Genetic variability and QTL for sunflower tolerance to premature ripening caused by *Phoma macdonaldii*

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• *Phoma macdonaldii* has been reported as the causal agent of the black stem disease on sunflower and premature ripening (PR). In France, PR is now regarded as one of the most widespread and detrimental diseases in sunflower. Genetic variability and QTL for partial resistance of sunflower to stem, collar and roots attacks have been reported since ten years. The aim of this work was to describe the genetic variability and to find QTL involved in PR resistance in sunflower.

• Four field trials were conducted at INRA, in Auzeville-Tolosane (Haute-Garonne, France) between 2009 and 2011. Genetic variability of PR resistance was assessed in 2009 and 2010 on 21 sunflower lines chosen among the 48 lines of the *Helianthus annuus* core collection. Four RIL population parents (XRQ, PSC8, FU and PAZ2) showed very different level of resistance to PR. In accordance, the PR resistance QTL analysis was conducted in 2010 and 2011 on two recombinant inbred lines populations: respectively XRQxPSC8 (115 lines), and FUxPAZ2 (120 lines). Plants were subjected to *Phoma macdonaldii* mycelium inoculation at the stem base. QTL analysis was based on a genetic map of the two RIL populations built respectively with 203 and 123 SSR and SNP markers.

• An important genetic variability was detected among the 21 inbred lines of the core collection: the percentage of PR plants ranges from 0 to 100% in 2009 and 0 to 68% in 2010, 49 days post-inoculation (DPI). On the two RIL populations, at 49 DPI, differences between inbred lines were highly significant ($P<0.0001$). Two QTL involved in PR sunflower tolerance have been detected at 49 DPI: one QTL on linkage group 10 (XRQxPSC8 population) and one QTL on linkage group 7 (FUxPAZ2 population) explaining respectively 22% and 29% of the phenotypic variability.

• The high percentage of stem base necrosis (up to 95%) observed before natural attacks occurrence on the four trials shows the effectiveness of the inoculation method. The absence of common molecular markers between our genetic map of the FUxPAZ2 population and the map previously used doesn't allow the comparison of QTL involved in PR resistance with those previously reported as involved in black stem disease.

• This work reveals for the first time the quantitative behavior of sunflower PR resistance and a high genetic variability in the *Helianthus* genus. These results open new prospects for sunflower genetic improvement.

Key words: Recombinant inbred lines – Mycelium – Core collection - Quantitative resistance.

INTRODUCTION

Premature ripening (PR) induced by *Phoma macdonaldii* Boerema (teleomorph: *Leptosphaeria lindquistii*) is one of the most severe and widespread sunflower disease in France. PR could be partly responsible for the yield stagnation around 2.5 $t.ha^{-1}$ during the two last decades. *P. macdonaldii* is also responsible of black stem disease. As chemical control is difficult, development of resistant varieties is an important breeding objective. Some cultivars appear more resistant than others to black stem disease (Larfeil *et al*., 2010) and PR (Seassau *et al*., 2010a) but no hybrid has been identified to be immune to this disease. Several studies dealing with the genetic control of sunflower resistance to *Phoma* attacks on petiole, stem base and roots of seedling in growth chamber have demonstrated the quantitative character of this resistance (Abou Al Fadil *et al*., 2007a,b ; Bert *et al*., 2004 ; Darvishzadeh and Sarrafi, 2007 ; Rachid Al-Chaarani *et al*., 2002). Recently, Seasseau *et al*. (2010b) demonstrated the clear role of aerial *Phoma* infection in PR compared with soilborne inoculums. They showed that artificial inoculation at the stem base with pycniospores or mycelium of *P. macdonaldii* could be used for screening genotypes having high levels of partial resistance to PR. The developed method provides a unique toolkit to investigate phenotypical variability and genetic control of premature ripening resistance in *Helianthus*.

MATERIALS AND METHODS

Sunflower lines: In 2009, the resistance level to PR of 42 genotypes was evaluated in a field trial in Auzeville: 40 lines were selected from the *Helianthus annuus* nested core collection of 48 lines (Coque *et al*., 2008) and two additional lines were included due to their high level of tolerance in preliminary observations : (Tub-1709-1)-1-6A is derived by selfing from the USDA accession TUB-1709-1 which results from an introgression of *H. tuberosus* , and 97B7 is derived from a cross involving *H. argophyllus.* According to the results, a representative subset of 21 lines was chosen to confirm their resistance level in a second trial in 2010 (Table 1).

Table 1. Description of the 21 lines used (19 from the nested core-collection). 'Core collection ID' indicates the line code used in this study and in Coque et al., (2008), 'Type' indicates if lines restore the male sterility on PET1 cytoplasm (R) or maintain the PET1 cytoplasmic sterility (B), 'Origin' indicates the line pedigree.

Core collection ID	Type	Other name	Origin
SF056	B	FU	INRA line, quantitative resistance to downy mildew, tolerance to sclerotinia head rot
SF060	B	-	INRA line
SF061	B	-	INRA line
SF063	B	-	INRA line
SF085	B	CD	close to HA89 /USDA
SF107	B	92B6	INRA line, derived from a cross with *H.argophyllus*
SF110	B	NF	INRA line
SF193	B	XRQ	INRA line, *Pl5* , quantitative resistance to downy mildew
SF263	R	-	INRA line, quantitative resistance to downy mildew
SF278	R	OQP7	INRA line, high oleic, *PlArg*
SF292	R	PRS5	INRA line
SF302	R		INRA line, parent of the RIL population PAC2*RHA266
SF306	R	PAZ2	INRA line
SF308	R	PAC1	INRA line
SF310	R	PST5	INRA line
SF326*	R	PSC8	INRA line
SF330	R	RHA801	USDA
SF334	R	-	INRA line
SF336	R	-	INRA line
(Tub-1709-1)-1-6A	?	-	derived from Tub-1709-1, USDA, *H. tuberosus* introgression
97B7	B	-	INRA line, derived from a cross with *H. argophyllus*

*SF326: missing phenotyping data in core collection trial in 2010

The two RIL populations were obtained by single seed descent from a cross between INRA lines from the nested core collection. The first RIL population (115 F8 lines) was obtained from a cross of XRQ and PSC8 parental lines (Vear *et al*., 2008). XRQ showed a higher level of resistance to PR than PSC8. The

second RIL population (120 lines F7-F10) results from the cross of FU and PAZ2 (Bert *et al*., 2004), these two parental lines appear respectively susceptible and resistant to PR.

Experimental design: Four field experiments were carried out to evaluate the resistance level of sunflower lines against PR over three years (2009, 2010 and 2011) at INRA, Auzeville-Tolosane, near Toulouse (Haute Garonne, South-Western France).
Before sowing, one level of N fertilization was applied on each trial (60 $kg.ha^{-1}$). The crop was sown at the beginning of May in 2009 (late due to rainy conditions) and at the beginning of April in and 2010 and 2011. The experiments were conducted in a randomized block design, with two replications in 2009 and three replications in 2010 for core collection trials, and with two replications for XRQxPSC8 (2010) and FUxPAZ2 (2011) RIL populations trials. Each plot consisted of 3 rows of about 25 plants. Plant density was 7 $plants.m^{-2}$ after thinning. According to rainfall, irrigation (30 mm) was performed before inoculation in 2011. As *Phomopsis* is regularly present in Auzeville-Tolosane, Corbel (fenpropimorph, 0.8 $l\ ha^{-1}$, BASF) was applied in June to control this disease in all experiments.

Plant inoculation: A single *P. macdonaldii* monopycniospore strain (MPH2), coming from natural stem base attack in Toulouse area and previously selected for its severe aggressiveness, was used in all experiments. Mycelium conservation, inoculum production and mycelium inoculation were performed according to Seassau *et al*. (2010b). Mycelium inoculation occurred before natural attacks, at bud stage (E1 to E5).

Disease assessment: Development of necrosis at the stem base and premature ripening induced by *P. macdonaldii* was assessed from 14 days post-inoculation (DPI) over the three years. The disease was scored using a 1–5 scale: 1 = healthy plant, 2 = less than 3/4 of the stem base circumference black, 3 = necrosis girdling the stem base, 4 = all leaves wilted but the stem green, 5 = plant completely dry. A PR plant is defined as completely dry before physiological maturity with necrosis girdling the stem base.
Disease symptoms were observed weekly on the 15 infected plants per replication. A few plants affected during the cropping season by other fungal diseases (*Phomopsis* stem canker, *Verticillium* wilt, *Alternaria* leaf spot and blight) were ignored and only *Phoma* symptoms were observed on the remaining infected plants. Disease assessment was performed up to physiological maturity on the four experiments. *Alternaria* leaf spot and blight was assessed in July 2010 on XRQxPSC8 RILs population (two replications) using a 1-4 scale: 1= healthy plant, 2 = foliar symptoms in the lower part of the plant, 3= foliar symptoms on the whole plant, 4= blighted plant.

Statistical analyses: For each experiment, mean disease score and percentage of PR plants of each plot and each genotype were calculated (Microsoft Excel software). The genotype effect on PR resistance was assessed by analysis of variance on mean disease scores according to a general linear model (GLM procedure, SAS software, SAS Institute Inc.) for each disease assessment date.

QTL analysis: The two RIL population genetic maps were built with genotyping data developed in others projects (data available upon request). The FUxPAZ2 and XRQxPSC8 maps contain respectively 123 and 203 markers, including morphological markers, SSR markers and gene-based markers. These maps, covering 879 and 1230 cM respectively, present 7 and 12 makers on average per linkage group with an average distance between makers of 7 and 6 cM. QTL detection was performed on adjusted mean disease scores with MCQTL® software (Jourjon *et al*., 2005) under the *forward* algorithm and *iQTL* option (Charcosset *et al*., 2001). A genome scan of 2cM was used and the level of significance (detection threshold) was determined through 2000 permutations for each trait.

RESULTS AND DISCUSSION

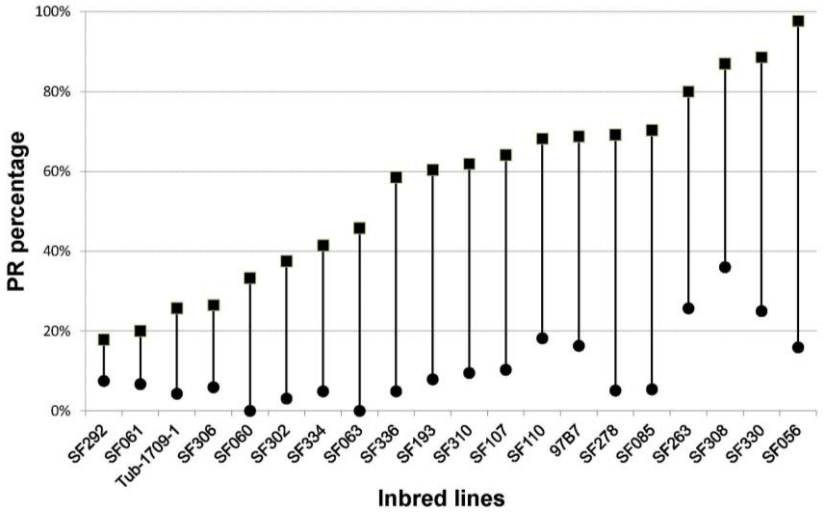
Disease development: On the four experiments, the mean percentage of plants showing *Phoma* necrosis at the stem base (disease score $\geq$ 2) ranged from 97,9% to 98,7%. These high percentages showed the effectiveness of the inoculation method. Such result has already been obtained in field and greenhouse trials by Seassau *et al*. (2010a, b) : over a total of six experiments, 100% of infected plants showed stem base necrosis and 92% to 100% reached a disease score $\geq$3 (girdling necrosis).

On all four experiments, stem base necrosis increased from disease score 2 to disease score 3 during the first 35 DPI. All genotypes presented at least one plant with a girdling *Phoma* necrosis at the stem base (disease score =3). The first premature ripened plants appeared in each trial at 42 DPI and between 17%

and 33% of plants showed PR at 49 DPI according to the experiments. One week later, this percentage ranged between 35% to 51%. This evolution is consistent with previous results obtained on sunflower hybrids in field trials (Seassau, 2010) and greenhouse trials (Seassau *et al*., 2010b), where the premature ripened plants started to appear at 43 DPI.

Phenotypic variability in the core-collection: An important genetic variability has been detected among the 20 inbred lines of the core collection. Due to large phenological differences among the 20 lines, PR percentage data are given at the same phenological stage for each genotype: the M1.2-M1.3 stage allows a good evaluation of PR resistance, avoiding confusion of premature ripening with natural senescence. According to the genotypes, this stage is reached between 35 and 63 DPI.
The PR percentage at M1.2-M1.3 stage (disease score ≥ 4) ranged from to 0% to 100% in 2009 and from 17.9% to 97.7% in 2010. As the total number of plants per inbred line is low in 2009 (mean=13.6), the variability for PR resistance among the 20 lines is presented on 2010 data (mean number of plants = 54.3). Analysis of variance of the mean disease score at M1.2-M1.3 stage showed a highly significant genotype effect for PR resistance in the 2010 trial (mean=3.74; CV=18.5; F=16.4; P<0.0001). Fig. 1 presents the comparison of PR percentage during the critical period around M1.2-M1.3 stage of the 20 lines in 2010.

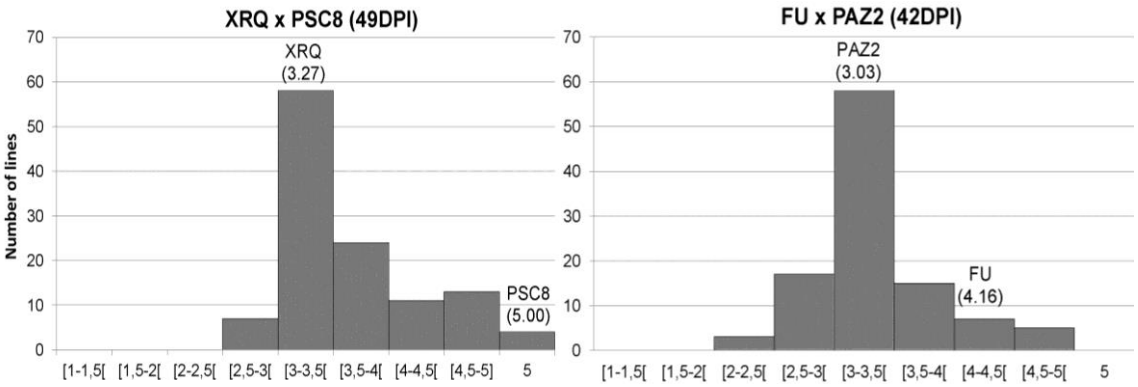
Fig.1. Evolution of PR percentage (disease score ≥4) on the 20 lines, assessed in 2010 during a 7-day period around the M1.2-M1.3 phenological stage (first disease scoring ● and second diseases scoring 7 days later ■).



Between the two disease assessment dates around the M1.2-M1.3 stage, the mean PR percentage of the 20 lines increased from 10.6% to 56.1%. This increase in PR severity showed a very high variability among the 20 lines : from +10 to +82%. Several lines seemed to have a high level of PR resistance: SF292, SF061, (Tub-1709-1)-1-6A and SF306, with a final PR percentage less than 30% and lines such SF263, SF308, SF330 and SF056 appeared quite susceptible, with more than 80% of premature ripened plants. It seems that these lines are characterized by a higher level of PR severity just before M.2-M1.3 stage.

Disease observations on RILs: In the two populations, the phenological stage M1.2-M1.3 is reached between 42 and 49 DPI. Figure 2 presents frequency distributions for PR resistance observations. At this phenological stage, the global mean disease score of XRQxPSC8 and FUxPAZ2 RILs populations is respectively 3.90 and 3.29. On the two RIL populations, this mean disease score varied from 2.30 to 5.00. These data confirmed the behaviour of the four parental lines: PSC8 and FU appeared quite susceptible (5.00 and 4.16 respectively), whereas disease severity remained low on XRQ and PAZ2 (3.27 and 3.03 respectively). Transgressive lines are observed in XRQxPSC8 and FUxPAZ2 populations with respectively 2 and 8 lines showing higher mean disease score than the susceptible parental line, and 48 and 3 lines showing lower mean disease score than the resistant parental line.
The mean disease score analysis of variance showed highly significant differences between inbred lines (P<0.0001) on the two populations.

Fig. 2. Frequency distributions of mean disease score for PR resistance in the XRQxPSC8 (a) at 49DPI and FUxPAZ2 (b) RIL populations at 42 DPI. Phenotypic values of parental lines are indicated.



QTL detection: Quantitative trait loci associated with sunflower PR resistance on the two RILs populations are presented in Table 2. For XRQxPSC8 population, five QTL were identified, one on linkage group 16 at 14 DPI and four on the linkage group 10 at 42, 49, 56 and 63 DPI. These QTL explained from 15% to 23% of the observed phenotypic variance. The QTL located on the linkage group 16 is detected only two weeks after inoculation : this can be explained by the fact that the stem base of the RILs carrying the XRQ allele was more rapidly girdled by *P. macdonaldii* necrosis (disease score 3) than those carrying the PSC8 allele, then these lines showed better resistance during disease development. Taking into account the disease severity level from 42 to 63 DPI, the position and the confidence interval of the four QTL identified on the linkage group 10, these QTL are probably the same QTL, which is involved in PR resistance. The effect of the resistance was given by tolerant parental line XRQ. After flowering, the RIL population has been affected by *Alternaria* leaf spot and blight. The 120 RILs showed a very important variability for disease severity, the genotype effect was highly significant (Kruskal-Wallis test : P<0.0001). Among the three QTL identified, one very significant QTL is detected on the linkage group 10 and explained more than 60% of the phenotypic variance. This QTL is located at the same position that the QTL involved in PR resistance, and the parental line XRQ provided the favourable allele for the two diseases. These results suggest that a major gene involved in *Alternaria* leaf spot and blight resistance probably exists in this linkage group and the existence of a QTL involved in PR resistance in the same position may be discussed.
Concerning FUxPAZ2 population, three QTL were detected on the linkage group 7 (at 42, 49 and 56 DPI), explaining 22 to 29% of the phenotypic variability. For the same reasons as the XRQxPSC8 population, these three QTL are probably the same QTL, involved in PR resistance.

Table 2. Significant QTL for premature ripening resistance in XRQxPSC8 and FUxPAZ2 RIL populations.

RIL population	QTL name	Linkage group	Local position	Interval support	LOD	R^2 (%)	Parental effect value
XRQxPSC8	PmJ14	16	75.7	[66.8;85.1]	4.6	15.9	PSC8 : (0.052)
	PmJ42	10	15.3	[1.7;39.6]	5.3	17.8	XRQ : (0.102)
	PmJ49	10	13.3	[2.8;24.3]	7.1	22.3	XRQ : (0.168)
	PmJ56	10	20.4	[12.1;30.7]	6.5	21.0	XRQ : (0.170)
	PmJ63	10	22.4	[12.5;40.8]	4.2	14.5	XRQ : (0.113)
	Alternaria	2	36.4	[9.0;48.6]	3.6	9.0	XRQ : (0.107)
	Alternaria	10	13.3	[12.8;13.8]	58.9	64.2	XRQ : (0.429)
	Alternaria	11	13.4	[0;38.1]	1.8	5.2	XRQ : (0.075)
FUxPAZ2	PmJ42	7	0	[0.0; 4.8]	6.2	22.3	FU : (0.137)
	PmJ49	7	0	[0.0; 4.1]	8.0	27.0	FU : (0.193)
	PmJ56	7	0	[0.0; 5.2]	6.2	22.3	FU : (0.144)

Bert *et al*. (2004) have detected four QTL involved in *Phoma* resistance to stem attack on 19-day-old seedlings on FUxPAZ2 F2-F3 families. The absence of common molecular markers between our genetic

map of this population and the map previously used doesn't allow the comparison of QTL involved in PR resistance with those previously reported. Nevertheless, the resistance mechanisms involved in black stem disease resistance at seedling stage and premature ripening resistance at adult stage are probably different. The absence of correlation between genotype behaviour at early stages and adult stage has already been demonstrated for *Sclerotinia sclerotiorum* sunflower resistance and seem to exist also in the case of *Phoma macdonaldii*, where no relationship could been established between resistance level of plantlets and grown-up plants (data not shown).

In this study, no common QTL has been detected between the two RILs populations. A high percentage of the phenotypic variation of PR severity remains unaccounted for. New molecular markers should be added on the genetic maps of the two RILs populations to detect new QTL and to get more precise information (localization, effects, specificity) about them.
This work reveals for the first time the quantitative behaviour of sunflower PR resistance and a high genetic variability in the *Helianthus* genus. Although phenotyping for PR resistance appears hard because it must be done on adult plants in field conditions, QTL involved in resistance to premature ripening have been successfully detected. These results open new prospects for sunflower genetic improvement.

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