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Genotype-isolate interaction for resistance to black stem in sunflower (*Helianthus annuus*)

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Two experiments were undertaken to determine the partial resistance of sunflower genotypes to seven isolates of *Phoma macdonaldii*. In the first experiment, 28 genotypes, including recombinant inbred lines and their parents, M6 mutant lines developed by gamma irradiation, and some genotypes from different geographical origins, were used. The experiment consisted of a split-plot design with three replications, each with 12 seedlings per genotype per isolate, in controlled conditions. Seven days after inoculation, plantlets were scored on a 1–9 scale for percentage necrotic area. Highly significant differences were observed among genotypes, isolates and their interactions. The presence of a differential interaction between genotypes and *P. macdonaldii* isolates was confirmed in a second experiment using 12 genotypes representing large variability for partial resistance to *P. macdonaldii* isolates, as identified in the first experiment. Inbred lines B454/03, ENSAT-B5 and LC1064C were the most susceptible sunflower genotypes, whereas two American lines SDR19 and SDR18 presented high partial resistance to all *P. macdonaldii* isolates studied. The least and most aggressive isolates were MA6 and MP3, respectively. Isolates interacted differentially with sunflower genotypes. This study identified two genotypes (AS613 and PAC2) presenting specific resistance to isolate MP8. The results also showed a wide range of isolate-nonspecific partial resistances among the lines tested. The information presented here could assist sunflower breeders to choose parents of crosses for breeding of durable resistance to phoma black stem disease.

Keywords: black stem, genotype-isolate interaction, partial resistance, *Phoma macdonaldii*, sunflower

Introduction

Black stem, caused by the necrotrophic fungus *Phoma macdonaldii*, is one of the most important diseases of sunflower in the world. It is present in many European countries, including the former Yugoslavia, Italy, Romania and Bulgaria (Acimovic, 1984), China (Hua & Ma, 1996), Australia (Acimovic, 1984; Miric *et al*., 1999) and the USA (Acimovic, 1984). Black stem is the second-most important sunflower disease in France after downy mildew (Alignan *et al*., 2006). The disease is characterized mainly by black spots that appear on the stem at the base of leaf petioles and spread along stems. Black stem can occur at any time during the growing season and it is most severe when abundant moisture is available during and after flowering (Gulya *et al*., 1997). When the disease girdles the stem base, symptoms of premature ripening may occur (Donald *et al*., 1987; Sackston, 1992), resulting in small heads and seeds, and reducing seed and oil yields (Carson, 1991). Yield losses caused by the disease are moderate, ranging from 0·2 to 0·7 t ha^{-1} (Debaeke & Pérès, 2003). Infected plants are weak and more susceptible to lodging (Sackston, 1992).

Utilization of sunflower cultivars with improved partial resistance to phoma black stem in combination with appropriate crop management practices is an effective way to control the disease. Genetic variability for partial resistance to phoma black stem in sunflower was reported in both field (Pérès *et al*., 1994) and controlled conditions (Roustaee *et al*., 2000a; Rachid Al-Chaarani *et al*., 2002; Bert *et al*., 2004). Using parental genotypes and their F_1 hybrids, Roustaee *et al*. (2000a) showed that the variation for disease severity score among genotypes studied resulted from the additive and dominant effects of genes controlling black stem partial resistance. In two independent studies under controlled conditions using seedling tests, several quantitative trait loci (QTLs) for partial resistance to phoma black stem were identified (Rachid Al-Chaarani *et al*., 2002; Bert *et al*., 2004). All the above-mentioned genetic studies used plant materials inoculated with an aggressive French isolate of *P. macdonaldii* (MP6). Roustaee *et al*. (2000b) showed substantial differences among French *P. macdonaldii* isolates for growth rate, pycnidia morphology, pycnidiogenesis, pigmentation

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Table 1 Sunflower lines and their country of origin used in experiments for partial resistance to *Phoma macdonaldii* isolates

Sunflower line	Type[a]	Origin	Sunflower line	Type	Origin
C81	RIL	France	SDB1	BL	USA
C43	RIL	France	SDB3	BL	USA
C79	RIL	France	F651/1	BL	Hungary
LR64	RIL	France	F1250/03	BL	Hungary
RHA266	BL	USA	B454/03	BL	Hungary
PAC2	BL	France	ENSAT-B5	BL	France
M6-54-1	M	France	H565R	BL	France
M6-133-2	M	France	ENSAT-R5	BL	France
M6-85-3	M	France	H543R	BL	France
M6-894-2	M	France	RT931	BL	France
AS613	BL	France	AS5305	BL	France
RHA274	BL	USA	ENSAT-R4	BL	France
SDR18	BL	USA	ENSAT-B4	BL	France
SDR19	BL	USA	LC1064C	BL	France

[a]BL, breeder's line; RIL, recombinant inbred line; M, gamma-irradiation-induced mutant line.

and aggressiveness. The high genetic variability for pathogenicity in *P. macdonaldii* requires simultaneous incorporation of several genes for resistance to remain effective in cultivars used over a large area. The lack of information on the interactions between resistance genes and pathogen populations places a limitation on the effective deployment of resistance. This paper reports the results of experiments on partial resistance of some sunflower lines to different *P. macdonaldii* isolates.

Materials and methods

Sunflower genotypes

Twenty-eight sunflower genotypes were selected on the basis of their agricultural characteristics and levels of susceptibility to *P. macdonaldii* (Table 1). Recombinant inbred lines (RILs) derived from a cross between PAC-2 and RHA266 were selected for their high partial resistance to the MP6 isolate of *P. macdonaldii* (Rachid Al-Chaarani *et al*., 2002). Mutant lines were developed by irradiation of the AS 613 genotype with gamma rays and advanced by modified single-seed descent (SSD) with no prior selection for resistance to the disease (Sarrafi *et al*., 2000). A population of 120 mutant lines (M6) was evaluated against *P. macdonaldii* isolate MP6 in controlled conditions. Several mutants were identified that consistently showed altered black stem resistance and four of them were used in this investigation. Other genotypes used in this study were inbred lines introduced from the United States Department of Agriculture (USDA) and Hungarian and French seed companies, or pure lines from the department where this study was carried out.

Fungal isolates

Seven *P. macdonaldii* isolates were used for inoculation (Table 2). Six of these were collected from different regions in France; their characteristics were previously described by Roustaee *et al*. (2000b). The seventh isolate, MP11, was obtained from stem lesions in sunflower fields in Godolos, Hungary. Stem fragments showing characteristic symptoms were cut into 5- × 5-mm pieces, surface-sterilized for 5 min in a sodium hypochlorite solution (6 chlorometric degrees), washed three times (5 min) in sterile distilled water, transferred to Petri dishes containing potato dextrose agar (PDA, 39 g L^{-1}) and incubated for 10 days at 25 ± 1°C in the dark to allow mycelial growth. The dishes were incubated for a further 10 days under alternating periods of illumination (12 h, 37 μEm^{-2} s^{-1}; Philips TLD 15W, 33 lamps) and darkness. Single-pycnidiospore cultures were prepared by serial dilutions (Roustaee *et al*., 2000b) and maintained on PDA for further subculture. Conservation of isolates was achieved using the method described by Roustaee *et al*. (2000b). A healthy sunflower stem fragment, sterilized at 110°C for 25 min, was placed on a culture of an isolate (from a single spore) on PDA. Pycnidia were visible on the stem fragment after incubation for 15 days at 25 ± 1°C under alternating 12-h periods of illumination (37 μEm^{-2} s^{-1}) and darkness. When these fructifications developed, the stem fragment was placed in a sterile haemolysis tube. Tubes were closed with an absorbent cotton plug, covered with aluminium foil and kept in the dark at 4°C.

Experimental design

First experiment

The responses of 28 sunflower genotypes were evaluated with the seven isolates of *P. macdonaldii* under controlled conditions [14-h photoperiod and 25 ± 1°C/18 ± 1°C light/dark temperature, with a light intensity of 200 μEm^{-2} s^{-1} provided by NAV-T 600W lamps (Osram-Vialox), under 75–80% relative humidity]. The experiment was designed as

Table 2 *Phoma macdonaldii* isolates used in partial resistance experiments

Isolates	Year of collection	Country of origin	Locality
MP3	1996	France	Tour de Faure
MP6	1996	France	Castanet
MP8	1996	France	Saint Lys
MP10	1996	France	Il de France (Saint Pathus)
MA6	1997	France	Saint Lys
MA7	1997	France	Castanet
MP11	2003	Hungary	Godolos

a split plot with three blocks. Main plots were *P. macdonaldii* isolates and subplots were sunflower genotypes. Each subplot consisted of 12 plantlets of each genotype per replication. Seeds were sterilized for 5 min in a sodium hypochlorite solution (6 chlorometric degrees) and washed in sterile distilled water. Two rows of six seeds per genotype per replication were sown in plastic containers filled with horticultural substrate (Hawita Flor) and transferred to the growth chamber. *Phoma macdonaldii* isolates were separately grown on PDA medium at 25 ± 1°C with a 12-h photoperiod (37 μE m^{-2} s^{-1}). After a 10- to 12-day incubation period, pycnidiospore suspension was obtained by the addition of sterile water to the surface of each culture and mechanical mixing. Twenty microlitres of spore suspension containing 10^6 pycnidiospores mL^{-1} in water, 0·5% orange juice and 0·25% gelatine were deposited at the intersection of the cotyledon petiole and hypocotyl of two-leaf-stage sunflower plantlets. During the first 48 h following inoculation, plantlets were covered with a transparent cover (Plexiglas) to maintain nearly saturated humidity, favourable for fungal development. Both cotyledon petioles of each plantlet were scored 7 days after inoculation according to the percentage of the petiole area exhibiting disease symptoms. A score of 1 (resistant) to 9 (susceptible) was given in relation to the proportion of petiole area showing necrosis, as proposed by Roustaee *et al*. (2000a), where: 1 = 0–5%, 2 = 6–10%, 3 = 11–20%, 4 = 21–30%, 5 = 31–40%, 6 = 41–60%, 7 = 61–80%, 8 = 81–99% and 9 = 100%, with necrosis spreading down the stem.

Second experiment

Twelve sunflower genotypes identified in the first experiment as representing extensive variability for partial resistance and susceptibility to *P. macdonaldii* isolates were sown and inoculated with the same *P. macdonaldii* isolates. The experimental design and pathogen inoculation were the same as for the first experiment.

Statistical analysis

Analysis of variance was performed for the first experiment, as well as for the 12 genotypes used in both experiments, using the general linear model (GLM) procedure in the SAS software (SAS Institute). The main effects of genotypes and isolates, as well as their interactions, were determined.

Specific interactions between genotypes and isolates were identified by a robust statistical technique, median polish (Tukey, 1977; Arraiano & Brown, 2006). Median polish was used to fit an additive model of genotype plus isolate to the mean disease severity score for each genotype-isolate pair. Median polish operated by iteratively calculating the median scores for the isolates across the set of genotypes and those for the genotypes across the set of isolates. If there was no 'genotype × isolate' interaction, residuals of the observed mean disease severity score from the genotype-plus-isolate additive model fitted by median polish were normally distributed. On the other hand, a median polish residual for a genotype-isolate pair which was an outlier from a normal distribution, indicated that the genotype was specially resistance to the isolate if the residual was negative, or susceptible if it was positive. A negative outlier indicated that the amount of disease caused by an isolate on a genotype was exceptionally low considering the median amount of disease caused by all seven isolate on that host genotype and the median disease caused by that isolate on all 28 genotypes. Combinations of genotypes and isolates were not replicated equally, so the median polish residual for each genotype-isolate pair was multiplied by the square root of the number of plantlets scored, to ensure that all residuals, with the exception of outliers, were sampled from a single normal distribution (Chartrain *et al*., 2004). The statistical significance of the deviation of the median polish residual from a normal distribution was tested by Grubbs' method (Sokal & Rohlf, 1981; Brown *et al*., 2001). This part of the analysis was completed using Microsoft EXCEL 2002.

Results

The main effects of genotypes, isolates and their interactions were highly significant in both experiments, whereas the main effect of experiment and the interactions between experiments and other sources of variation were not significant (Table 3). Median disease severity scores across isolates for each genotype and across genotypes for each isolate were calculated omitting data for isolates with which the genotypes had specific interactions. These medians therefore reflected the general level of susceptibility of a genotype to the isolates tested, as well as the general aggressiveness of an isolate to the genotypes tested. The inbred sunflower lines B454/03, ENSAT-B5 and LC1064C were the most susceptible genotypes, showing high susceptibility to all isolates tested (Table 4). The most resistant genotypes were the two American lines SDR19 and SDR18, followed by RIL-C81 and RHA274/R, in which no specific resistance was identified (Table 4). SDR18 and SDR19 showed good partial resistance to all isolates tested.

Isolates also showed differences in their aggressiveness for the 28 genotypes tested. MP3 was the most aggressive isolate and MA6 showed the lowest level of aggressiveness (Table 4). Isolate genotype interacted differentially with host genotype. MP8 was the only isolate for which two sunflower genotypes with specific resistance were identified. AS613 and PAC2, which are recognized as sources of specific resistance to isolate MP8, were more susceptible to most other isolates. RIL-C81 was identified as specifically susceptible to isolate MP11. This line had good partial resistance to most isolates tested, except MA7 (Table 4).

Discussion

Scoring the damage caused by the pathogen in naturally infected genotypes under field conditions can be reliable, but it is not always possible to expose plants to the pathogen evenly and so achieve uniform infection. Homogeneous infection of each genotype is essential for the precise

Table 3 Analysis of variance for disease severity score in sunflower genotypes infected by *Phoma macdonaldii* isolates in two experiments

Experiment I			Experiment II		
Source of variation	d.f.[a]	MS[b]	Source of variation	d.f.	MS
Genotype	27	44·30***	Genotype	11	128·82***
Isolate	6	21·51***	Isolate	6	20·49***
			Experiment	1	1·78^{ns}
Genotype × isolate	162	5·71***	Genotype × isolate	66	14·24***
			Genotype × experiment	11	0·49^{ns}
			Isolate × experiment	6	0·34^{ns}
			Genotype × isolate × experiment	66	0·20^{ns}
Replication	2	2·07^{ns}	Replication (experiment)	4	0·67^{ns}
Replication × isolate	12	1·95	Replication × isolate (experiment)	24	0·65
Residual	378	0·56	Residual	308	0·56

Coefficients of variation were 15·00% and 15·53% for experiments I and II, respectively.
[a]d.f. = degrees of freedom.
[b]MS = mean of squares.
*** = Significant at 0·001probability level; n.s. = non significant.

Table 4 Mean disease severity score[a] of 28 sunflower genotypes inoculated with seven *Phoma macdonaldii* isolates under controlled conditions

Sunflower genotype	MA6	MA7	MP10	MP11	MP3	MP6	MP8	Median[b]
AS5305	4·19	6·15	6·83	4·34	8·15	4·70	4·74	4·87
AS613	6·74	7·57	3·63	4·01	6·80	7·37	**2·24 (−4·42)**[e]	6·79
B454/03	7·77	7·42	7·27	7·60	7·71	7·46	7·28	7·40
ENSAT-B4	7·62	6·96	5·83	6·33	7·19	6·83	7·33	6·80
ENSAT-B5	6·78	6·72	7·50	6·25	7·54	7·41	7·76	7·21
ENSAT-R4	3·14	3·59	3·94	3·92	7·70	2·20	5·58	3·89
ENSAT-R5	2·21	4·81	7·00	3·72	4·05	2·10	1·71	3·66
F1250/03	2·88	5·01	3·73	5·97	5·82	2·13	2·15	3·68
F651/1	3·60	7·13	5·25	3·61	5·95	6·72	5·43	5·56
H543R	1·56	3·08	4·03	3·34	6·99	6·15	3·89	3·98
H565R	7·40	4·66	7·16	6·36	7·10	7·40	5·44	6·71
LC1064C	6·81	7·35	7·44	7·14	7·59	6·13	6·43	7·20
M6-133-2	5·27	4·77	6·52	4·28	3·72	5·44	5·90	5·39
M6-54-1	1·24	4·07	2·21	6·88	5·96	2·78	6·46	3·91
M6-85-3	7·16	7·62	6·99	6·94	5·83	6·21	6·85	6·98
M6-894-2	6·87	6·54	6·27	5·27	7·63	7·52	6·64	6·77
PAC2	5·14	6·71	7·10	5·83	7·55	6·92	**2·38 (−4·20)**	6·71
RHA266	2·48	6·00	3·04	4·31	3·71	6·06	6·33	4·66
RHA274/R	1·12	6·15	2·90	3·55	2·67	3·78	2·21	2·85
RIL-C43	3·76	4·80	5·43	3·23	6·94	4·07	1·88	4·19
RIL-C79	4·60	6·19	7·06	3·65	7·88	5·97	4·53	5·92
RIL-C81	2·32	5·40	1·57	*6·96 (4·50)*[c,d]	3·27	3·28	1·20	2·81
RIL-LR64	6·94	5·11	5·25	3·68	2·22	7·19	1·64	4·95
RT931	3·24	5·39	5·68	5·14	3·70	4·66	4·36	4·61
SDB1	4·02	5·29	4·90	4·24	4·48	3·10	4·32	4·45
SDB3	4·37	3·71	6·48	4·05	4·98	5·71	4·15	4·59
SDR18	1·77	1·90	1·32	3·85	2·49	2·05	1·89	2·02
SDR19	2·15	2·49	2·56	2·52	2·81	3·08	2·06	2·50
Overall median	4·82	5·41	5·31	4·90	5·64	5·30	5·12	

[a]Means estimated by general linear modelling.
[b]Medians for each genotype calculated from mean scores with seven isolates, omitting any isolate for which the 'genotype × isolate' interaction effect was significant ($P = 0·05$) in the median polish (MP) analysis.
[c]Italics indicate specific susceptibility.
[d]Round brackets indicate residuals (difference of observed and fitted values) for statistically significant interactions ($P = 0·05$) in MP analysis. A larger negative residual indicates greater isolate-specific resistance and a positive residual indicates isolate-specific susceptibility.
[e]Bold type indicates specific resistance of genotype to an isolate ($P = 0·05$ in MP analysis).

identification of the level of susceptibility for each inbred genotype. For this reason, the artificial inoculation method developed by Roustaee *et al*. (2000a) was used in the present studies. The seedling petiole test used in this study allowed the inoculum load per plant to be controlled, thus minimizing the number of plants escaping infection and consequently reducing the potential for false negatives, as up to now reports on black stem resistance in sunflower indicate the lack of full resistance in cultivated sunflower (Roustaee *et al*., 2000a; Debaeke & Pérès, 2003). Moreover, all plantlets with no visible lesion on the petiole were defined as not infected, and excluded from the present experiments. Roustaee *et al*. (2000a) reported that the disease severity score at the seedling stage was the same as that at the adult plant stage.

Genotypes used in this study differed considerably for black stem resistance (Table 4). Among 28 sunflower genotypes used in the experiments, three lines, B454/03, ENSAT-B5 and LC1064C, were susceptible to all isolates of *P. macdonaldii*, whereas two American lines, SDR18 and SDR19, showed partial resistance to all *P. macdonaldii* isolates (Table 4). Other genotypes showed intermediate responses across *P. macdonaldii* isolates (Table 4). These results confirm the genetic variability for partial resistance to phoma black stem that was previously reported in both field (Pérès *et al*., 1994) and controlled conditions (Roustaee *et al*., 2000a; Rachid Al-Chaarani *et al*., 2002; Abou Al Fadil *et al*., 2004; Bert *et al*., 2004).

This study showed significant differences in aggressiveness among isolates of *P. macdonaldii* (median ranged from 4·82 to 5·64). The least and most aggressive isolates were MA6 and MP3, respectively (Table 4). This variation can be attributed predominantly to genetic causes. In this study the infection of plants at the same growth developmental stage, grown under similar conditions, helped to minimize the influence of environmental factors. The constancy of ranking of isolates over the two experiments in this study provides further strong evidence for this claim. Similar variation in aggressiveness was demonstrated among isolates of *P. macdonaldii* originating from different geographical locations based on pathogenicity tests on cotyledon petioles (Roustaee *et al*., 2000b; Larfeil *et al*., 2002).

The results provide strong indications of the existence of specificity between *P. macdonaldii* isolates and sunflower genotypes for partial resistance. The presence of differential interaction between sunflower genotypes and *P. macdonaldii* isolates was confirmed in the second experiment using a genetically diverse group of genotypes (Table 3). These results are in agreement with those of Larfeil *et al*. (2002), who also found large differences between French *P. macdonaldii* isolates and those of other countries in response to sunflower genotypes for partial resistance to black stem infection. The median polish analysis allowed individual interactions to be identified clearly. Two genotypes (AS613 and PAC2) presenting specific resistance to isolate MP8 were identified (Table 4). Specific interactions can be used to postulate the presence of resistance genes that operate partially in compatible interactions as the percentage of petiole area exhibiting disease symptoms (necrosis) in partially compatible interactions (partially resistant genotypes) corresponds to a very localized necrosis and not to a hypersensitive response, as it spreads slightly with time. Zenbayashi-Sawata *et al*. (2005) reported a new gene-for-gene relationship between a partial resistance gene in rice and a gene for aggressiveness in the fungus *Magnaporthe grisea*. They concluded that the gene-for-gene relationship between host and pathogen might operate not only for complete resistance, as expressed in incompatible combinations, but also for partial resistance in compatible interactions. In the present experiments AS613 and PAC2 did not show good levels of resistance across the isolates tested, whereas they were resistant to isolate MP8 (Table 4). This indicates that field resistance to phoma black stem in these genotypes may be controlled by isolate-specific partial resistance genes rather than by isolate-nonspecific partial resistance genes. The list of isolate-specific interactions identified here is not necessarily exhaustive. The statistical methods used identified outliers, so that for each isolate a line with a score that contrasted with those of other lines was identified as significant and, similarly for each line, a response to an isolate which contrasted with the responses to other isolates was identified as significant. However, if a line was resistant to all isolates no interaction could be detected, and if one isolate had low aggressiveness on all lines tested no interaction could be detected either. SDR18 and SDR19, the most resistant lines, had high partial resistance to all isolates (Table 4), but the possibility that they had weak specific resistance to all seven isolates cannot be excluded. RIL-C81 was especially susceptible to isolate MP11, but it was the most resistant genotype overall. This genotype probably lacks a gene carried by the majority of genotypes that confers resistance to isolate MP11.

'Genotype-isolate' interactions for partial resistance were also observed in other pathosystems, such as sunflower-*Phomopsis* (Viguié *et al*., 1999), potato late blight (Flier *et al*., 2003), rice leaf blast (Zenbayashi-Sawata *et al*., 2002), wheat leaf rust (Broers, 1989) and dry bean anthracnose (Havey *et al*., 1988).

In this study, mutant line M6-54-1 showed strongly enhanced partial resistance towards three *P. macdonaldii* isolates (MA6, MP10 and MP6) compared to the original line AS613 (Table 4). This might have been caused by the lack of some susceptibility factors in the mutant line. The same mutant line also possessed enhanced susceptibility for a further fungal isolate (MP8) to which the parental line was partially resistant (Table 4). Plant resistance towards a pathogen is often correlated to receptor-mediated perception of the pathogen, which triggers a fast and efficient defence response in the host (Montesano *et al*., 2003). A possible hypothesis to explain the phenotype of mutant M6-54-1 is that a mutation modified a putative receptor involved in resistance towards isolates MA6, MP10 and MP6. This modification would have changed ligand specificity so that these three isolates were more readily perceived, but MP8 was not. It would be interesting

to further identify the mutation and the genes responsible for resistance to these isolates.

Recombinant inbred lines (RILs) used in the present study showed different reactions to seven isolates of *P. macdonaldii* when compared with their parental lines (PAC2 and RHA266) (Table 4). This is in agreement with the work of Bert *et al*. (2004), who observed that the susceptibility of sunflower genotypes in F_3 families infected by an isolate of *P. macdonaldii* varied in both directions when compared with their parents. This phenomenon, considered as transgressive segregation, is the result of accumulation of alleles with positive or negative additive effects in the offspring (Zhang *et al*., 2001).

The isolate-specific and -nonspecific partial resistance genotypes identified in these experiments should be used in crossing programmes for breeding of durable resistance to phoma black stem disease.

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