

## **Genotypic responses and diallel analysis for an early resistance test to *Sclerotinia sclerotiorum* in sunflower**

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**Résumé** : L'évaluation de la performance des génotypes du tournesol aux infections de *Sclerotinia sclerotiorum* permet d'identifier des nouvelles sources de résistance aux différents types d'attaque produits par ce pathogène. Les tests de résistance, menés la plupart du temps au champ, peuvent fournir des renseignements pertinents sur le niveau de résistance du matériel testé. Ce dernier pouvant ensuite permettre l'obtention de variétés hybrides. Malheureusement les protocoles d'évaluation de résistance actuellement sont coûteux et difficile à mettre en place, donc l'utilisation de tests ayant le but du rétrécissement des coûts et travaux menés doit être considéré d'intérêt. Dans ce contexte cet article décrit le comportement du germplasm de tournesol à base génétique large par moyen d'un test d'évaluation de résistance à *S. sclerotiorum* réalisé en conditions contrôlées de serre à un stade précoce du développement de la plante. Un plan de croisements diallele, sans les réciproques, a été suivi en utilisant quatre populations d'origine argentine. Les dix génotypes, c'est-à-dire les 4 populations et leurs 6 descendants, ont été infectés sans blessure en déposant une graine d'avoine contaminée par du mycélium de *S. sclerotiorum* au niveau du sol et à la base de la jeune tige des plantules au stade quatre paires de feuilles. La proportion de plantules mortes est estimée 25 jours après l'inoculation. L'analyse des données a détecté des réponses significativement différentes parmi les génotypes infectés ainsi que des effets significatifs des aptitudes (générale et spécifique) à la combinaison des populations évaluées. La méthodologie usagée a permis de détecter des populations parentales du tournesol qui pourraient être utilisées en sélection pour leur performance à *S. sclerotiorum*. L'utilité de ce matériel et du test d'évaluation de résistance à *S. sclerotiorum* dans les programmes d'amélioration génétique du tournesol est discutée.

**Summary** : The evaluation of sunflower genotypic performance against *Sclerotinia sclerotiorum* infections is important for understanding its usefulness as source of resistance. In the field, artificial and natural resistance tests provide important information that can be used in the selection of the best materials, however the procedures for resistance tests are usually both expensive and laborious. This work describes the performance of sunflower genotypes of broad genetic base at an early stage of plant development using a resistance test carried out under controlled conditions in the greenhouse. Statistical analysis detected highly significant genotypic responses and the combined abilities effects among the evaluated populations and their offspring, obtained by a diallel crossing system method 2 for dead seedlings (%) at 25 days after *S. sclerotiorum* infection on the basal stem.

The methodology allowed superior parents to be detected and crosses to be furthered in the selection for *S. sclerotiorum* resistance. The usefulness of the evaluated genetic materials and the early resistance test in sunflower breeding plans is discussed.

**Mots-clés** : sélection, résistance aux maladies, test précoce, pourriture blanche, *Helianthus annuus*.

**Keywords** : breeding, disease resistance, early testing, *Helianthus annuus*, white rot.

## ARTICLE

*Sclerotinia sclerotiorum* Lib. de Bary is one of the most important pathogens in the sunflower (*Helianthus annuus* L.) crop because it can severely reduce the seed yield, particularly in temperate and humid environments [15]. Although all organs of the sunflower plant can be infected, *S. sclerotiorum* attacks on the capitula (known as white rot) are considered the principal fungal disease in Argentina and Europe [6, 7], whilst basal stem rot is frequent in central and northern Italy, especially with early spring sowing [29].

Different methods have been proposed to limit damage caused by *S. sclerotiorum* infections but at present sowing hybrids with a good resistance level to white rot is considered the most promising method for reducing the risk [7].

Sunflower breeders normally use different populations as sources of genetic variability [10]. These populations must be genetically characterised in order to develop parental inbred lines with high combining ability for agronomic traits such as white rot resistance. *S. sclerotiorum* resistance of sunflower germplasm resources is poorly described in literature. Therefore it seems to be useful to sample some sunflower populations, because of interest in possible differences of genetic variability among them, and to evaluate their usefulness as resistance sources against *S. sclerotiorum* infections.

In breeding programmes, field tests carried out with natural or artificial infections remain the most widely used method for detecting moderately resistant genotypes to *S. sclerotiorum*. However resistance evaluation to the different infection types are laborious, expensive and time-consuming. It would therefore be of great interest to use other *S. sclerotiorum* resistance tests more rapid and easy, particularly in those sunflower breeding programmes with a lack of resources. Research has been developed to successfully produce *S. sclerotiorum* infections on sunflower seedlings of inbred lines and hybrids under controlled conditions [3, 14, 17, 22], but segregating and heterogeneous populations have not yet been used. The interest of such studies lies in the possibility of their direct use in sunflower breeding programmes for white rot resistance.

The aim of this work is to evaluate the performance of sunflower populations and their offspring against *S. sclerotiorum* artificial infection on seedlings grown under greenhouse conditions and to estimate the genetic components of complex population variance.

## Materials and methods

### *Sunflower genotypes*

Germplasm of Argentinean origin was used: synthetic B1, the open-pollinated (OPP) populations Norkinsol 2, Pehuén INTA, Antilco and their offspring, obtained by a diallel genetic crossing system with Griffing method II (without reciprocals). Synthetic B1 was characterised by having no resistance genes for the local races of *Plasmopara halstedii* [23], but its performance against *S. sclerotiorum* is unknown. Antilco is one of most popular selected varieties for its relatively high seed yield; its *S. sclerotiorum* behaviour is also unknown. The last two OPP have contrasting resistance levels to white rot in the capitula [12]: Pehuén INTA is fairly resistant and Norkinsol 2 is highly susceptible.

### *Experimental design and infection method*

An infection test was carried out at the University of Udine, Italy, following the general procedure described by Tahmasebi-Enferadi *et al.* [22]. Twenty seeds of the 10 genetic materials (*i.e.* the four sunflower populations and their six offspring) were sown in aluminium pots (30 cm long, 20 cm wide and 6 cm deep) filled with a sandy loam soil. Pots were maintained under controlled conditions in a greenhouse, with daytime temperatures of 22 and 18° C at night, and 60 and 70% relative humidity, respectively. A constant 16 hour photoperiod, provided by a timer connected to 10 lamps (Philips® SGR 200/400), assured a regular minimum photon flux density of about 900  $\mu\text{Em}^{-2}$  above the seedlings. Soil moisture content was maintained at approximately field capacity until the end of the experiment. The experimental scheme was a randomised complete block design with three replicates (pots).

*S. sclerotiorum* inoculum was produced from sclerotia harvested from stems of sunflower plants naturally infected in a previous season. Disinfected sclerotia and sterilised oat grains were placed together on a potato dextrose agar medium in Petri dishes, which were incubated in the dark at 25° C. After a three-four weeks delay, the oat seeds infected with the pathogen mycelium were removed, air dried and stored in sealed Petri dishes.

Infecting was done 28-days after sowing, at the V8 stage of sunflower plant development [20] or its homologous B8 [9], by placing at soil level an infected oat grain on the basal stem of the young plants. At least a dozen sunflower seedlings were infected per pot. The inoculum was then covered with moistened cotton and a plastic film in order to maintain humidity. A fourth plot with non-infected oat grain placed on the basal stem of seedlings provided a control material. The percentage of dead seedlings at 25-days after infection was calculated. An average value of dead seedlings (%) by genetic material and pot was obtained.

### *Data analysis*

Data were first analysed to test the significance of genotypic differences by an analysis of variance for a two-way comparison of means. The next step was to calculate the separation of the means by the least significant difference test (LSD); the genetic materials were then ranked in descending order according to their mean performance against *S. sclerotiorum*. A degree of

genetic determination approach was used in order to understand the extent to which individuals' phenotypes are determined by their genotypes.

Secondly, analysis of variance for a fixed model using Griffing's method II was done (*i.e.* the parents *per se* and the one set of F1 crosses' responses to *S. sclerotiorum* infections) to estimate the significance of both general (GCA) and specific (SCA) combining abilities effects. The values of GCA and SCA effects and their respective standard errors were also obtained following standard procedures.

To test the significance of association between the dead seedlings values (in %) of parents and their offspring, Spearman's nonparametric test was done analysing the data by ranking the sunflower genotypes and calculating the coefficient of rank correlation.

Statistical and genetic analysis was done according to Reza Hosmand [18], and Singh and Chaudary [21], respectively. The software MSTATC was used.

## Results

### *Genotypic responses after S. sclerotiorum infection*

In order to evaluate the feasibility of the early resistance test and its power of discrimination among populations, the 10 sunflower genotypes were evaluated under greenhouse conditions for their responses to *S. sclerotiorum* infection on the basal stem. *Table 1* shows the mean of dead seedlings for every infected genotype. A mean of 58.3% indicates that, 25-days after the infection date, less than half the infected seedlings survived. The range of the 10 sunflower genotypes for dead seedlings was 64.6%. The coefficient of phenotypic variability calculated (CV = 7%) suggested a high precision in scoring the experimental data.

Analysis of variance showed highly significant ( $p = 0.001$ ) genotypic effects; the null hypothesis of no difference among the infected sunflower genotypes was thus rejected. No significant replicate effects were detected.

The least significant difference was estimated in order to calculate the difference of means that are just significant between any pairs of means at a probability of committing a type I statistical error of  $\alpha = 5\%$ . The LSD value of 7% allowed genetic materials to be classified in three groups according to their dead seedling values. Group 1 (G1) involved only the cross Antilco x Pehuén INTA that has the highest dead seedling value (88.8%) in the experiment. Group 2 (G2) was formed by the OPP Pehuén INTA (27.8%) that does not differ from the cross Norkinsol 2 x B1 (24.2%), both with the lowest dead seedling value. Group 3 (G3) covered the rest of the genetic materials.

The degree of genetic determination (DGD) was 98%, suggesting that only 2% of the phenotypic variability among the sunflower populations was due to non-genotypic effects. The coefficient of genetic variability (CGV) was 34%, indicating not-negligible genotypic differences that can ease the selection for disease resistance.

### *Diallel analysis*

A diallel study was done to check if this early resistance test could be applied to heterogeneous sunflower germplasm and whether it might be of interest in breeding programmes for this oleaginous crop. A diallel cross analysis was therefore done for a fixed model, using Griffing Method II with parents but without including reciprocal crosses.

Analysis of variance showed that GCA and SCA effects were statistically significant ( $p = 0.001$ ), suggesting that considerable genetic variability exists among these sunflower populations and crosses. This indicates that both additive and non-additive effects were involved in the expression of the dead seedlings after *S. sclerotiorum* infection on the basal stem, although the non-additive variance was six times higher. Therefore, the genes affecting the evaluated trait in the assessed genotypes have mainly non-additive effects; nevertheless, the additive effect is far from being unimportant.

The estimated GCA and SCA effects are shown in *table 2*; the negative values are to be considered as favourable since they indicate the superior performance of both parents *per se* and in crosses. Antilco and B1 populations showed significant ( $p = 0.05$ ) GCA effects. Because the synthetic B1 had the most favourable GCA effect (3.72%), it can be considered the best combiner in this experiment and has potential for improving *S. sclerotiorum* resistance in this species.

All SCA effects were significantly different from zero and vary from 25.20% (Antilco x Pehuén INTA) to 28.75% (Norkinsol 2 x B1); the latter population has the most favourable SCA effect because it had a higher percentage of survived seedlings than expected from the GCA effects from its parents. The population crosses Antilco x B1 and Norkinsol 2 x Antilco have intermediate values of surviving seedlings after *Sclerotinia* infection.

The Spearman rank correlation coefficient calculated ( $r_s = 0.35$ , not significantly different from zero) suggests that the degree of closeness of the linear association between the disease parental rank and that of the mean of their crosses was negligible. Therefore the ranking of sunflower crosses cannot be predicted from the *per se* rank of their parents because of the significant non-additive effects.

### **Discussion**

A successful and efficient resistance test is important in breeding for disease resistance. This work uses an *S. sclerotiorum* methodology on sunflower populations at an early stage of development, grown under greenhouse conditions.

The infection procedure showed a series of advantages that could be considered as a useful tool for disease improvement. The experiment occupied a small physical space inside a greenhouse and the infection test was easily carried out, suggesting that few human and economic resources are necessary. The data were obtained with a relatively high precision and early in the sunflower development (*i.e.* less than two months after sowing). Early knowledge on genotypic performance (e.g. before flowering) is of interest in sunflower breeding given that selection for *S. sclerotiorum* resistance could be twice as effective as selection after pollination because a parental control could be done efficiently (*i.e.* both parents, the male and female plants, could be selected) during the recombination process if, for example, a recurrent selection programme for

disease resistance is done. The sunflower seedlings are grown in a greenhouse under controlled conditions, permitting an off-season environment to be used where resistance performance and breeding operations (*i.e.* emasculation, hybridisation, inbreeding, seed increase) can be conducted. Multiple generations of sunflower crop can therefore be produced, with an increased genetic gain per year in breeding programmes.

The early resistance test allowed populations to be differentiated according to their resistance level to *S. sclerotiorum* infection at seedling stage. Although the high performance of Pehuén INTA and the relatively low one of Norkinsol 2 (*Table 1*) show an interesting agreement with their field behaviour against white rot in capitule [12], the performance of only two OPP is not enough argument to conclude that the early resistance test could be usefully utilized to estimate the genotype behaviour against the *S. sclerotiorum* attacks in heads. Further experiments including additional genotypes of well-known field resistance level to capitulum infection could help to know whether the results of this early resistance test are associated with.

The high degree of genetic determination agrees with that shown by Gallo *et al.* [11] when a set of commercial sunflower hybrids were evaluated for their mycelium extension resistance on the principal vein of leaves infected with *S. sclerotiorum*, and by Castaño *et al.* [4] when a dozen F1 single crosses were evaluated for white rot severity in the capitula. The fact that only one experiment was done suggests however that more studies involving the same sunflower materials will allow a more precise estimation of genetic parameters to be made

The selection of sunflower populations with acceptable resistance levels against *S. sclerotiorum* infections is an arduous task. Some variables, such as available resources (*i.e.* funding and access to an experimental field and auxiliary personnel) and the polygenic nature of *Sclerotinia* resistance in sunflower [5, 7, 8], affects the decision-make process to determine which genetic material is the most suitable to be furthered in a breeding programme.

In this work, Griffing's diallel analysis provided useful information on the potential of the sunflower populations to produce superior offspring by hybridisation and the magnitude of gene action. In this sense, the results on genetic variability and combining ability indicated the significance of mostly non-additive effects but also of additive ones as components in the genetic control of the dead seedling performance after *S. sclerotiorum* infection.

There are few reports on the sunflower-*S. sclerotiorum* pathosystem where the host response is mainly conditioned by non-additive gene effects. Most papers show genetic effects preponderantly additive for disease resistance when single hybrids were artificially infected by *S. sclerotiorum* on plant organs such as capitula [13, 19, 27], leaves [5], terminal buds [1], basal stem [14] and cotyledons [24]. Conversely, Vrânceanu *et al.* [28] showed strong SCA effects for resistance to mycelium infection of *Sclerotinia* basal attack in sunflower germplasm and Pîrvu *et al.* [16] detected two inbred lines with a single recessive gene controlling mechanical resistance to initial penetration of the stem-surface tissues by *Sclerotinia* mycelium. In our work, the populations would have enough additive genetic variance to show significant responses from selection for *S. sclerotiorum* resistance at the seedling stage but these responses could be significantly enhanced in the offspring if the population resistance genes were combined by crossing.

Sunflower breeders use extensively intra- and inter-population recurrent selection schemes to develop improved source populations for different characters of agronomic interest from which lines for hybrid production can be derived and selected [10]. Traditional methods such as recurrent phenotypic selection have been effective in improving sunflower populations for white rot resistance in the capitula [25, 26] and it is considered that in the near future these selection methods will incorporate non-traditional techniques such as molecular markers for *Sclerotinia* resistance in order to improve the efficiency of plant breeding programmes [2]. Our results showed that the synthetic B1 and the population cross Norkinsol 2 x B1 (*Table 2*) had the most favourable GCA and SCA effects, respectively. These genetic materials should therefore be kept in mind for use in breeding programmes for *Sclerotinia* resistance.

The results show the practical interest in sunflower improvement for *S. sclerotiorum* resistance that the used test would have. Nevertheless, further experiments must be developed to confirm the repeatability of results, the performance of evaluated populations, and the utility of some sunflower germplasm as a source of resistance.

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## CONCLUSION

The early resistance test under greenhouse conditions detected differential responses among the segregating and heterogeneous sunflower populations. This test can be considered as a technique that reduces the complexity and operative costs of sunflower breeding for disease resistance to *S. sclerotiorum* infections.

The synthetic B1 has resistance genes against *S. sclerotiorum* infection that can be used in breeding programmes either for *per se* selection or for inter-population selection when combined in a specific cross with the Norkinsol 2 population.

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## Illustrations

Table 1. Mean of dead seedlings (in %) at 25 days after *Sclerotinia sclerotiorum* artificial infections on basal stem of sunflower seedlings grown in a greenhouse.

Sunflower genotypes (Code)	Mean of dead seedlings (%)
Antilco x Pehuén INTA (AxP)	88.8
Norkinsol 2 x Pehuén INTA (NxP)	76.1
Pehuén INTA x B1 (PxB)	70.6
Antilco (A)	62.7
Norkinsol 2 (N)	62.5
B1 (B)	60.7
Norkinsol 2 x Antilco (NxA)	56.5
Antilco x B1 (AxB)	52.8
Pehuén INTA (P)	27.8
Norkinsol 2 x B1 (NxB)	24.2
Mean	58.3
CV (%)	7.0
LSD (p < 0.05)	7.0

Table 2. Combining abilities (general, in diagonal, and specific, off diagonal)  $\pm$  standard errors of 10 sunflower genotypes evaluated by dead seedlings (in %) 25 days after at basal stem infection.

Genotypes (codes <sup>a</sup> )	N	A	P	B
N	-1.59 $\pm$ 1.44	-5.54* $\pm$ 2.57	19.46** $\pm$ 2.57	-28.75** $\pm$ 2.57
A		5.36* $\pm$ 1.44	25.20** $\pm$ 2.57	-7.10* $\pm$ 2.57
P			-0.04 $\pm$ 1.44	16.10** $\pm$ 2.57
B				-3.72* $\pm$ 1.44

<sup>a</sup>: See table 1.

\*: p = 0.05

\*\* : p = 0.01