

## 15e CONFERENCE INTERNATIONALE TOURNESOL

### 15th International Sunflower Conference Horizontal resistances in sunflower: a review of a workshop at the 15th International Sunflower Conference

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**Résumé** : Un atelier sur la résistance horizontale, dans le cadre de la 15e Conférence sur le tournesol tenue à Toulouse, France du 12 au 16 juin 2000, a traité de l'évaluation, la stabilité et l'héritabilité des résistances du tournesol à *Sclerotinia sclerotiorum*, *Diaporthe helianthi*, *Alternaria helianthi* et *Verticillium dahliae*. On a pu remarquer que les recherches sur les meilleurs moyens d'évaluer la résistance continuent, en même temps que les études de génétique quantitative, la recherche de marqueurs moléculaires et de QTL, et les essais d'amélioration de la résistance par transgénèse.

**Mots-clés** : *Alternaria helianthi*, génie génétique, *Helianthus annuus*, *Phomopsis helianthi*, *Sclerotinia sclerotiorum*, sélection, *Verticillium dahliae*.

**Summary** : A workshop on horizontal resistance at the 15th International Sunflower Conference, held in Toulouse, France, from June 12 to 16, 2000, covered topics including the evaluation, stability and inheritance of resistance of sunflower to *Sclerotinia sclerotiorum*, *Diaporthe helianthi*, *Alternaria helianthi* and *Verticillium dahliae*. It was notable that research continues into evaluation methodology at the same time as quantitative genetics, use of molecular markers, search for QTL and resistance improvement by genetic engineering.

**Keywords** : *Alternaria helianthi*, breeding, genetic engineering, *Helianthus annuus*, *Phomopsis helianthi*, *Sclerotinia sclerotiorum*, *Verticillium dahliae*.

#### ARTICLE

The 15th International Sunflower Conference held in Toulouse, France, from June 12 to 16, 2000, included a number of workshops dealing with the most important research subjects on sunflower. The present account provides a review of the results presented concerning Horizontal Resistance. The term Horizontal Resistance was proposed by Van der Plank [1] after analysis of diagrams, which showed the quantity of blight that several potato cultivars suffered when they were subjected to 16 races of *Phytophthora infestans*. These graphs showed the presence of a constant ranking of cultivars and pathogen races in order of resistance or aggressiveness, respectively, and the absence of significant interactions between host and pathogen genotypes. Sunflowers show Horizontal Resistance to a wide range of pathogens in different parts of the world [2] and since control is

polygenic, considerable work is necessary both to determine genetic control and to obtain significant selection responses. The pathogen, which appears to give rise to most research, is *Sclerotinia sclerotiorum*, followed by *Phomopsis (Diaporthe helianthi)* and *Alternaria helianthi*, and then *Verticillium dahliae*. At the Toulouse conference, there were no reports on *Botrytis cinerea*. The following three main objectives appear in work on Horizontal Resistance in sunflower.

### **Development of methods for measuring horizontal resistance**

One of the keys to study sunflower-pathogen interactions and to develop control methods is the development of strategies that allow quantitative measurement of disease symptoms. Techniques making it possible to quantify disease with precision are essential for effective breeding programmes and to make good use of the molecular markers now available, so that the genomic region carrying factors controlling Horizontal Resistance can be determined with minimum error. It is thus indispensable to study what, how, when and where to evaluate disease performance.

Although studies of techniques to infect sunflowers with *S. sclerotiorum* started more than 25 years ago [3], they can still be improved. Millet grains infected with *S. sclerotiorum* mycelium were introduced in capitula through a wound by Hahn [4]. Differences in the mycelium extension among the inoculated inbred lines were found to be mainly determined genetically. However, the author indicated that the use of this test in order to detect differential performances to white rot resistance did not always give useable results and that further work is necessary both to improve their repeatability and to compare them with results obtained under natural infections. Infected grains (this time barley) were also used to infect the root system of young sunflower plants by Pérès *et al.* [5]. Basal stem symptoms began to appear 25 days after planting. Since this test can detect differential responses of genotypes, is not very expensive, needs little space to be developed and its results are correlated with natural infection, it could be used in selection.

Pérès [6] also reported improvements in the efficiency of a terminal bud test, at present made in the field. Terminal buds of hybrid seedlings were infected in the greenhouse with aqueous suspensions of *S. sclerotiorum* ascospores and active apothecia were present. Maximal hygrometry was maintained by situating the infected seedlings under a small polythene tunnel. Disease symptoms on buds appeared 16 to 48 days after inoculation. This method showed different responses according to genotype and results obtained in greenhouse were generally correlated with those obtained in the field although greenhouse conditions enhanced the susceptibility of genotypes showing a medium resistance level in the field. Since the physiology of seedlings in greenhouse does not appear modified, the author suggests the possibility of using this system in breeding for terminal bud resistance.

Răducanu *et al.* [7] used culture filtrates, isolated from different parts of sunflower plants infected with *S. sclerotiorum*, to make *in vitro* and *in vivo* inoculations of inbred lines and hybrids. There was an effect of filtrates on some agronomic traits such as seed weight and oil percent and the electrophoretic spectrum of helianthines was modified. *In vitro* results were correlated with those *in vivo*, so the methodology proposed could be used in breeding since it is also economic and easy to carry out. Tahmasebi-Enferadi *et al.* [8] also inoculated seedlings of inbred lines and hybrids with the mycelium and culture filtrate of *S. sclerotiorum*. Their results indicated that the culture filtrate induced reactions similar to those following mycelium inoculation and both tests increased the oxalic acid (OA) level in the leaves. The last trait could be therefore successfully used in breeding for

resistance to *S. sclerotiorum*. An increased activity of shikimate dehydrogenase enzyme in the inoculated plants was observed after mycelium and culture filtrate treatments. The authors suggested that this enzyme was triggered by a more specific reaction between sunflower-*S. sclerotiorum* relationship and it is not simply induced by the increase of OA in the host tissue.

A decade of investigations on relations between sunflower and *Diaporthe helianthi* and breeding for *Phomopsis* resistance were summarised by Viguié *et al.* [9]. Two methodologies are used to evaluate levels of resistance. Firstly, under semi-natural infection, infected stems with perithecia are placed between plots, irrigation is applied if necessary and the numbers of plants showing lesions at least 5 cm long at physiological maturity are counted. Secondly, agar disks containing *Phomopsis* mycelium are placed in the tips of leaves or cut petioles and lesions lengths are measured. Over 10 years, the disease incidence increased more than 30% with the leaf test, probably because *Phomopsis* isolates are kept at - 80° C and their aggressiveness is checked in the greenhouse before use in the field; the inoculations are made when plants are in the star-bud stage and a given genotype is inoculated on one day and its disease incidence is defined in comparison with the control inoculated the same day. Mycelium infections are successful every year, in contrast to semi-natural infections, but they do not cover the whole infection cycle and their cost is greater. Leaf infections should be preferred in breeding programmes since it is better correlated with semi-natural infection. The latter must be carried out in regions where pathogen exists to confirm genotype ranking.

The methods described so far all concern whole plant reactions. The study of defence mechanisms could provide an useful analysis of these reactions since Horizontal Resistance is determined by numerous and complex mechanisms that reduce the rates of infection and colonisation in the plant and of reproduction of the pathogen. When well known, defence mechanisms could be used as selection characters. Biochemical analyses are a first step in this direction and Prats-Pérez *et al.* [10] studied Horizontal Resistance determined by phenolic compounds, preformed or induced after *S. sclerotiorum* capitulum infection. It is known that, after infection, phenols are deposited on the cell walls of resistant cultivars and these compounds become melanized and lignified. Necrotic lesions were observed in anthers, bracts and receptacle and showed that mycelium was present in the anthers of all inoculated genotypes, indicating that there was no impediment to the ascospore germination on the capitulum surface. Susceptible genotypes had necrosed ovaries and abundant lesions in corollas, bracts and capitula whereas the resistant ones had only limited necrosis in the ovary and corolla and no symptoms in the other organs, so the presence of a defence mechanism stopping the pathogen growth beyond the anthers was suggested. Analysis of total phenols in healthy tissue around lesions in bracts and corollas indicated that the quantity of phenol varied with genotype, time from inoculation and tissue. The most resistant genotypes had more total phenols before and after inoculation and the same was true for some specific compounds (7-hydroxylated simple coumarins). Detection of genetic factors coding for phenolic compounds by molecular tools could make possible markers assisted selection.

### **Heritability and stability of horizontal resistance and breeding methods**

In studies of the inheritance of Horizontal Resistance, several factors need to be considered in order to make accurate genetic interpretations necessary for breeding: i) the scale to be used, ii) how many times during the plant-growing season the evaluations will be made? iii) which pathogen races or isolates should be used if the epiphytotic is induced by artificial inoculations? iv) what types of

parameters can be used to estimate genotype responses (e.g. severity, incidence, incubation period), v) what statistical analysis will give the best characterisation of inheritance (general means, diallel, factorial analysis, etc.)?

Inbred lines and hybrids were evaluated by Langar *et al.* [11] for their reaction to different *Phomopsis* isolates. Genotype and isolate effects explained almost all variability observed. The appearance of genotype x environment (G x E) interaction, suggested that it was necessary to study the stability of resistance because the environmental conditions affects mycelium growth. The same authors studied the genetic variability of *D. helianthi* responses in F3 and F7 populations obtained from a two-parent (susceptible x resistant) cross [12]. Experiments were carried out under semi-natural and controlled conditions. Heritability coefficients showed an important range of values but since trials were carried out in a single year, these may be over-estimated because there was no estimation of G x E interaction. Some F3 families showed greater symptoms than the most susceptible parental line under semi-natural infection, whereas a transgression effect toward the resistant inbred line was not detected. This suggests the presence of genetic factors diminishing the resistance level in the susceptible parent. The distribution of individual frequencies for the lesion length in leaves of F7 lines was between the parents, suggesting the existence of a segregating major gene. Low but significant correlations were found between the necrosis growth in controlled conditions and disease incidence under semi-natural infection, perhaps due to the presence of different resistance components, which could act at different sunflower development stages. The authors indicated that some lines can be considered as a good germplasm for breeding for *Phomopsis* resistance and that selection efficiency can be maximised using molecular markers.

Breeding new sunflower varieties is a very expensive business and the requirements demanded of the product of this breeding work - the new variety - are constantly increasing. There is thus a real need for all breeding plans that have to maximise genetic improvement by disease resistance. Therefore it is necessary to have information concerning the availability of resistance sources and genetic and environmental factors which can affect the magnitude and expression of the resistance. Masirevic [13] evaluated sunflower germplasm for its reaction to *Phomopsis* stem canker. Trials carried out in Europe and North America showed high levels of resistance (from 3 to 30% of disease attack under natural infections) in commercial cultivars, Yugoslavian hybrids, crosses between Yugoslavian and French inbred lines and Plant Introductions. All this material can be used as resistance sources in breeding programmes. It was suggested that studies of stability of resistance should be made in order to manage more efficiently the effects of G x E interaction.

The evaluation of inbred lines for *per se* and hybrid performance is of interest in breeding plans for white rot resistance in capitula. Experiments in different environments allow the assessment of both inbred lines with good combining ability and the G x E interaction. *S. sclerotiorum* inoculations of a set of inbred lines and their offspring were made by Godoy *et al.* [14]. Results indicated that some inbred lines show-ed good general combining ability. The relative disease incidence of F1 hybrids was significantly different in two locations, with G x E interactions. Field evaluation of new genotypes is one of the most laborious and expensive steps in breeding plans and these results show the need both to use more environments and to study G x E interaction to maximise effective allocation of resources. The results also indicated that large sunflower cultivation areas should be divided into sub-areas in order to enhance the efficiency of the white rot evaluation.

These papers have shown how the phenomenon of G x E interaction for a quantitative trait such Horizontal Resistance can: i) reduce the usefulness of subsequent analyses, ii) restrict the significance of inferences that would otherwise be valid, iii) seriously limit the feasibility of selecting superior genotypes. An increase in G x E interaction variances decreases the correlation between genotypic and phenotypic values, and subsequently the response to selection. Thus, it is important to study the phenotypic stability of cultivars for disease resistance. Six experiments, carried out by Escande *et al.* [15] under natural infection of *Verticillium dahliae*, were made to evaluate the adaptability of cultivars to these environments. Disease intensity was measured as foliage symptoms and stem break and four different methods to analyse cultivar stability to *Verticillium* wilt were compared. Results showed that cultivars changed in relative ranking from one environment to another. Regression analysis of phenotypic values on an environment index was the most appropriate to detect stable cultivars. Standard deviation of cultivars in one of the environments helped to detect different levels of stability.

To improve Horizontal Resistance by conventional breeding, the genomes of two or several sunflower plants has to be combined and mixed and the descendants evaluated and selected. Population improvement has the objective of increasing the frequency of resistant. Gonçalves-Ungaro [16] showed that mass selection was not effective for increasing the level of resistance to *Alternaria helianthi* (producing leaf and stem blight) in a complex sunflower population. In contrast, recurrent selection based on half-sib families was efficient to develop less susceptible genotypes in two populations: irradiated or not with gamma rays. Vear *et al.* [17] presented results of evaluation of *S. sclerotiorum* attacks on capitula of F3 and F4 families obtained by pedigree selection in crosses with a susceptible test-er. The system of comparing these hybrids with those obtained from crosses of the same tester and the parents of the original cross should help in breeding programmes by defining the F3 families with significantly improved resistance. Artificial infections should be considered as a complementary step to semi-natural infections during pedigree selection programmes.

Ravikumar [18] described the results obtained after a cycle of gametophytic selection by *Alternaria helianthi* resistance. Genotypes with different resistance levels were crossed and an *A. helianthi* culture filtrate was applied, one hour before pollination, on the stigma and style of those plants used as females. The F1 hybrids obtained were evaluated in the field under natural infection and showed less disease intensity than the checks. This implies that stigmas stressed with pathogen toxins allowed only the pollen grains having resistance genes to germinate and then produced selective fertilisation. After to a cycle of gametophytic selection, there appeared to be preferential transmission of the resistance character. Although the genetic gain was not very high, the author suggested that the efficiency *A. helianthi* resistance breeding could be increased combining this methodology and phenotypic selection on adult plants.

### **Use of molecular tools in horizontal resistance genetics and breeding**

In addition to the use of various breeding methods and of chemical, physical and biological measures, modern plant protection in sunflower makes increasing use of biotechnology. One approach for integrating biotechnology with traditional sunflower breeding is marker-assisted selection.

High-density linkage maps of molecular markers are now available and statistical associations between alleles at quantitative trait loci (QTL) can be used to select indirectly, but with very high accuracy, for DNA segments containing favourable alleles, increasing the heritability of Horizontal Resistance.

Bert *et al.* [19] presented a programme comparing QTL for *S. sclerotiorum* and *D. helianthi* resistance. Two inbred lines were crossed to obtain a F2 population and F3 families, which were evaluated for disease resistances. The *S. sclerotiorum* test showed the best polymorphism. Use of both RFLP and AFLP markers will make possible the construction of a saturated genomic map and it will be possible to compare these QTL with those detected in other populations and to study the genetic control of certain agronomic characters. Jouan *et al.* [20] found that some QTL coding for agronomic and resistance traits were located near the gene *b1* which controls an apical branching, such that branched plants often possessed higher resistance levels to *S. sclerotiorum*. In this study, the same sunflower populations as Bert *et al.* [19] were used and results showed that a large proportion of phenotypic variability for oil content and for seed yield per plant was explained by the *b1* gene, whose effects could be pleiotropic. In contrast, although branched plants showed better reactions to the ascospore test they were more susceptible than unbranched plants to the mycelium test, suggesting that although QTL for resistance appear located close to the *b1* gene they should be separate genes.

Genetic engineering by the transfer of segments of foreign DNA to a chosen receiver genotype could provide new developments in resistance breeding. Genes from various sources (bacteria, fungi, etc.) could be constructed in such way that, following their transfer, they become active in a chosen organ or tissue at a specific time during plant growth. This would allow the breeder to work more rapidly and to envisage new levels of Horizontal Resistance. Concerning *S. sclerotiorum* resistance, it is known that cell walls in sunflower plants are weakened by the action of *S. sclerotiorum* toxins because the OA they contain acidifies host tissues and causes chelating of calcium. An interesting strategy to increase the resistance to *S. sclerotiorum* could be the selection of plants not susceptible to the toxins. Transfer from wheat into cultivated sunflower of a gene coding for an enzyme detoxifying *S. sclerotiorum* toxins - oxalate oxidase (OxOx) -, reported in three papers can be considered as an original step in the search for increased resistance to this pathogen.

Lu *et al.* [21] demonstrated that the gene coding for the OxOx enzyme can have continuous expression throughout the life cycle of sunflower cells and perhaps also in all tissues, so it could be useful against all types of *S. sclerotiorum* infection. Results showed that constitutive promoters of the OxOx gene are active during different stages of sunflower development. The omega' element enhanced the promoter activity in transgenic sunflower. The oxalate transgenic sunflower expressed oxidase activity in various plant tissues and conferred increased resistance to *S. sclerotiorum*. Scelonge *et al.* [22] detailed the methodology to transfer the OxOx gene into sunflower using *Agrobacterium tumefaciens*. Using meristem transformation, the authors recovered 2 and 46% percent of T0 and T1 events respectively. Leaves of T1 young sunflower plants were evaluated for OxOx enzyme assay and ELISA, and ELISA positive T1 plants were evaluated for Southern analyses at a later development stage to confirm the stable transgene integration. In transgenic plants lesions from inoculated petioles stopped at the stem whereas in control plants, lesions elongated steadily

and girdled the stem. The number of sclerotia was significantly lower in transgenic plants. Higher OxOx protein levels and lower numbers and weights of sclerotia were scored when the transgenic locus was homozygous. However, the absence of correlation between stem resistance level and the homozygous or heterozygous state of oxalate locus suggests that OxOx gene is not the only one controlling *S. sclerotiorum* resistance in sunflowers. Bazzalo *et al.* [23] carried out inoculations on capitula of transgenic inbred lines and their hybrids. Results showed that transgenic inbreds and hybrids had less disease than their isogenic equivalents. The resistance level attained was similar to moderately resistant checks. It should be highlighted that the OxOx gene effect was similar in all hybrids, so its effect could be considered independent of the genetic background into which it was inserted.

## CONCLUSION

The reports in this workshop showed that horizontal resistance is an important objective in sunflower research and that considerable effort is necessary to answer the questions raised. It is notable that research continues on resistance observations at the same time as genetic and molecular studies. Accurate measurements of resistance that take into account environmental interactions and possible variation of the parasite, almost systematically require definition of several different characters that are less complex to analyses than overall resistance levels. At present, measurements of phenotypic reactions are imperfectly correlated with resistance genotypes, but they have made possible first QTL and gene analyses. In the future, such identification of the genes and biochemical pathways involved should help, in return, to provide improved definition of the exact characters that are the most important in horizontal resistance in sunflower. Breeding programmes will then use combinations of marker assisted genotypic selection and observations of the most useful phenotypic characters. Finally, it may be suggested that the best use of genetic engineering would be if extremely effective genes, giving resistance levels that cannot be obtained from *Helianthus annuus* or its wild relatives, become available.

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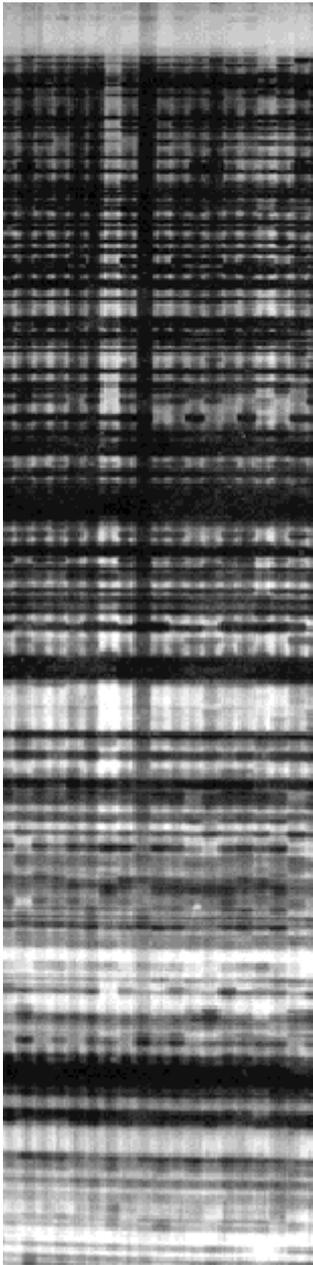
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## Illustrations



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*Photo. Numerous techniques of molecular biology are now widely used for studying genetic resistance of sunflower. Obtaining various molecular markers allows high-density linkage maps construction and Quantitative Traits Loci detection. An illustration of molecular analysis is the AFLP™ technology (Vos et al., 1995). Based on PCR amplifications of DNA fragments after specific primers fixation, this techniques generate a very high rate of polymorphic bands as can be seen on photo (Bert et al., UMR INRA-UBP : « ASP », Clermont-Ferrand).*

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