

The resistance response of sunflower genotypes to black stem disease under controlled conditions

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Summary. Phoma black stem, caused by *Phoma macdonaldii*, is one of the most important diseases of sunflower in the world. The sources of resistance to Phoma black stem were investigated. A total of 184 genotypes, including some recombinant inbred lines (RILs), several M6 mutant lines obtained by gamma irradiation of seed of the genotype AS 613, and other genotypes from different countries, were evaluated against an aggressive French isolate (MP6) in controlled conditions. The study was carried out in a randomized complete block design with three replicates. Each replicate consisted of 10–12 seedlings. Twenty μL of spore suspension (10^6 pycnidiospores mL^{-1}) were deposited on the intersection of the cotyledon petiole and the hypocotyl of sunflower plantlets at the two-leaf stage. The percentage of the area exhibiting disease symptoms was scored on the two cotyledon petioles of each of the plantlets three, five and seven days after inoculation. The disease progress rate (r_d), as the slope of the regression line for disease severity against time, was also calculated. Analysis of variance detected significant differences among sunflower genotypes for disease severity 7 days after inoculation, as well as for the disease progress rate. A strong correlation ($r=0.96$, $P<0.01$) was found between disease severity 7 days after inoculation and the disease progress rate. The inbred lines F1250/03 (origin: Hungary), M5-54-1, M6-862-1 (mutant lines), SDR 18 (origin: USA) and two wild *Helianthus* accessions, 1012 Nebraska and 211 Illinois, (wild type) were highly resistant to Phoma black stem. These findings will assist breeders in choosing parent plants for breeding durable resistance to Phoma black stem.

Key words: *Helianthus annuus* L., mutant lines, *Phoma macdonaldii*, recombinant inbred lines, wild accessions.

Introduction

Black stem, caused by *Phoma macdonaldii*, is one of the most serious diseases of sunflower in the world (Sackston, 1992). The fungus infects various sunflower tissues but most often the lower leaves, and its most conspicuous symptom is a dark lesion at the base of the petioles (Pérès *et al.*, 1994). Infection is initiated when the fungus directly or indirectly penetrates the plant through wounds or natural openings such as the lenticels or the stomata (Roustae *et al.*, 2000a). Infected sunflowers exhibit premature ripening and loss in yield

of 10 to 30% (Penaud, 1996). Other symptoms are a reduction in oil contents and in thousand seeds weight (Carson, 1991), and premature death (Donald *et al.*, 1987). Sunflower genotypes with different levels of resistance have been found, but none that had complete resistance (Roustae *et al.*, 2000b; Rachid Al-Chaarani *et al.*, 2002, Bert *et al.*, 2004).

Besides conventional breeding techniques, genetic mutation is an alternative means to obtain desired characters in a field crop. In mutation, desired genotypes are created, selected, evaluated and multiplied. Much work has been done on mutations brought about in various plant species using irradiation, chemicals and other mutagenic agents. One of the most important means to bring about mutations is by nuclear techniques, and

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these have become established to breed new varieties (Ahloowalia *et al.*, 2004). Sixty-four per cent of all radiation-induced mutants are currently produced by gamma irradiation, followed by X-rays (22%) (Maluszynski *et al.*, 2000). Radiation mutagenesis causes various chromosomal DNA alterations in plants, including deletions, point mutations and inversions. Mutants with greater resistance to fungi have been recovered from some crops such as rice plants that were more resistant to rice blast (Han *et al.*, 2004), and chickpea plants that were more resistant to *Ascochyta* blight and *Fusarium* wilt (Ahloowalia *et al.*, 2004). Besides the economic benefit, some mutants are also important in the study of genetics and plant growth (Bretagne-Sgnard *et al.*, 1996).

Wild *Helianthus* accessions are rich sources of genes for disease resistance and have been used successfully to produce appropriate genetic variations in sunflower (Seiler, 1992). Several wild *Helianthus* species have been reported as being potential sources of genes conferring resistance to *Sclerotinia sclerotiorum* (Seiler and Rieseberg, 1997) and have been used to produce interspecific hybrids (Kräuter *et al.*, 1991). Cerboncini *et al.* (2002), examining wild sunflower gene pools, found several genotypes resistant to *S. sclerotiorum*.

Segregation of multiple genetic factors most often leads to quantitative inheritance (Kearsey and Pooni, 1996). Earlier studies suggested that the inheritance of resistance to *P. macdonaldii* was polygenic (Rachid Al-Chaarani *et al.*, 2002; Bert *et al.*, 2004). Polygenic characters are strongly influenced by environmental conditions (Kearsey and Pooni, 1996). Artificial infections under controlled conditions are helpful for the evaluation of inbred lines and hybrids in order to select individual plants and families in segregating generations. Screening tests have been developed to evaluate inbred sunflower lines for their susceptibility to *P. macdonaldii* (Pérès and Lefol, 1996; Roustae *et al.*, 2000b). There is evidence that disease severity in sunflower plantlets is similar to that in the adult plants (Roustae *et al.*, 2000b). Larfeil (2003) reported that the susceptibility of sunflower to *Phoma* black stem under field and controlled conditions is much the same. A high correlation between seedling and adult plant resistance has also been found in other pathosystems such as

Leptosphaeria maculans/oilseed rape (*Brassica napus*) (Newman and Bailey, 1987; McNabb *et al.*, 1993; Bansal *et al.*, 1994), *Pyrenophora teres* f. sp. *teres*/barley (Gupta *et al.*, 2003) and *Didymella rabiei*/chickpea (Chen *et al.*, 2004). A correlation between the disease reaction at the plantlet stage and at the adult plant stage is an important consideration when breeding for disease resistance. A high correlation indicates that the resistance genes are expressed throughout the life cycle of the plants, in which case disease screening can safely be carried out on the plantlets. Screening plantlets tends to be more cost-effective and will keep populations in breeding programmes to a more manageable size (Plaisted *et al.*, 1984).

The aim of this study was to look for sources of resistance to *Phoma* black stem and to evaluate the resistance of a great number of genetically diverse inbred sunflower lines.

Materials and methods

Sunflower genotypes and *Phoma macdonaldii* isolate

A total of 184 sunflower genotypes of diverse origin were evaluated for resistance to *Phoma* black stem. Genotypes included: 1. some mutant lines developed by irradiating genotype AS 613 with gamma rays at the Laboratoire de Biotechnologie et Amélioration des Plantes, Castanet-Tolosan, France, followed by single seed descent (SSD) with no prior selection (Sarraf *et al.*, 2000), and 2. some recombinant inbred lines (RILs) coming from crossings between PAC-2 and RHA266 that were kindly provided by INRA (France). Other genotypes were obtained from the US Department of Agriculture (USDA), from Hungary and French seed companies, and some were pure lines from the collection of our department. Seeds of wild *Helianthus* accessions were pre-treated with 1 mM GA3 in acetone to break down dormancy (Seiler, 1996).

Pure pycnidiospores of *P. macdonaldii* isolate MP6, collected from naturally infected plants in south-western France were used for the pathogenicity tests (Roustae *et al.*, 2000c). This fungal isolate is moderately aggressive so partial resistance could not be concealed by the rapid development of severe symptoms (Roustae *et al.*, 2000c).

Pathogenicity tests and disease assessments

Genotypes were evaluated in a randomized complete block design with three replicates. Each replicate consisted of 10–12 seedlings. Seeds were sterilized for 5 min in a sodium hypochlorite solution (6 chlorometric degrees) followed by washing in sterile distilled water. Two rows of six seeds per genotype per replicate were sown in plastic containers (50×38×10 cm) filled with horticultural soil (Hawita Flor, Germany; <http://www.hawita-gruppe.de>) and transferred to a growth chamber with a 14 h day, 75–80% relative humidity and a day/night temperature of 25±1/18±1°C with a daylight intensity of 200 mEm⁻² s⁻¹, provided by NAV-T 600W lamps (Osram-Vialox, Molsheim, France). The fungal isolate was grown on potato dextrose agar (PDA) and incubated at 25±1°C with a 12 h day (37 μEm⁻² s⁻¹). After 10–12 days, 20 μL of a pycnidiospore suspension containing 10⁶ spores/mL, 0.5% orange juice and 0.25% gelatine was inoculated at the intersection of the cotyledon petioles and the hypocotyls of sunflower plantlets at the two-leaf stage. In the first 48 h after inoculation, seedlings were covered with a transparent cover (Plexiglas) to maintain nearly saturated humidity which favours fungal growth. The two cotyledon petioles of the plantlets were scored for percentage of petiole area showing *Phoma* black stem three, five and seven days after inoculation. Petioles were scored from 1 (resistant) to 9 (susceptible) as proposed by Roustae et al. (2000b), where 1, 0–5% petiole area with necrosis spreading downward into the stem; 2, 6–10%; 3, 11–20%; 4, 21–30%; 5, 31–40%; 6, 41–60%; 7, 61–80%; 8, 81–99%; and 9, 100%. The disease progress rate

(r_d) was also calculated as the slope of regression line for disease severity against time by means of the $\ln[9/(9-DSS)]$ formula, which is derived from $\ln[1/(1-DSS)]$, as described by Rapilly (1991), and taking account of the theoretical asymptote of the disease scale at score 9.

Statistical analysis

The normality of disease severity and the disease progress rate data were assessed with the Shapiro-Wilks test (Proc Univariate; SAS Institute Inc., Cary, NC, USA). Analysis of variance (ANOVA) on disease severity was carried out using the general linear model (GLM) by SAS software. The disease progress rate was transformed (square of r_d+1) and analysed. However, the mean values for the disease progress data are shown in Table 2 as non-transformed data. The correlation between the disease severity 7 days after inoculation and the disease progress rate was also investigated.

Results and discussion

Analysis of variance detected significant differences among sunflower genotypes for disease severity 7 days after inoculation as well as for the disease progress rate (Table 1). A strong correlation ($r=0.96$, $P<0.01$) was found between the disease severity 7 days after inoculation and the disease progress rate (Figure 1). A disease assessment requires a considerable amount of time, space, and resources. The strong correlation already found between the two sunflower traits after 7 days sug-

Table 1. Analysis of variance for the disease severity score (DSS) and the disease progress rate (DPR) in 184 sunflower genotypes inoculated with *Phoma macdonaldii* isolate MP6 in controlled conditions.

Source of variation	df	MS	
		DSS	DPR
Replication	2	0.85 ^{ns}	0.02 ^{ns}
Genotype	183	4.89 ^{***}	0.06 ^{***}
Error	366	0.71	0.02
Total	551	2.10	0.032

***, Significant at $P<0.001$.

^{ns}, Not significant.

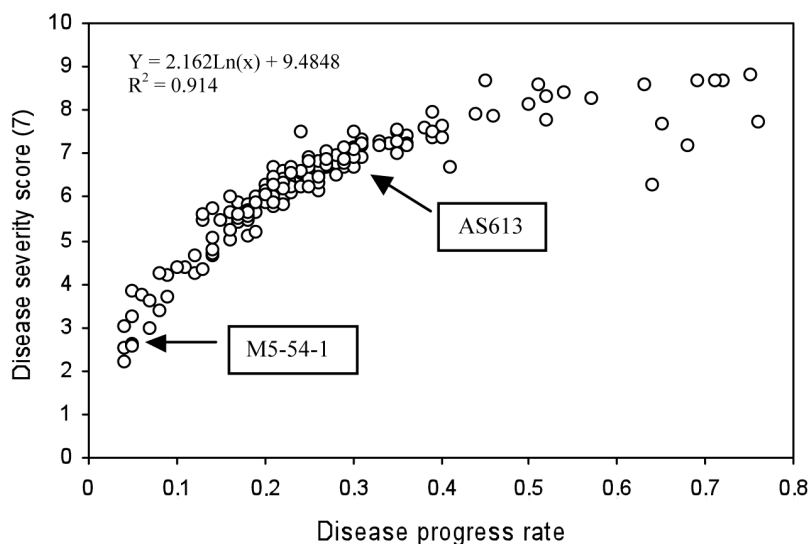


Figure 1. Correlation between the disease severity scores seven days after inoculation and the disease progress rate for 184 sunflower genotypes inoculated with *Phoma macdonaldii* isolate MP6 in controlled conditions. Arrows show the resistant mutant line M5-54-1 and the original line AS613.

gested that further assessments were unnecessary and that even after such a short time the disease severity score identified the sources of resistance to Phoma black stem. Mean disease severity at 7 days after inoculation was 6.30, and levels of genotype resistance to black stem varied from highly resistant to partially resistant. Almost 2.7% of genotypes were highly resistant, and 12.5% were moderately or partially resistant (Table 2). The inbred lines F1250/03 (from Hungary), M5-54-1, M6-862-1 (mutant lines), SDR 18 (from USA), and two wild *Helianthus* accessions, 1012 Nebraska and 211 Illinois (from USA), were highly resistant to black stem (Table 2).

Some mutant lines such as M5-54-1 presented highly enhanced partial resistance to black stem as compared with their original line, AS613 (Table 2). Plant resistance to a pathogen is often correlated with the receptor-mediated perception of the pathogen, which triggers a fast and efficient defence response in the host (Montesano *et al.*, 2003). One way to explain the phenotype of resistant mutant lines is that the mutation modified a putative receptor involved in resistance to the disease. Mutants produced by gamma irradiation that were resistant to fungal pathogens have been found in a number of other crops. For example, in wheat such mutants conferred resistance to stem and stripe

rust (Skorda, 1977), in sesame they produced resistance to *Phytophthora* blight (Pathirana, 1992), in rice they created resistance to rice blast (Zhang *et al.*, 2003; Han *et al.*, 2004) and in chickpea they made that plant more resistant to *Ascochyta* blight and *Fusarium* wilt (Ahloowalia *et al.*, 2004). In sunflower, gamma-irradiation produced genetic variations modifying morphological characteristics (Nabipour *et al.*, 2004) and germination traits (Alejo-Jaimes *et al.*, 2004).

In our study, the parental RILs (PAC-2 and RHA266) were moderately susceptible to Phoma black stem. However, with some RILs the disease was less severe than it was with their parents, while with others it was more severe (Table 2). Once the alleles for the increasing as well as decreasing quantitative traits in the parental lines disperse, the higher levels of favourable alleles in the progeny may result in better traits than in the parental lines because of a phenomenon known as transgressive segregation, in which the accumulation of alleles with positive or negative additive effects accumulate in the offspring (Zhang *et al.*, 2001). Transgressive segregation has been reported for resistance to Phoma black stem by Rachid Al-Chaarani *et al.* (2002) and Bert *et al.* (2004); for partial resistance to *S. sclerotiorum*, also in sunflower, by Micic *et al.* (2005a, b); for resistance

Table 2. Origin, disease severity scores^c (DSS) and disease progress rate (DPR) in some sunflower genotypes used in a partial resistance test against *Phoma macdonaldii* isolate MP6 in controlled conditions.

Sunflower genotype	Type ^a	Origin ^b	DSS Mean	SD ^d	DPR
211 Illinois	W	USA	2.23	0.80	0.04
F1250/03	BL	H	2.54	0.14	0.04
1012 Nebraska	W	USA	2.56	0.55	0.05
M5-54 -1	M	F	2.60	0.32	0.05
M6-862 -1	M	F	2.61	0.77	0.05
665 Iowa	W	USA	3.00	0.19	0.07
M5-39 -2 -2	M	F	3.03	0.74	0.04
SDR18	BL	USA	3.28	0.78	0.05
SDR19	BL	USA	3.61	1.61	0.07
ENSAT-R4	BL	F	3.70	0.34	0.09
1016 Nebraska	W	USA	3.77	0.62	0.06
M5-663 -1	M	F	3.82	0.17	0.05
M5-796 -2	M	F	4.21	0.73	0.09
RHA274	BL	USA	4.25	0.42	0.12
510 Kansas	W	USA	4.25	1.08	0.08
C81	RIL	F	4.33	1.87	0.13
AS86-1	BL	USA	4.37	0.93	0.10
M5-502 -2 -2	M	F	4.39	0.99	0.11
RT931	BL	F	4.66	0.93	0.14
M5-352 -2 -2	M	F	4.67	1.01	0.12
AS5305	BL	F	4.70	0.29	0.14
M5-357 -1	M	F	4.80	0.69	0.14
RHA266	BL	USA	6.57	1.23	0.29
PAC2	BL	F	6.76	0.80	0.30
AS613	BL	F	7.19	0.58	0.31
C134	RIL	F	7.19	0.80	0.36
C138	RIL	F	7.29	1.15	0.35
M5-522 -1	M	F	7.88	0.75	0.46
M5-214 -1	M	F	7.94	0.19	0.44
SDB1	BL	USA	7.96	0.54	0.39
27/2n-p	BL	EE	8.67	0.50	0.72
76/1n	BL	EE	8.82	0.40	0.75

^a BL, breeder's line; RIL, recombinant inbred line; M, gamma-irradiated induced mutant line; W, wild type.

^b F, France; USA, the United States of America; H, Hungary; EE, Eastern Europe.

^c Disease severity score of 36 seedlings per genotype infected with *Phoma* black stem isolate MP6, seven days after petiole inoculation. A score of 1 (resistance) to 9 (susceptible) was given depending on the proportion of petiole area showing necrosis, where: 1, 0–5% necrotic area; 2, 6–10%; 3, 11–20%; 4, 21–30%; 5, 31–40%; 6, 41–60%; 7, 61–80%; 8, 81–99%; and 9, 100% petiole area with necrosis spreading downward into the stem.

^d Standard deviation.

to powdery mildew in oat lines derived from a cross between cv. Maldwyn and the susceptible cv. Mostyn by Jones (1983); and for resistance to powdery mildew in bread wheat by Lillemo and Skinnes (2006).

In this study, two wild *Helianthus* accessions (1012 Nebraska and 211 Illinois, both from the USA) showed high partial resistance against Phoma black stem. Several wild *Helianthus* accessions have been described as potential sources of genes conferring resistance to *S. sclerotiorum* (Seiler and Rieseberg, 1997) and this is the first report that these accessions also confer resistance to Phoma black stem. The significance of wild accessions in sunflower breeding is well known (Serieys, 1987), and interspecific hybridization is used as a potential source of cytoplasmic male sterility (Serieys, 1995), fertility restoration, resistance to insects and disease, early ripening, and improved oil and protein quality (Seiler, 1992).

In conclusion, significant differences were found between sunflower genotypes in their partial resistance to Phoma black stem. New sources of resistance to Phoma black stem were identified in the genotypes selected from the mutant sunflower population as well as in the gene pools of wild genotypes. These genotypes may therefore contribute significantly to the diversity of the gene pool that is available to sunflower breeders and that confers resistance to Phoma black stem.

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