European Journal of Plant Pathology **102:** 421–429, 1996.

Resistance of sunflower (*Helianthus annuus* L.) to terminal bud attack by *Sclerotinia sclerotiorum* (Lib.) de Bary

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Accepted 5 December 1995

Key words: additive genes, ascospores, infection, inheritance, spore germination

Abstract

Resistance of sunflowers to terminal bud attack by *Sclerotinia sclerotiorum* was studied by microscopical observations of infection processes and by genetical analyses of trials showing natural infections. Electron microscope studies showed that there were no differences in leaf morphology between susceptible and resistant genotypes, and that both were contaminated by ascospores. Only on the susceptible genotype was considerable ascospore germination observed, followed by mycelial development and leaf penetration. On the resistant genotype, there was little ascosopore germination and no further development. The genetical studies of percentage natural attack observed on eight inbred lines representing a range of reactions, and the hybrids from a factorial cross of these lines, indicated that inheritance is mainly additive, with few interactions.

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary attacks most parts of the sunflower plant: roots (Putt, 1958), leaves (Cuk, 1978), terminal buds (Peres et al., 1989) and capitula (Lamarque et al., 1985), causing white rot or wilt. Attacks on terminal buds were first identified in France in 1980 (Tourvieille et al., 1992); they reappeared in 1984 and there have been economic losses in recent years in many parts of the country. Ascospores infect the young leaves around the terminal bud; these leaves then become discoloured and show necrotic lesions. Under humid conditions, the rot spreads to the petiole and stem, destroying the terminal bud and thus causing considerable yield loss.

Chemical control is possible (Peres et al., 1991) but costly, so that under present economic conditions it cannot be recommended. Genetic control is of greater interest since almost complete resistance has been found in genotypes of good agronomic value. In breeding for this type of resistance, it is necessary to reproduce disease symptoms artificially, with the same genotype reactions as under natural conditions, and to have some knowledge of the heredity of resistance.

Achbani et al. (1994) reported difficulties in developing breeding tests which represented the normal disease cycle. Some infection methods were significantly correlated with natural attacks, but they represented only part of the infection cycle. It was therefore necessary to make detailed studies of the reactions of susceptible and resistant plants to ascospore contamination of the terminal bud in order to determine the important infection stages. Earlier microscopical studies of the reaction of sunflower capitula to contamination by *S. sclerotiorum* ascospores (Lamarque et al., 1985; Says-Lesage and Tourvieille, 1988) made it possible to define the infection processes involved, so similar techniques were used to study the reaction of sunflower terminal buds to *S. sclerotiorum* contamination.

Studies of the inheritance of resistance to other forms of attack by *S. sclerotiorum* have shown that resistance is partial and polygenic and that additive genes are generally the most important (Robert et al.,

Table 1. Origins of the sunflower inbred lines used as parents of the factorial cross

Inbred line	Origin	Breeder
Females		
IF1	Provided by Prograin-Génétique	Confidential
CD(HA89)	Population VNIIMK8931	USDA
2603	Moroccan population	INRA
CC40	Canadian material (CM408)	INRA
Males		
PAC1	HA61 (USDA) × *H.petiolaris* restorer	INRA
IR1	Provided by Prograin-Génétique	Confidential
RHA801	Pool of USDA restorers	USDA
IR2	USDA line RHA274	Confidential

1987; Vear and Tourvieille, 1988), with a continuous variation in levels of resistance. Parent-progeny correlations are significant except in the case of observations of semi-natural attack on capitula. Tourvieille et al. (1992) showed that the ranking of genotype reaction to natural attack of terminal buds was stable, so that it appeared most useful to make genetical studies based on such observations.

Materials and methods

Sunflower genotypes

Four female (cytoplasmic male sterile) and four male (male fertility restorer) lines were observed (Table 1), together with the 16 hybrids obtained from a factorial cross using these parents. The two inbred lines with the most extreme reactions, PAC1 (resistant) and CC40 (susceptible) (Castaño et al., 1993) were used for the microscopical observations.

Fungal isolate

A mixture of ascospores produced from sclerotia collected from infected sunflower capitula was used for the artifical infections. The sclerotia were induced for ascospore production by placing them for three winter months in damp perlite out of doors, then replacing them in damp soil-less compost in an unheated greenhouse at 12°–18°C (Tourvieille et al., 1978). Apothecia appeared after two weeks.

Experimental design

For the microscopical observations following artificial infections, plants were grown in pots of soil-less compost. For the genetical studies, the 24 genotypes (8 inbred lines and 16 hybrids) were sown in randomized block design trials, in two locations: St. Florent /Cher, (Cher, France) and Lussat, (Puy-de Dôme, France) in 1992 and 1993. The 1992 Puy de Dôme trial was discarded as there was insufficient *S. sclerotiorum* attack. The trials had four replications in the Cher and two in the Puy-de Dôme and 30 to 50 plants per plot.

Artificial infection methods

The terminal buds of plants at the susceptible stage (6–12 leaves) were either infected with dry ascospores discharged directly from ripe apothecia (Says-Lesage and Tourvieille, 1988) or sprayed with suspensions of ascospores in distilled water (Vear et Tourvieille, 1988). The plants were then transferred to a humid chamber. A saturated atmosphere was maintained continuously for 38 h using a medical 'ultrasonic nebuliser' apparatus (Europe Medical NU 52), normally used to treat respiratory disorders. This apparatus produced a fine mist resulting in the slow deposition of water on the leaves.

Microscopical techniques

Observations of spore germination were made by the method of Preece (1959) using a light microscope. Samples mounted in water showed excellent colour contrast between the ascospores and lesions which appeared red, and the rest of the leaf, which was green. Epifluorescence microscopy was used to determine ascospore viability. These observations were made using the fluorochromatin test (FCR) of Heslop-Harrison et al. (1983), developed to determine pollen viability. Live *S. sclerotiorum* ascospores appeared green under blue light (495nm) whereas the dead ascospores were colourless. A scanning electon microscope, Philips SEM 505, with a Hexland cryogenic stage was used to observe leaf structure and ascospore contamination with the technique of Says-Lesage and Tourvieille (1988). Ilford HP5 400 ASA film was used for photography.

Observations

Under the microscope a scale of ascospore concentration was defined for each leaf zone: 0 = no ascospores; 1 = 1 to 20 ascospores mm^{-2}; 2 = 21 to 50 ascospores mm^{-2}; 3 = 51 to 100 ascospores mm^{-2}; 4 = >100

ascospores mm^{-2}. In the field, observations were made of the percentage of plants infected by *S. sclerotiorum* on terminal buds, just before flowering.

Statistical analyses

One-way and factorial analyses of variance were made to determine genotype, parental and interaction effects. The relative importance of additivity in genetic control was estimated from the ratio:

$$\frac{\text{generalcombiningabilityvariance}(\sigma^2\text{G.C.A.})}{\text{specificcombiningabilityvariance}(\sigma^2\text{S.C.A.})}$$

This was calculated from the method of Robert et al. (1987), adapted from Falconer (1981):

$$\frac{\sigma^2\text{G.C.A}}{\sigma^2\text{S.C.A}} = \left[\frac{\text{female mean square} - \text{interaction mean square}}{\text{nb.rep} \times \text{nb.males}} + \frac{\text{male mean square} - \text{interaction mean square}}{\text{nb.rep.} \times \text{nb.females}}\right] / \frac{\text{interaction mean square} - \text{error mean square}}{\text{nb.rep}}$$

Results

Microscopical observations

Contamination sites. The morphological structure of the small leaves making up the terminal buds of the two sunflower genotypes, CC40 (susceptible) and PAC1 (resistant), appeared identical. The apical part of the leaves showed fewer epidermal hairs than the other zones, with leaf tips having almost none (Figure 1a). There were no apparent differences in epidermal hairs (Figure 1b, 1c), cell architecture or stomata number (Figure 1d, 1e) between the two lines. A magnification of at least × 312 enabled ascospores to be observed, without confusion with other structures (Figure 1f). On plants sprayed with ascospore suspensions, few ascospores were observed whereas all leaves showed inoculum received from the dry apothecial discharge, but with considerable differences in the ascospore concentrations on different leaf parts (Table 2). The large leaves showed the greatest numbers of ascospores. On the small leaves (length <0.8cm), which were very close together, with most of their surfaces covered by other leaves, ascospores were mainly limited to the extremities (Figure 2a). In contrast, it was the central part of the larger leaves (length >1.5cm) which showed the greatest concentrations (Figure 2b), few ascospores being observed on their tips (Figure 2c).

Table 2. Ascospore concentration indices on leaves around the sunflower terminal bud

Inbred line	CC40	PAC1
Leaves of lengths:		
<0.8 cm	0–1	0
0.8–1.5 cm	1–2	1–2
1.6–2.0 cm	2	4
2.1–3.0 cm	4	4
3.1–4.0 cm	4	4

0 = 0 ascospores; 1 = 1–20 ascospores/mm^{-2}; 2 = 21–50 ascospores/mm^{-2}; 4 = >100 ascospores/mm^{-2}

Ascospore germination and mycelial development. No infections were obtained on plants sprayed with ascospore suspensions. When direct apothecial discharge was used on the susceptible genotype CC40, the first sign of infection was a discoloration of the zone around the ascospores, especially when they were in high concentrations; this effect was visible when there was a water film on the leaf. Certain leaf regions appeared more favourable for germination than others, in particular the tips and the bases of young leaves. At these sites, there was considerable development of *S. sclerotiorum* mycelium (Figure 2d). Nevertheless, germination did occur on other parts of the leaf. A mucilagenous substance was observed around the ascospores. In Figure 2e, it is difficult to distinguish the frontier between the ascospore exudate and leaf tissues. Hyphae were not attracted to the stomata (Figure 2f) and no penetration was observed through these openings. In contrast to CC40, observations made on the resistant genotype PAC1 showed an ascospore exudate but only very rare ascospore germination, and no further development of mycelium.

Observations of natural attack in the field

Hybrids. The percentages of plants attacked are presented in Table 3. Levels of attack in the Cher varied from 0 to 47% in 1992 and 0 to 22% in 1993. The 1993 Puy de Dôme trial showed 0 to 58% attack, but with about half the genotypes showing no symptoms. The analyses of variance showed significant genotypic effects in all trials. CC40 × IR2 and 2603 × IR2 were the most susceptible with mean attacks of 29.0% and 41.6%, respectively. In contrast, certain genotypes showed almost no symptoms: IF1 × PAC1 (0%), CD × PAC1 (0.3%), IF1 × IR1 (0.5%) and IF1 × RHA801 (0.5%). Some hybrids showed varying levels of attack, for example CD × IR2, CC40 × RHA801 and CC40 ×

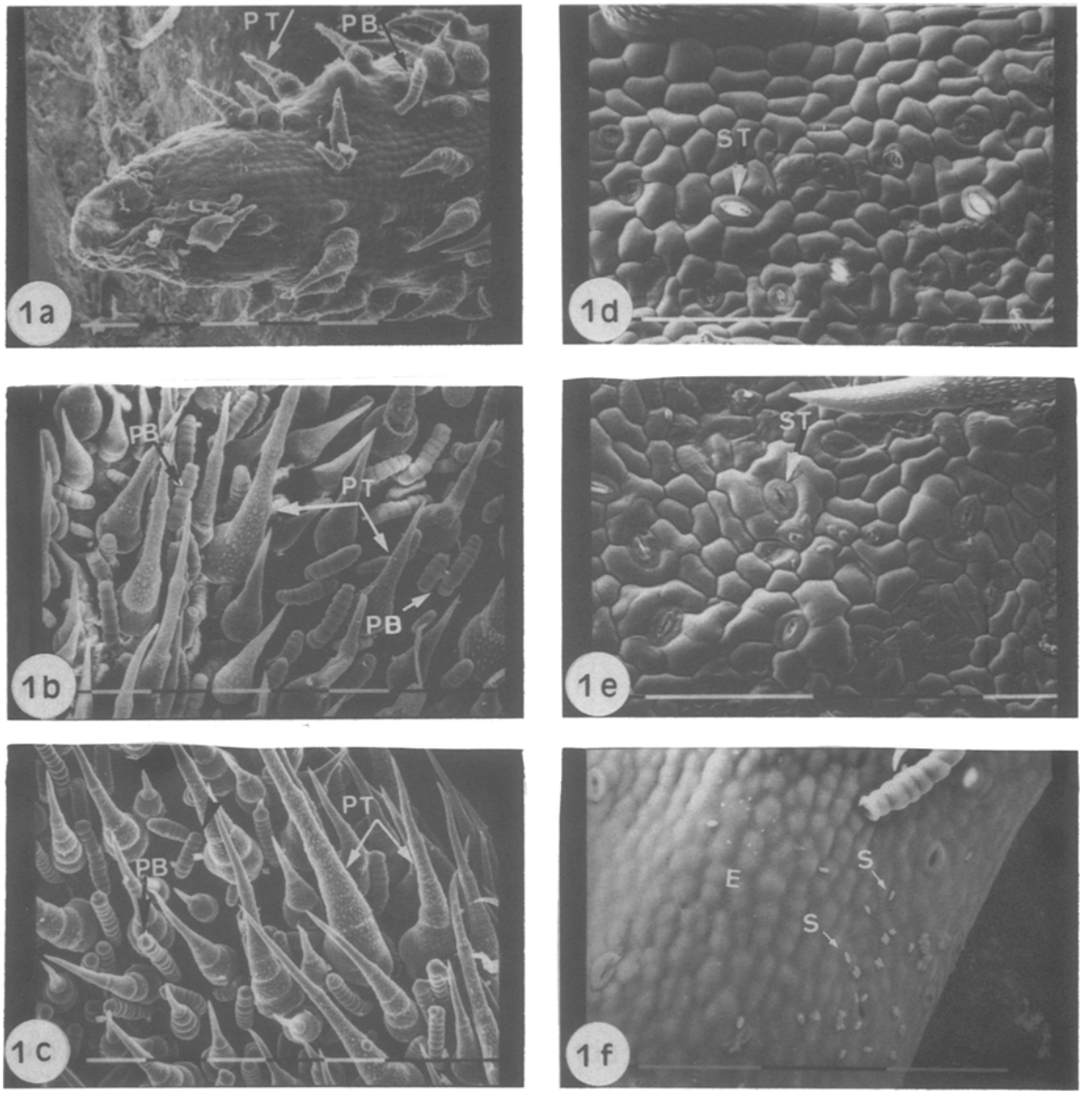

Figure 1. Scanning electron microscopy observations of the surface of leaves forming the terminal bud of two inbred sunflower lines (bar scale = 100 μm). 1a. tip of a leaf of CC40; 1b. central region of a leaf of CC40; 1c. central region of a leaf of PAC1; 1d. tip of a leaf of CC40; 1e. tip of a leaf of PAC1; 1f. *Sclerotinia sclerotiorum* spores on the epidermis of a leaf of CC40. PT: pointed hair; PB: rounded hair; ST: stomata; S: *Sclerotinia sclerotiorum* ascospores; E: epidermis.

IR2. However, their ranking compared with the other genotypes did not vary; the correlation coefficients between the three trials were all highly significant ($P < 0.01$): Cher92–Cher93: $r = 0.89$; Cher92–PdD93: $r = 0.82$; Cher93–PdD93: $r = 0.90$.

Between years, there were differences in climatic conditions. The mean temperature in May and June was higher in 1992 (16.5°C) than in 1993 (14.1°C) and the number of hours of sunshine was also greater in 1992. However, precipitation (rainfall and irrigation) during May, the period that coincides with production of *S. sclerotiorum* apothecia, and the plant stage most susceptible to *S. sclerotiorum* bud attack (6 to 10 leaves) was greater in 1992 than in 1993 (76 mm and 37 mm, respectively). In the 1992 Cher trial, dates of appearance of the sixth leaf were noted for each hybrid. These

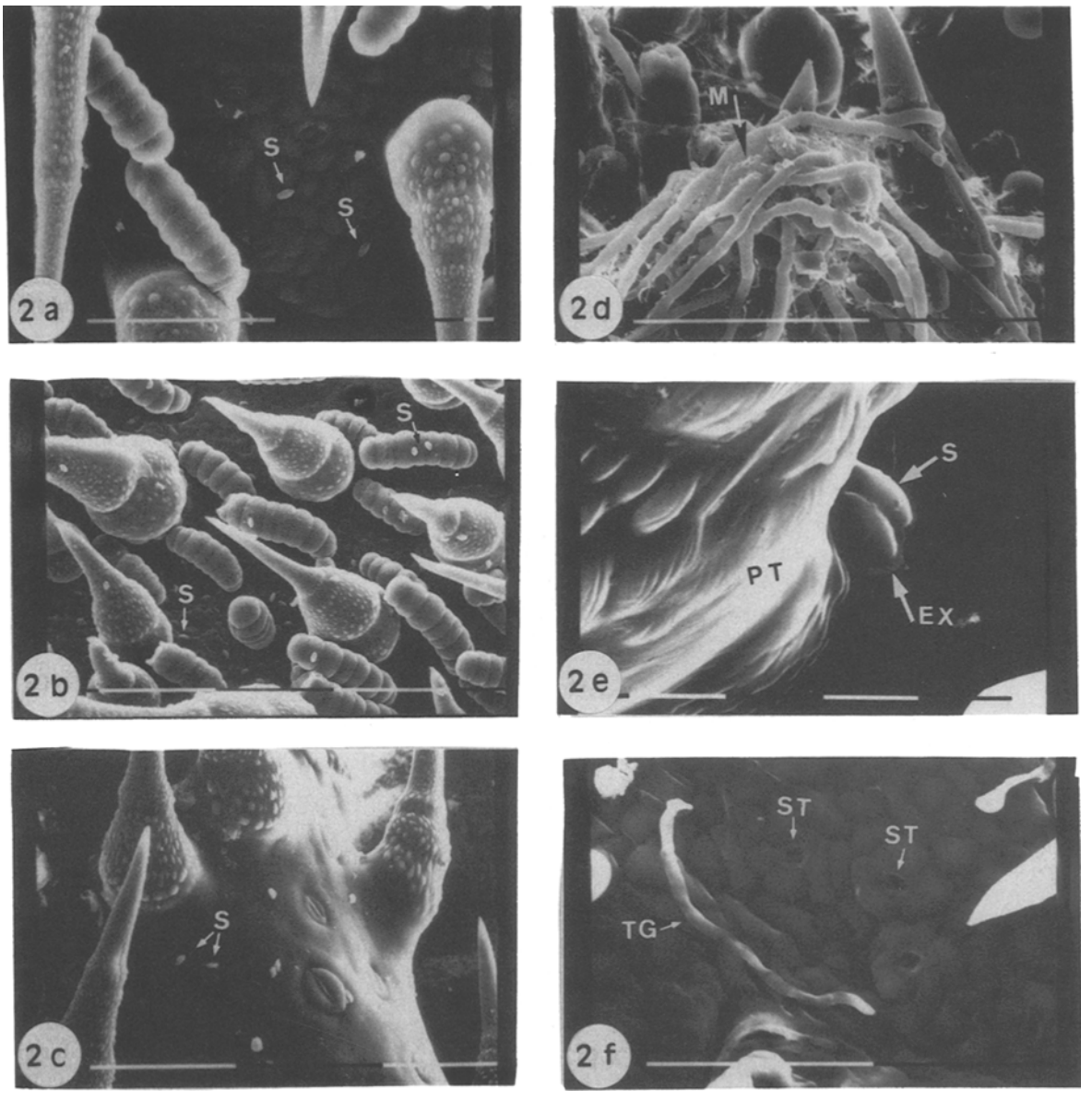

Figure 2. Scanning electron microscopy observations of the sites of contamination by *Sclerotinia sclerotiorum* ascospores on the leaves making up the terminal bud of the inbred line CC40 (bar scale = 100 μm). 2a. tip of a <0.8cm leaf; 2b. central region of a >1.5cm leaf; 2c. tip of a >1.5cm leaf; 2d. tip of <0.8cm leaf; 2e. presence of an exudate in contact with the spores (bar scale = 10 μm); 2f. germinated ascospores. M: mycelium; S: *Sclerotinia sclerotiorum* ascospores; TG: germination tube; PT: pointed hair; ST: stomata; EX: exudate.

varied very little: for IF1 × IR2, 2603 × IR2, CD × IR2, IF1 × IR1 and CC40 × IR1, it was 28 days after sowing and for all the other hybrids it was 29 days.

Inbred lines. The percentages of plants attacked in the different trials are given in Table 4. As for the hybrids, in all cases there were significant differences between the inbred lines, and results in the different trials were also correlated significantly ($P < 0.01$) (Cher92–Cher93: $r = 0.72$; Cher92–PdD93: $r = 0.93$; Cher93–PdD93: $r = 0.89$). The results for the female lines indicate that IF1 and CD are resistant and 2603 and CC40 susceptible. In contrast the male lines showed few symptoms, less than 5% in the Cher in 1992, up to

Table 3. Percentage *Sclerotinia sclerotiorum* attack on the terminal buds of 16 sunflower hybrids observed under conditions of natural infection

Year			1992	1993		Mean
Location			Cher	Cher	Puy-de-Dôme	
Hybrids						
IF1	×	PAC1	0.0	0.0	0.0	0.0
CD	×	PAC1	0.9	0.0	0.0	0.3
2603	×	PAC1	2.9	3.5	0.0	2.1
CC40	×	PAC1	11.9	4.9	11.9	9.6
IF1	×	IR1	0.0	1.5	0.0	0.5
CD	×	IR1	8.6	0.5	0.0	3.0
2603	×	IR1	0.0	3.4	0.0	1.1
CC40	×	IR1	15.7	3.6	3.6	7.6
IF1	×	RHA801	1.6	0.0	0.0	0.5
CD	×	RHA801	5.5	0.5	0.0	2.0
2603	×	RHA801	6.7	8.4	6.0	7.0
CC40	×	RHA801	24.6	7.7	6.0	12.8
IF1	×	IR2	9.0	8.3	2.1	6.5
CD	×	IR2	16.4	4.5	6.0	9.0
2603	×	IR2	43.8	22.6	58.4	41.6
CC40	×	IR2	47.4	16.4	23.0	29.0
Mean			**12.1**	**5.4**	**7.3**	**8.3**
CV			57.0	60.4	49.2	
SD			8.5	5.8	5.4	
LSD(5%)			11.4	8.2	11.1	

10% in the Puy de Dôme in 1993 and only up to 20% in the Cher in 1993. Overall, IR2 appears susceptible, RHA801 intermediate and PAC1 and IR1 resistant.

Heredity of resistance. Factorial analyses of variance were carried out on the results of the 16 hybrids (Table 5). They showed significant parental effects in all trials. The means of the hybrids with each parent are given in Table 4. In both the Cher trials, the female effect was greater than the male effect, whereas the reverse was true in the Puy de Dôme. In addition, in the first two trials, there were no significant interactions and the general/specific combining ability variances are presented in Table 5 simply to show the importance of additive effects. In contrast, in the Puy de Dôme, there was a significant male × female interaction and the GCA/SCA variance ratio (0.98) indicated dominance effects equivalent to those of additivity. This difference compared with the Cher trials resulted from the behaviour of hybrids with the inbred 2603 as parent: 2603 × IR2 was considerably attacked (58.4%), 2603 × RHA801 slightly attacked (6.0%) whereas the other two hybrids showed no symptoms (Table 3). In spite of this difference, the rankings of parents according to the mean of their hybrids remained constant; IF2, CD, IR1 and PAC1 were the most resistant whilst CC40, 2603, IR2 and RHA801 were more susceptible.

Correlations between the percentage attacks on inbred lines and the means of their hybrids are given in Table 4. For the female lines, the correlations are always significant, whereas for the male lines this was the case only in 1993. The 1992 Cher trial showed no significant correlation between male inbreds and their hybrids due to the absence of symptoms that might have been expected on the inbred lines IR2 and RHA801.

Discussion

Microscopic studies on a susceptible sunflower genotype provided information on the beginning of the infection process of *S. sclerotiorum* ascospores on young sunflower leaves. The dry ascospores used in this study, freshly discharged by apothecia, adhered solidly to the leaf epidermis. In contrast, when

Table 4. Percentage *Sclerotinia sclerotiorum* attack on the terminal buds of the eight inbred sunflower parental lines and the means of their hybrids, observed under conditions of natural infection

Year	1992		1993			
Location	Cher		Cher		Puy-de-Dôme	
	Parent value	Hybrid mean	Parent value	Hybrid mean	Parent value	Hybrid mean
Females						
IF1	7.3	5.5	2.4	5.3	0.0	1.0
CD	0.0	11.1	2.0	3.9	0.0	2.4
2603	17.0	14.7	27.9	14.9	38.4	14.6
CC40	31.0	28.1	22.5	14.2	54.1	18.9
Correlation parent values/hybrid means						
	$r = 0.88$**		$r = 0.99$**		$r = 0.99$**	
Males						
PAC1	5.0	6.1	0.0	4.5	0.0	4.6
IR1	0.0	8.6	0.0	5.8	0.0	2.5
RHA801	0.0	13.4	2.1	8.1	5.9	6.4
IR2	0.0	31.2	16.2	19.9	9.4	23.4
Correlation parent values/hybrid means						
	$r = 0.51$ NS		$r = 0.99$**		$r = 0.84$*	
LSD (5%)	11.4		8.2		11.1	

**: $P < 0.01$; *: $P < 0.05$; NS: not significant

ascospore suspensions in water were applied, they did not adhere; such a suspension may dilute substances necessary for adhesion, as reported by Azmeh (1976) for *Puccinia* spp. spores on barley. The presence of an exudate secreted by spores was observed, a phenomenon which may be necessary for recognition between the fungus and its host (Achbani, 1993).

Germination of *S. sclerotiorum* ascospores requires the presence of senescent tissues or some other nutritive support (Mellinger and Hooker, 1969). The present observations showed that infections of the susceptible genotype always occurred at the tip of the young leaves around the terminal bud. On older leaves, infection generally starts at the base, near the main vein, where a leaf exudate is produced (Sedun and Brown, 1987). For *S. sclerotiorum* attacks on rapeseed, senescent petals provide a nutritive base (Brun et al., 1983) and on sunflower capitula, pollen and nectar are present at the susceptible stage (Says-Lesage and Tourvieille, 1988). No leaf exudate was observed on the very young sunflower leaves, but the cuticle may not have formed, or may be very thin at the tip, permitting direct absorption of nutrients by *S. sclerotiorum* mycelium.

Table 5. Factorial analyses of variance of the percentage *Sclerotinia sclerotiorum* attacks on terminal buds of 16 sunflower hybrids

Year	1992	1993	
Location	Cher	Cher	Puy-de-Dôme
F hybrids	10.9**	7.9**	15.4**
F females	28.7**	21.9**	7.2**
F males	20.6**	14.6**	14.1**
F interaction	1.8 NS	1.0 NS	4.3**
Var.GCA/Var.SCA	(13.6)	(393.0)	0.98

**: $P < 0.01$; NS: not significant

Rapilly (1991) reported that ascospores of *S. sclerotiorum* will not infect sunflower if they are present in very small numbers. According to Purdy (1958), *S. sclerotiorum* ascospore germination will be followed by colonisation only if a sufficient mass of mycelium is produced. Here, it was observed that direct discharge of an apothecium generally gave high concentrations of ascospores on the young sunflower leaves, and this was visible by the discoloration of the contaminated tissues. If the concentration of ascospores was low, no discoloration was visible, and in this case no infections were observed.

The frequency of ascospore germination was the main observation distinguishing resistant and susceptible genotypes, with a very low rate on the resistant inbred. A hypothesis to explain this would be that the leaves of this genotype do not produce sufficient nutritive exudate to stimulate ascospore germination. As a result, few ascospores germinate and do not provide the mass of mycelium necessary for infection. Achbani (1993) observed that ascospores could infect leaves of PAC1 if these were covered with a nutritive suspension such as a vegetable extract. In addition, mycelial explants always infect sunflower leaves, whatever the genotype, although the mycelium extends more slowly on resistant genotypes than on susceptible ones (Achbani et al., 1994). This suggests that there could also be production of some fungistatic compound by leaves of resistant genotypes. Analyses of leaf surface exudates, not only for nutrients, but also for compounds which could hinder spore germination, are necessary to determine the validity of these hypotheses.

The observations of natural attacks of sunflower terminal buds by *S. sclerotiorum* made it possible to distinguish the behaviour of different genotypes. Extreme genotypes, such as 2603 × IR2 or CC40 × IR2 (both very susceptible) and IF1 × PAC1 (resistant), showed up to a 47% difference in plants attacked. Generally, ranking of genotypic reaction was constant between trials and between years, in agreement with Tourvieille et al. (1992) who showed that more than 90% of the rankings in 49 trials carried out from 1984 to 1991, were significantly correlated.

Observations of natural attack have also been used to distinguish sunflower genotypes for their resistance to *S. sclerotiorum* leaf attack (Cuk, 1978) and root attack (Putt, 1958). Although significant differences were observed for capitulum attack, Vear and Tourvieille (1987) showed that flowering date played a considerable role and that irrigation was necessary to ensure that all genotypes were subjected to the same conditions at the stage of maximum susceptibility, whatever their earliness. The difference between terminal bud and capitulum attack may arise from the fact that the former occurs earlier in the season when developmental differences between genotypes are smaller. In addition, the duration of the susceptible stage is much greater for terminal bud attack than for capitulum attack.

The studies of inheritance indicate that resistance to terminal bud attack is mainly controlled by genes with additive effects, which are expressed both in inbred lines and hybrids. The general absence of any female/male interaction and the large differences between the most suceptible and most resistant genotypes suggest that genetic control is simpler than that for other forms of *S. sclerotiorum* attack. This would agree with the observation that spores do not germinate on the terminal buds of resistant genotypes, whereas for other forms of attack, there are sufficient nutrients for ascospore germination and resistance depends on other, perhaps more complex, plant characteristics. However, since there are intermediate levels of terminal bud attack, it is likely that resistance is polygenic, as when other parts of the sunflower plant are involved.

In certain cases, symptoms were not observed when they might have been expected from overall results. This was the case for several hybrids with 2603 as female parent in the Puy de Dôme trial, and for the inbred lines IR2 and RHA801 in the 1992 Cher trial. It is possible that on these intermediate reaction genotypes, ascospore germination is slower than on the very susceptible types, so that conditions have to be favourable for longer for infection to occur, and this may not always have been the case.

In conclusion, natural terminal bud attacks by *S. sclerotiorum* may be used to judge resistance or susceptibility of sunflower genotypes and the behaviour of hybrids can generally be predicted from direct observations of inbred lines or from their general combining abilities. To improve breeding efficiency, it will be necessary to develop further tests using dry ascospores, followed by a misting system which does not wash them off the plants. The most efficient method on a large scale may well be a system of "semi-natural" attack, with provision of apothecia and misting for a sufficiently long period to give conditions for the natural disease cycle.

Acknowledgements

We thank F. Serre and P. Walser for their technical assistance. The first author was supported by a French Foreign Ministry Doctoral Studentship, as part of a collaboration programme between INRA-France and INRA-Morocco. We thank Prograin Génétique for the use of their inbred lines and CETIOM and PROMOSOL for their support of this programme.

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