 a	16.7 b	39.2
PM	%	100.0 a	96.6 a	70.0 b	81.8

Lesion% of the head at the end of flowering (EF) and at physiological maturity (PM) after *Sclerotinia* artificial head infection.
Means followed by the same letter are not significantly different at *p*<0.05 with a Student test

At both EF and PM periods, the inbred line 9304 and the F_1 hybrid showed significantly higher white rot lesions than the inbred R28. As expected, the latter
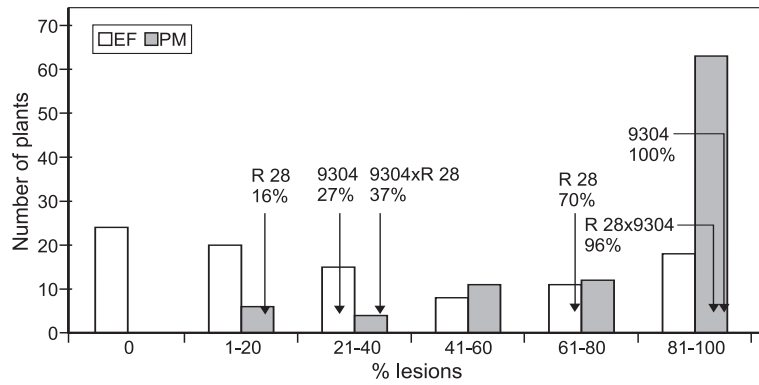
Figure 1: Distribution of lesions after Sclerotinia artificial infection in F$_2$ plants. Lesions are expressed as% of the head at end of flowering (EF) and at physiological maturity (PM). Values (in italics for PM) are for parental lines and hybrid.
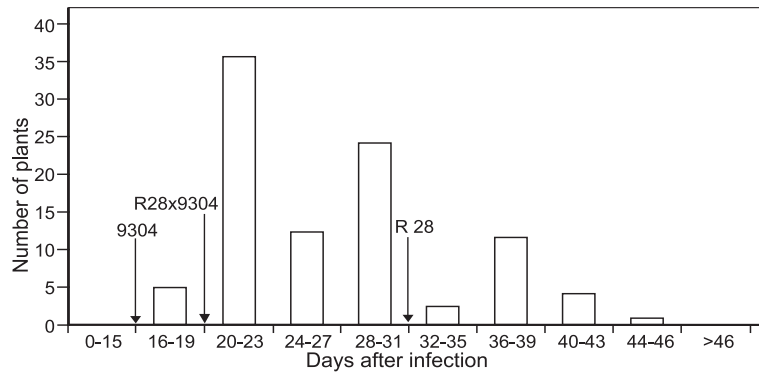

Figure 2: Distribution of the incubation periods, IP (days from infection to symptom appearance on the head) after Sclerotinia ascospore artificial infection in F$_2$ plants. Arrows indicate values for parental lines and hybrid.
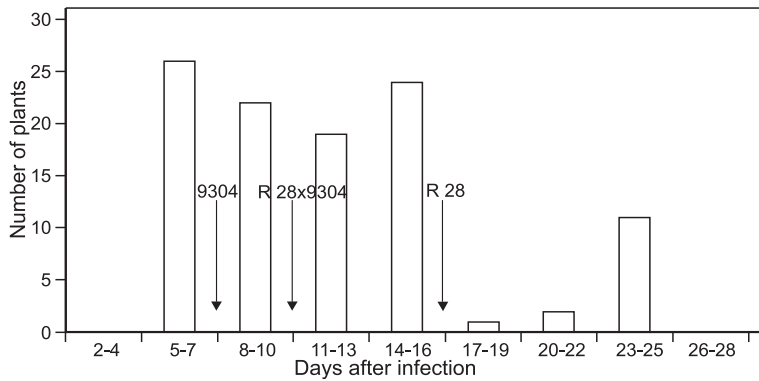

Figure 3: Distribution of the incubation periods, IP (days from infection and necrosis appearance) after basal stem Sclerotinia artificial infection in F$_2$ plants. Arrows indicate values for parental lines and hybrid.

inbred line confirmed its good performance against *S. sclerotiorum* infections (Table 2).

Regarding the incubation period (IP) for the head, the mean value of the F_2 population was 27.6 days, while the inbreds R28 and 9304 had 32 and 16 days, respectively, and their hybrid had 20 days (Figure 2). It is to note that 23% of F_2 plants had IP longer than R28 parental line, reaching 44-46 days. Conversely, 9304 was affected earlier than the other plants.

Regarding basal stem infection, 72.2% of 155 F_2 plants showed symptoms at the physiological maturity. All plants that showed the disease symptoms died subsequently. The mean value of IP for this resistance test was 12.5 days in the F_2 population. The inbred line R28 showed higher incubation period (IP 16 days) than the hybrid (IP 10 days) and the other inbred 9304 (IP 7 days) (Figure 3). The performance of the inbred R28 was in accordance to that reported previously by Baldini *et al.* (2002). Some segregant plants had IP longer than 23-25 days, showing a behavior resembling an escaping mechanism to the disease.

For all traits, some plants of the F_2 exceeded the mean value of the resistant parental line and in any case, with the exception of the EF trait, the F_1 mean did not show higher or lower values than mid-parent, confirming the presence of a genetic control controlled by quantitative genes, with the possibility to obtain interesting results from a selection program using the evaluated traits. The absence of non-additive effects suggests that selection might be done as early as possible on the basis of the performance of F_2 plants, according to Vear and Tourvieille (1984) and Castaño *et al.* (2001b).

The negative correlation (r=-0.76**) between IP and EF in the F_2 plants means that the greater the delay in first symptom appearance, the lower the percentage of lesions at end of flowering (i.e., an early stage of infection), suggesting a possible escape mechanism.

Conversely, the absence of a significant correlation between IP and PM (r=0.10 n.s.) indicates the disappearance of the former relationship by the progression of the mycelia into the tissues during the end of flowering-physiological maturity period. This situation, which suggests two different mechanisms involved in sunflower resistance against *Sclerotinia* infection (i.e., resistance to penetration into the tissue and the rate of mycelium extension into the inflorescence), is in agreement with the results of Castaño *et al.* (1993).

Both mechanisms involved in the sunflower resistance to *Sclerotinia* could be improved. In fact, with a high level of resistance to ascospore penetration. the number of diseased capitula in the crop will be reduced and the proportion of healthy harvested capitula will be indirectly increased. In the same way, if the mycelia growth into inflorescence tissues is slowed down, it could allow a satisfactory seed yield harvest.

The inbred line R28 showed a good reaction to the penetration and extension of *Sclerotinia* mycelia. This inbred line came from *H. argophyllus*, a wild species

which rendered many valuable genes (Hahn, 2002; Seiler and Reisemberg, 1997; Škorić, 1985), and it could be used as a source of resistance in breeding programs for *S. sclerotiorum* resistance in sunflower.

SSR genotyping

The 20 primer combinations published by Paniego *et al.* (2002) were tested on our material and can be summarized as follows: *Ha432-ar* and *Ha1237-ar* gave a very complex amplification profile; *Ha196-ar* and *Ha514-ar* produced a very low signal or no signal after amplification; *Ha95-ar, Ha140-ar, Ha239-ar, Ha360-ar, Ha494-ar, Ha806-ar, Ha1209-ar, Ha1626-ar, Ha1796-ar* were monomorphic. Among the remaining primer combinations, the best were *Ha991-ar, Ha1442-ar, Ha1608-ar.* The first one is a dinucleotide microsatellite with a GA motif, whereas the other two microsatellites contained a trinuclocleotide motif (ATT)n. These primers were tested on 150 F_2 plants and the distribution of alleles was in accordance with the Mendelian segregation.

Table 3: Amplification, number of loci and alleles, heterozygosity of PCR primer pairs selected among these of Tang *et al.* (2002)

Marker	Amplification	No. *loci*	Dominant - Codominant	No. alleles	Heterozygosity
ORS307	Monomorphic				
ORS340		2	2 - 0	2	-
ORS364		1	0 - 1	2	0.9865
ORS385	Unscoreable				
ORS409	Unscoreable				
ORS423		2	1 - 1	3	0.9964
ORS445	Many peaks				
ORS481		2	1 - 1	3	0.9886
ORS499		2	0 - 2	4	0.9999
ORS503	Not amplified				
ORS513	Many peaks				
ORS523		3	2 - 1	4	0.9804
ORS533		2	1 - 1	3	0.9962
ORS545	Many peaks				
ORS546	Monomorphic				
ORS552		2	1 - 1	3	0.9966
ORS557		4[†]	2 - 1	5	0.9955
ORS562	Many peaks				
ORS565		2[†]	1 - 0	2	-
ORS573	Not amplified				
ORS602		1	0 - 1	2	0.9898

[†]one locus was monomorphic

Capillary-sequencer generated data are summarized in Table 3. In our conditions, no amplicons were produced by ORS573 and ORS503 primer pairs, whereas

ORS385, ORS409, ORS445, ORS513, ORS545, ORS562 generated a complex and unpredictable pattern. ORS307 was monomorphic and was excluded from further analysis. The peak profile of the remaining primer combinations, with the exception of ORS364 and ORS602, was interpreted as postulating the amplification of more than one locus and the presence of null alleles. ORS340 and ORS565 amplifications (2 and 1 loci, respectively) were scored as dominant for the presence of only null alleles. The other primer pair amplifications were mutlilocular, with a combination of dominant and codominant markers. Only ORS364 and ORS602 were unilocular and codominant. In the analysis of variance, no significant associations were found with trait values, although it was expected to obtain a few of them.

More than 800 SSR markers were developed by Tang *et al.* (2002) and about half of them have been mapped. A further increase in map density and utility of molecular genetic linkage map of sunflower was reported by Yu *et al.*, (2003). These SSR markers, now in public domain, constitute a critical mass to develop a molecular-marker-assisted approach for *S. sclerotiorum* resistance in sunflower. However, this approach is expensive and time consuming as several markers have to be tested on segregant populations obtained after specific crossing. A consistent cutdown for SSR genotyping, both in time and money, may be obtained by high-throughput systems and PCR and post-PCR multiplexing. On the other side, the widespread use of multiplexing PCR for SSR genotyping in crop plants has been limited by several factors, including a limited number of polymorphic, single-locus SSR markers, recalcitrant primer combinations for multiplex PCR (Tang *et al.*, 2003). The ideal marker for multiplexing should be single locus and it should not produce non-target bands. To be more informative, it should be also codominat and should not produce null alleles.

Following these considerations, our results point out that SSR primers should be carefully selected, as not all among these tested had the ideal features for high-throughput multiplexing systems. The costs could be further reduced by the use M13-tailed primers as proposed by Boutin-Ganache *et al.* (2001). To study the practicability of this approach, the testing of about 200 primers is in due progress.

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CALIFICACIÓN DE LA VARIABILIDAD GENÉTICA PARA LA RESISTENCIA A PATÓGENO *Sclerotinia sclerotiorum* Lib. de Bary EN LA POBLACIÓN F_2 DEL CRUZAMIENTO ENTRE EL GIRASOL SENSIBLE Y EL RESISTIBLE

RESUMEN

La línea consanguínea R28, que tiene origen en la especie *Helianthus argophyllus*, posee baja sensibilidad a la infección artificial y a la infección natural de la base del tallo y de la cabeza (podredumbre húmeda del capítulo de girasol) tanto como al filtrado de hongos (ácido oxálico). Esta línea fue cruzada con la línea consanguínea sensible 9304. Las plantas de la generación F_2 y F_1 y las plantas parentales, fueron infectadas artificialmente, de la forma siguiente: i. Con micelio del patógeno, administrado en la base del tallo (ataque en la base del tallo), ii. Con ascosporas administradas en el capítulo (ataque de la podredumbre húmeda del capítulo).

Tras infectar la base del tallo, en 72.4% F_2 de las plantas, se desarrollaron los síntomas de enfermedad, marchitándose luego. El tiempo de aparición de los síntomas, es decir, el período de incubación (PI), expresado con el número de días de infección hasta la presentación de los primeros síntomas, variaba entre 5 y 25 días tras la infección, con el promedio de población de 12.5±6.1 días. El PI en la línea 9304, fue 7 días, y en las plantas F_1 y la línea R 28, 10 y 16 días respectivamente.

Tras la infección con ascosporas, también se determinaba el PI y el porcentaje de lesiones, y eso en el final del período de floración (PF) y en la madurez fisiológica (MF). En la prueba con ascosporas, en 60.7% de las plantas F_2 infectadas, se desarrollaron los síntomas de enfermedad en el capítulo. En este caso, el PI variaba entre 16 y 45 días, con el promedio de población de 27.6±6.4 días. El PI en la línea 9304 fue 18 días, y en las plantas de F_1 y de la línea R 28, 25 y 35 días respectivamente. De todas las plantas con lesiones en la época de MF, 24% eran totalmente seguros en la fase PF, lo que indica diferente reacción de los genotipos F_2 al patógeno *Sclerotinia*. Se estudiaron las relaciones entre PF, MF y PI, y los resultados obtenidos indican la presencia de dos mecanismos diferentes de ataque de este patógeno.

Estamos considerando la posibilidad de obtener resultados de los programas de selección clásicos, utilizando estos parámetros. También estamos considerando un acercamiento, utilizando los marcadores moleculares. Recientemente se crearon varias centenas de microsatélites para el girasol. Para este fin hemos elegido el primer conjunto de combinaciones de primers, a base de longitud de los fragmentos fortificados ("amplicones") para posibilitar la multiplexión. Los marcadores SSR fueron probados en la presencia de polimorfismos, utilizando los multiplexos tricolores.

EVALUATION DE VARIABILITÉ GÉNÉTIQUE SUR LE PATHOGÈNE *Sclerotinia sclerotiorum* Lib. de Bary DANS LA POPULATION F$_2$ DU CROISEMENT ENTRE LE TOURNESOL SENSIBLE ET LE TOURNESOL RÉSISTANT

RÉSUMÉ

La ligne cultivée R28 issue de l'espèce *Helianthus argophyllus*, montre une faible sensibilité à l'infection naturelle concernant la base de tige et la capitule (pourriture de capitule) aussi bien à l'infection artificielle concernant le filtrat fongique (acide oxalique). Cette ligne est croisée avec la sensible ligne cultivée 9304. Les plantes de générations F$_2$ et F$_1$ et les plantes parentales sont artificiellement infectées à la manière suivante: i. le pathogène mycélium est appliqué sur la base de tige (attaque de la base de tige), ii. l'ascomycète est appliqué sur la capitule (attaque de la capitule).

Après l'infection de la base de tige, 72,4% de plantes F$_2$ ont développé les symptômes de maladie et se sont fanées. La période d'apparition de symptômes, c'est-à-dire la période d'incubation (PI) présentée par le nombre de jours à partir de d'apparition de premiers symptômes, a varié de 5 à 25 jours après l'infection, avec la population en moyenne de 12,5±6,1 jours. La période d'incubation pour la ligne 9304 était de 7 jours, tandis que pour les plantes F$_1$ et la ligne R28 de 10 et à 16 jours respectivement.

Après l'infection d'ascomycète, la période d'incubation et le pourcentage de lésions ont été enregistrés à la fin de la phase de floraison et maturation physiologique. Les résultats montrent que 60,7% de plantes F$_2$ ont développé les symptômes de maladie. La période d'incubation, dans ce cas a varié de 16 à 45 jours, avec la population en moyenne de 27,6±6,4 jours. La période d'incubation pour la ligne 9304 a été de 18 jours, tandis que pour les plantes F$_1$ et la ligne R28 25 et de 35 jours respectivement. De toutes les plantes avec lésions pendant la phase de maturation physiologique, 24% de plantes ont été sécurisées pendant la phase de floraison, cela témoigne d'une réaction différente de génotype F$_2$ au pathogène *Sclerotinia*. La phase de floraison, la phase de maturation physiologique et la période d'incubation ont été étudiées et les résultats montrent la présence de deux différents mécanismes d'attaque de ce pathogène.

Cette recherche prend en considération la possibilité d'obtenir les résultats du programme de sélection classique utilisant ces paramètres. Une approche assistée par les marqueurs moléculaires est envisagée. Récemment, quelques centaines de marqueurs microsatellites ont été développés pour le tournesol. Afin de réaliser ce but le premier groupe de combinaisons de "primers" est sélectionné à la base de longueur des fragments ("amplicon") pour faciliter multiplexage. Les marqueurs SSR ont été testés à la présence de polymorphismes utilisant les multiplex tricolores.