The use of natural infections under controlled conditions and of artificial infections to estimate phomopsis resistance of sunflower hybrids: conclusions after ten years of trials

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Summary:

The reactions of sunflower hybrids to attack by *Diaporthe helianthi* have been studied in the field, with natural infections after provision of inoculum, conditions of favourable humidity (irrigation) and the presence of controls with different flowering dates (system denoted semi-natural attack).The frequencies of plants showing stem lesions in these conditions was compared with the results of artificial infections with *Phomopsis helianthi* mycelium, on leaves and petioles, where lesion lengths and frequencies were noted, along the main vein of the leaf and on the stem, respectively. In spite of homogenised conditions, the semi-natural attack trials showed large variations between locations and between years (from <5% to >60% for one hybrid). In contrast, the artificial infections always gave sufficient frequencies of measurable lesions to provide usable data. Percentage successful infection was always greater for the petiole test (>80%) than for the leaf test (average 70%). There was a closer correlation between natural attack and the leaf infections than with the petiole infections, but the two mycelium tests appear complementary, measuring different resistance factors. However, some genotypes which appear susceptible to the mycelium tests, show good resistance to semi-natural attack, perhaps because the artificial infections with mycelium do not measure resistance to fungal penetration into leaves; a test using *D.helianthi* ascospores would be necessary to demonstrate this type of resistance.

Résumé :

Les comportements d'hybrides de tournesol vis-à-vis de *Diaporthe helianthi* ont été analysés en infection naturelle sous conditions contrôlées : irrigation, apport d'inoculum sous forme de tiges infectées l'année précédente et témoins de floraison (appellé attaque semi-naturelle). Les taux d'attaques sur tiges observés dans ces essais ont été comparés avec réponses à des tests d'infection artificielle avec du mycéluim sur feuilles et sur petioles où on mesure la vitesse d'extension du parasite le long de la nervure principale de la feuille ou sur la tige, respectivement. Pour les essais en attaque semi-naturelle, malgré les conditions d'irrigation, les taux d'attaques observés different considérablement d'un lieu à l'autre et d'une année sur l'autre (de quelques % à plus de 60% d'attaques). Au contraire, les infections artificielles donnent toujours des taux d'attaque suffisant pour être informatifs. Il faut noter cependant des taux de réussite toujours supérieurs avec le test sur pétiole (> 80%) qu'avec le test sur feuille (70 % en moyenne). Les corrélations réalisées entre les essais montrent que le test sur feuille est mieux corrélé avec les infections naturelles. Cependant, les deux tests permettent de mesurer des facteurs de résistance complémentaires. Enfin, certains génotypes apparaissent sensibles aux tests qui font intervenir des implants mycéliens alors qu'ils présentent un bon comportement en infection naturelle. L'explication donnée est que les tests mis en œuvre ne permettent pas de mesurer les facteurs de résistance à la pénétration du champignon dans les feuilles, seul un test utilisant des ascospores permettrait de prendre en compte cet élément.

Introduction

Phomopsis stem canker (*Diaporthe helianthi* Muntanola-Cvetkovic *et al*, 1981) was first identified in France in 1985 (Lamarque and Perny, 1985). This disease caused severe yield losses in 1987 (Mestres, 1987), and resistance tests were developed at INRA in order to help resistance breeding programmes (Bertrand and Tourvieille, 1987). Two types of observations are now recommended in breeding programmes: firstly counts of natural infections after provision of inoculum, conditions of favourable humidity (irrigation) and the presence of controls with different flowering dates (system denoted semi-natural attack); secondly, measurements of the lesions caused by artificial infections with *P.helianthi* mycelium on leaves and petioles.

Among the mycelium tests studied by Bertrand, 1986, those on leaves and petioles were retained as they permitted distinction between sunflower genotypes according to their resistance to mycelium extension across the leaf (Bertrand and Tourvieille, 1987) and its passage onto the stem (Tourvieille de Labrouhe, 1988). The leaf test is used systematically in INRA resistance breeding programmes, the petiole test for certain specific genetical and pathological studies.

The aim of this paper is to show the best use of the artificial infections in breeding programmes, and the necessity of maintaining semi-natural attack trials. Improvements made to the testing procedures since their publication and their effects on breeding programmes are discussed.

Materials and Methods

Fungal isolates:

For semi-natural attack, inoculum is provided in the form of naturally contaminated sunflower stems showing perithecia, in addition to natural infection caused by ascospores which are transported from other fields. There is no control of isolates, which are certainly a mixture. For the artificial infections, the mycelium may be from one or several isolates, either obtained from stems naturally infected the previous year, or chosen among a collection made in 1995 from samples in all parts of France and on all sunflower plant parts.

Sunflower genotypes:

Trials and tests have been made with all types of breeding material: populations, generations of pedigree selection, inbred lines, commercial and experimental hybrids. Checks were used each year (Resistant: Agrisol (Pau Semences), Susceptible: Viki (Maïsadour) or 74F (experimental INRA hybrid).

Semi-natural attacks:

The methodology described by Tourvieille (1989) consists of placing infected sunflower stems regularly throughout the trial at the starbud stage, and of irrigating by sprinkler sufficiently to insure spore production, plant contamination and symptom expression. Several trials are carried out each year in Southwest France. Observations consist of counts of the percentages of plants in each plot (about 50 plants) showing either stem lesions >5cm or stem encircling lesions.

Mycelium test on leaves:

Developed by Bertrand and Tourvieille (1987), this test measures the rate of extension of a Phomopsis lesion along the main vein from a mycelial explant placed on the upper side of the leaf tip with the mycelium in direct contact with the leaf. The explant is covered with aluminium foil, stapled to the leaf, to avoid desiccation. Two leaves per plant are infected. It has appeared important (INRA, 1992) to use good quality aluminium foil and sufficiently aggressive isolates to give high levels of successful infections. In contrast, the exact composition of the culture medium does not appear to influence the success rate of the test.

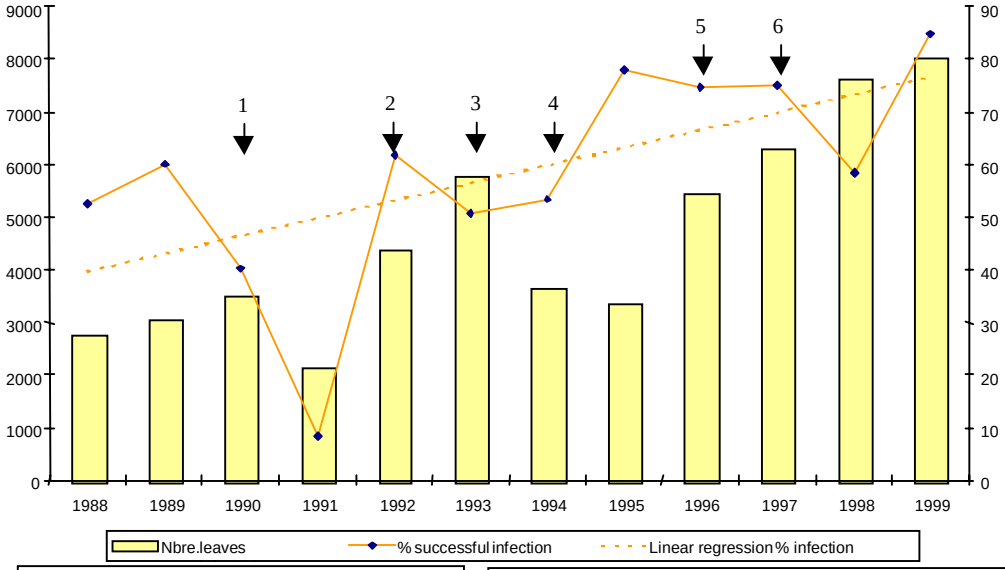
Mycelium test on petioles: (Bertrand and Tourvieille, 1987).

This test measures the rate of spread of mycelium from the petiole on to the stem and then its growth in the stem. The mycelium explant is placed on the cut end of the petiole and covered with aluminium foil. Infection success rate is generally nearly 100%, so only the lesion length is measured.

Results and Discussion

Improvements to the leaf test between 1988 and 1999 made it possible to increase the percentage success of the infections from 52.5% in 1988 to 84.9% in 1999 (Figure 1.)

Figure 1. Changes made to the mycelium leaf test methodology between 1988 and 1999 and the results obtained.



Method 1998
Inoculum : at least 2 isolates, obtained from stems contaminated the year before
Checks : segregating progenies
Trials sown in April, flowering beginning of July
Plants infected at star-bud stage,
several infection dates for one genotype/family
Infections from July 8th to August 9th
Irrigation : 10mm/day
Observations: lengths to nearest mm

Method 1999
Inoculum : one isolate, aggressiveness checked in greenhouse
Checks : Hybrids with well known reactions (74F (Sus) and Agrisol (Res))
Trials sown beginning of May, flowering mid-July
All plants of a genotype or progeny infected the same day, on young fully grown leaves
Infections from June 15th to July 1st : results before flowering
Irrigation: 5mm/day
Observations: lengths to nearest 5mm.

Improvements to the leaf test between 1988 and 1999 (1 to 6 on graph)

1. Infections made at an earlier stage, but under summer temperatures, to obtain results before flowering (permitting selection)
2. Use of homogeneous checks at each infection : allows calculation of an index to compare different infection dates
3. One isolate used for all tests in one year
4. Check of aggressiveness of isolate beforehand in the greenhouse
   Measurement of lesions to nearest 5mm: quicker and more standard between observers
5. Cryoconservationof isolates at -80°C
   Reduced amount of water for irrigation: 5mm/day in 3 periods of 20min '(giving better infection and more normal plant growth
6. All plants of one genotype or progeny infected on the same day, with choice of leaves at same growth stage:
   increased numbers of plants infected, or observed, in a given time.

The tests on leaves and petioles present several advantages compared with observations of semi-natural attack. First, they are successful each year (70% infection on leaves, 90% on petioles) and the results are quite precise, with coefficients of variation not exceeding 30% in general (Table 1.) The leaf test is better correlated with observations of semi-natural attack than the petiole test (Tourvieille de Labrouhe *et al.*, 1988; Viguié, 1999). Semi-natural attack trials are only possible in regions already contaminated by the disease, whereas the mycelium infections are possible in the absence of natural inoculum and since no spores are produced, if the

plants are destroyed at the end of the season, there is no danger of infecting a Phomopsis-free zone. The two tests avoid the problems of providing sufficient humidity for spore production and contamination, and of homogeneity of inoculum and they give repeatable results from one year to the next (Benomar, 1994; Vear *et al*., 1997). Measurement of lesion length after a given time appears to be the observation most adapted to these tests and to the resistance characteristics to be evaluated: the plants are observed only once, and since all the plants are observed at the same time, environmental effects are reduced to a minimum. Since the observations are length measurements, they should be completely objective.

Table 1. Percentage of successful artificial infections with the *Diaporthe helianthi* mycelium tests on leaves and petioles carried out since 1987, on non-selected genotypes and correlations with semi-natural attack.

Year	**Test**	**% succes**	**CV**	**Correlation coef. Ttests / semi-nat. Att.**	**Reference**
1987	Leaf	90.0	-	-	Bertrand et Tourvieille, 1987
1988	Leaf	71.9	-	0.87**	Tourvieille de Labrouhe *et al.*, 1988
	Petiole	94.9	-	0.13ns	
	Leaf	-	-	0.70**	Tourvieille, 1989
	Petiole	-	-	0.48ns	
1989	Leaf	89.0	-	0.51* à 0.56**	INRA, 1989a
1993	Leafe	-	15.0 à 29.7	0.36ns à 0.40*	INRA, 1994
1994	Leaf	92.6		0.447*	Benomar, 1994
	Petiole	94.4		-0.125ns	
1993-95	Leaf	-	-	hybrids: 0.73** liness: 0.56ns	Vear *et al.*, 1997
1996-98	Leafe	73.3	15.8 à 23.1	0.59** à 0.74**	Viguié *et al.*, 1999a
	Petiole	97.4	5.5 à 15.1	0.32ns à 0.45*	

The two tests are of combined interest compared with observations of semi-natural attack since they measure different resistance factors: the leaf test gives an indication of resistance to extension of mycelium in the leaf whereas the petiole test indicates resistance to spread of the fungus from the petiole into the stem and within the stem (Tourvieille de Labrouhe *et al*., 1988) A given genotype may be susceptible to one test and resistant to the other (Viguié *et al*, 2000). Observations of semi-natural attack do not give this possibility. These tests were found to be useful in breeding by Tourvieille *et al*.(1988) and Vear *et al*. (1997), who suggested that they could be used to eliminate the most susceptible genotypes in early breeding generations, both individual plants and families.

However, these two tests are not entirely satisfactory, in particular, they are costly in labour, both for the infections and the observations. Teams of at least two people are necessary to infect a series of plants, and the number of leaves or petioles to be infected is often very large, in order for the results to be dependable (for example, for one genotype, 2 leaves/plant and 2 replications of 10 plants are necessary = 40 infections). An average team of 3 people can infect 200 leaves/hour.

In addition, they are not sufficient to cover all types of resistance to Phomopsis. To be used alone, a resistance test must reproduce the natural disease cycle, including all the pollution and infection stages (Tourvieille and Guiard, 1992). The petiole test is an example where the leaf infection stage is not taken into account. In addition, it has been noticed that some genotypes appear susceptible to the mycelium tests but are resistant to semi-natural attack, or vice-versa (Viguié *et al*, 2000). It is probable that such genotypes are particularly resistant or susceptible to ascospore infections of the leaf tips, and a test using ascospores would be necessary to demonstrate this type of resistance.

Thus, although the artificial infection tests provide more regular information each year, observations of semi-natural attack remain essential to demonstrate all types of resistance. It also is also necessary, for the mycelium tests, to check that the results are in agreement with natural attack, that the fungal isolate gives the same reaction as the population of isolates that make up the natural populations in infected regions (Viguié *et al*,

1999). To obtain a valid judgement of a sunflower genotype in one year, it is useful to have several locations; this is possible for semi-natural attack, but would be very costly for mycelium tests. Table 2 shows that, each year, the results of some semi-natural attack trial locations cannot be used, most often because the level of attack is too low (with a mean attack of less than 10%), or it is too irregular (a coefficient of variation above 50%). Thus, a large number of trials must be planned to make possible general conclusions about the inheritance of resistance or the level of resistance of a new variety.

Table 2. Variations in percentage attack by *Diaporthe helianthi* in some semi-natural attack trials in Southwest France since 1988.

Year	% attack	Genotype	Nbre.location	Nbre.location analysed	References
1988	86.4	15 hybrids, Official trials	-	4	Tourvieille, 1989
1989	36.7	(attack from 20 to 50% on 4 sites)	9	4	INRA, 1989b
1990	5.6 61.0	Agrisol Viki	-	-	INRA, 1991
1993	31.3 96.6 62.0	Agrisol Viki 5F x 5M factorial	-	1 (Auzeville) 1 (Auzeville) 1 (Auzeville)	Vear *et al.*, 1997 INRA, 1994
1994	52.4 31.4	Factorial 1993	- 3	1 2	Vear *et al.*, 1997 INRA, 1995 Garreyn, 1995
1995	14.9 83.2 50.3 58.1 47.4	Agrisol (Factorial 1) Viki (Factorial 1) Factorial 1 Factorial 2 Factorial 3	- - - - -	1 (Auzeville) 1 (Auzeville) 1 (Auzeville) 1 (Auzeville) 1 (Cargill)	Vear *et al.*, 1997 INRA, 1996
1996	18.8	Factorial A 4Fx4M	7	4	Viguié *et al.*, 2000a
1997	26.9	Factorial B 5Fx6M	7	4	Viguié, 1999
1998	-	Factorial A	3	0	

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