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Aggressiveness in *Plasmopara halstedii* (sunflower downy mildew)

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Aggressiveness was studied in seven *Plasmopara halstedii* (sunflower downy mildew) pathotypes: 100, 300, 304, 314, 704, 710 and 714. Aggressiveness criteria including percentage infection, latent period, sporulation density and reduction of hypocotyl length (dwarfing) were analysed in one sunflower inbred line showing a high level of quantitative resistance. Genetic relationships were detected between the seven pathotypes using 12 EST-derived markers. Pathotypes 100, 300, 304 and 314 were characterized with shorter latent period and higher sporulation density than pathotypes 710, 704 and 714. All pathotypes showed high percentage infection values and caused a large reduction in seedling size except for pathotype 314 involved in dwarfing. Pathotypes 714, 704 and 314 had an intermediary genetic position between the pathotypes 100 and 710. No correlation was detected between aggressiveness traits and EST genotypes.

Keywords : EST-derived markers, *Helianthus annuus*, quantitative resistance

Sunflower downy mildew is caused by *Plasmopara halstedii* (Farl.) Berl. et Toni), an Oomycete specific to sunflower (*Helianthus annuus* L.), infected by soil- and seed- borne dissemination. Different physiological races (pathotypes) can attack a variable range of sunflower genotypes (Tourvieille de Labrouhe et al., 2000). To date, there are at least 35 races in different parts of the world (Gulya, 2007). Disease resistance in sunflowers to *P. halstedii* can be classified into one of two categories. The first is qualitative resistance which is conferred by the major *Pl* genes and tends to produce a disease-free plant (Tourvieille de Labrouhe et al., 2000). The second is quantitative resistance which is controlled by minor genes and tends to impact the rate of disease development (rate reducing) rather than producing a disease-free plant (Tourvieille de Labrouhe et al., 2008).

Concerning this pathosystem, several studies have been carried on virulence (Delmotte et al., 2008; Gulya, 2007; Spring et al., 2006; Tourvieille de Labrouhe et al., 2000, 2010) and more recently on aggressiveness (Sakr, 2009; 2010). Virulence has been defined as specific, disease-causing abilities and aggressiveness as non-specific, disease-causing abilities (Van der Plank, 1968). Sakr (2009) found differences between two groups of 100, 300, 304 and 314 races and 710, 704 and 714 races by using two criteria of aggressiveness: latent period and sporulation density. Moreover, Sakr (2010) noted that percentage infection and dwarfing could be used to differentiate aggressiveness in *P. halstedii*, but these criteria played a limited role in defining *P. halstedii* isolates according to their aggressiveness. Regarding genetic research in *P. halstedii*, the interaction of avirulence genes of *P. halstedii* and sunflower genotypes carrying *Pl* effective genes led a new virulence to appear in pathogen isolates. Today, the total *P. halstedii* races can overcome the resistance genes in seven of the nine downy mildew differential lines of *H. annuus*, and the *Pl2* and *Pl6* genes have each been overcome by eight races (Tourvieille de Labrouhe et al., 2000). Moreover, Spring et al. (2006) differentiated between populations of pathotypes 100, 310 and 330 and a group of populations representing pathotypes 700, 701, 703, 710 and 730. Also, Delmotte et al. (2008) identified three genetically differentiated groups of isolates organized around the first three races described in France: 100, 710 and 703. However, no studies on the correlation between aggressiveness traits and groups based on molecular markers for *P. halstedii* have been carried out. Therefore, our aim was to analyze the variation of aggressiveness in seven *P. halstedii* pathotypes belonging to several races in order to generate information about aggressiveness variability of *P. halstedii*. The genetic relationships between the seven pathotypes were also analyzed using 12 EST-derived markers (Giresse et al., 2007) to clarify the correlation between aggressiveness traits and EST genotypes.

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Materials and Methods

Fungal pathotypes. The seven *P. halstedii* pathotypes used in this study were collected in France and maintained at INRA, Clermont-Ferrand. Manipulation of this quarantine

parasite was in compliance with European regulations (No 2003/DRAF/70). All pathotypes samples were taken from naturally infected sunflower plants. There were three replications for each differential line (10 plants in each replication) and the entire experiment was repeated twice for each *P. halstedii* pathotype. Pathotypes 100, 300, 304, 314, 710, 704 and 714 were determined using the method reported by Tourvieille de Labrouhe et al. (2000) (Table 1).

Measurement of aggressiveness in *P. halstedii* pathotypes.

To characterize aggressiveness of *P. halstedii* pathotype, one INRA inbred line FU was used. It carried no *Pl* gene, but is known to have a high level of quantitative resistance (Tourvieille de Labrouhe et al., 2008). Percentage infection was considered as successful when the seedlings showed sporulation of the pathogen on the shoot surface. Observations were made 13 days after infection and expressed as the percentage of seedlings showing sporulation, whatever the plant parts concerned and the amount of the sporulation observed. Latent period was defined as the number of days of incubation necessary to obtain the sporulating pathogen on 80% of the plants. Sporulation density was defined as the number of zoosporangia of the pathogen produced on a cotyledon. Reduction of hypocotyl length (dwarfing) corresponded to the distance from the stem base to cotyledon insertion and was measured after 13 days of infection on diseased plants showing sporulation of the pathogen on the shoot (Sakr, 2009; 2010). All the pathogenic tests were carried out in growth chambers regulated at 18hrs of light, 18 °C ± 1 and RH of 65–90%. All statistical analyses of the aggressiveness data were performed using Stat Box 6.7® (GimmerSoft) software. The values obtained were submitted to a one-way analysis of variance (ANOVA). The Newman-Keuls test (Snedecor and Gochran, 1989) was used to compare the means at $P = 0.05$. The sample correlation coefficients (Pearson r) were calculated at $P = 0.01$ and $P = 0.05$.

DNA extraction and molecular typing. The 12 EST-derived markers were used because the other molecular markers were non-specific, insufficiently polymorphic within *P. halstedii* and no genetic structure in *P. halstedii* populations was identified by using these markers (Delmotte et al., 2008). For each pathotype, DNA was isolated from infected plant tissue as previously described for *Plasmopara viticola* by Delmotte et al., (2006). The 12 polymorphic EST-derived markers (Giresse et al., 2007) were then used to genotype the *P. halstedii* pathotypes. The polygenetic relations between the seven pathotypes were obtained by building a Neighbour-joining (NJ) tree (Jin and Chakraborty, 1993) and by using Populations 1.2.28 Software (Langella, 1999). A Bootstrap analysis was performed on 10.000 replicates. It is possible that no genetic structure in *P. halstedii* populations was identified in previous studies

Results

Analysis of aggressiveness criteria

Percentage infection. The analysis of variance indicated highly significant differences (Probability = 0.00001; Variation coefficient = 4.52%) between *P. halstedii* pathotypes (Table 2). The Newman-Keuls test showed that the pathotypes formed two very distinct groups. The first group containing the pathotypes 300, 304 and 314 showed a higher infection level than the second group containing the pathotypes 100, 710, 704 and 714.

Latent period. Analysis of the relation between sporulation percentage based on incubation period (Fig. 1) showed differences in behaviour among *P. halstedii* pathotypes. There were two main groups from day 8 onwards: pathotypes 100, 300, 304 and 314 sporulated faster than pathotypes 710, 704 and 714. All infected plants with pathotypes 100 and 3xx showed more than 80% sporulation 9 days after incubation, whereas pathotypes 7xx needed 11

Table 1. Virulence of seven *Plasmopara halstedii* isolates on nine sunflower differential lines

<i>P. halstedii</i> pathotype	Year isolated	Differential lines								
		D1 Ha-304	D2 Rha-265	D3 Rha-274	D4 PMI3	D5 PM-17	D6 803-1	D7 HAR-4	D8 QHP1	D9 Ha-335
100	1960	S	R	R	R	R	R	R	R	R
300	2005	S	S	R	R	R	R	R	R	R
314	2005	S	S	R	S	R	R	R	R	S
304	2005	S	S	R	R	R	R	R	R	S
710	1988	S	S	S	S	R	R	R	R	R
714	2005	S	S	S	S	R	R	R	R	S
704	2005	S	S	S	R	R	R	R	R	S

S: susceptible, sporulation on cotyledons. R: resistant, no sporulation data from Tourvieille de Labrouhe et al. (2000).

Table 2. Aggressiveness among seven *Plasmopara halstedii* pathotypes measured on the sunflower inbred line FU

Pathotypes	Percentage infection ^a (%)	Latent period ^b (days)	Sporulation density ^c (10 ⁵ zoosporangia per cotyledon)	Hypocotyl length ^d (mm)
Pathotype 100	96.0b	9.22b	14.32b	30.7b
pathotype 300	99.1a	8.09c	17.00a	32.4b
Pathotype 314	99.3a	8.42c	13.53b	40.7a
Pathotype 304	99.8a	8.25c	14.10b	29.0b
Pathotype 710	93.2b	10.80a	7.03c	28.7b
Pathotype 714	95.2b	11.06a	5.10c	27.5b
Pathotype 704	96.4b	11.29a	6.31c	27.9b
	P = 0.00001 VC = 4.52%	P = 0.0 VC = 6.01%	P = 0.0 VC = 21.86%	P = 0.0 VC = 10.01%

^aNumber of replications = 3, 60 plants per replication, ^bNumber of replications = 3, 10 plants per replication, ^cNumber replication = 2, 18 counts per replication and ^dNumber of replications = 3, 10 plants per replication (Sakr, 2009; 2010); according to the Newman-Keuls test, means followed by the same letter are not significantly different at P = 0.05, Probability (P), Variation Coefficient (VC).

Fig. 1. Sporulation of seven *Plasmopara halstedii* pathotypes of different races on the sunflower inbred line ‘FU’, based on incubation period.

days after incubation to reach the same level of sporulation.

There were highly significant differences between *P. halstedii* pathotypes (Probability = 0.0; Variation Coefficient = 6.01%). The Newman-Keuls test classified the pathotypes into three distinct groups (Table 2). Those with the shortest length of latent period (< 9 days) were the pathotypes 300, 304 and 314. Pathotype 100 was intermediate. The pathotypes 710, 704 and 714 were grouped together and showed longer latent periods (> 10 days).

Sporulation density. Fig. 2 shows that the quantities of zoosporangia produced increased with time. There were two main groups from day 9 onwards: pathotypes 100, 300, 314 and 304 produced more zoosporangia than pathotypes 710, 714 and 704 (Fig. 2). The quantity of zoosporangia produced was at a maximum 12 days after incubation.

There were large differences between the pathotypes; sporulation density varied from 5×10^5 zoosporangia per ml for pathotype 714 to 17×10^5 zoosporangia per ml for

Fig. 2. Sporulation density of seven *Plasmopara halstedii* pathotypes of different races on sunflower inbred line ‘FU’, based on incubation period.

Table 3. Correlation coefficients on inbred line FU among criteria of aggressiveness for seven pathotypes of *Plasmopara halstedii*

	Sporulation density	Latent period	Hypocotyl length	Percentage infection
Sporulation density	1.000	−0.959**	0.533 ^{ns}	0.743 ^{ns}
Latent period		1.000	−0.609 ^{ns}	−0.832*
Hypocotyl length			1.000	0.222 ^{ns}
Percentage infection				1.000

*P = 0.05, **P = 0.01, ns = no significant

pathotype 300 (Table 2). Differences were highly significant (Probability = 0.0; Variation Coefficient = 21.86%). The Newman-Keuls test classified the pathotypes into three very distinct groups (Table 2). Pathotypes 710, 704 and 714 showed the lowest sporulation density ($< 7.05 \times 10^5$ zoospores per cotyledon). Pathotypes 100, 304 and 314 were intermediate. Pathotype 300 had the highest sporulation density.

Reduction of hypocotyl length. Diseased plants had hypocotyls with only one third the mean lengths of healthy plants (30.9 ± 2.9 mm and 90.0 ± 2.3 mm respectively) whatever the pathotype of *P. halstedii*. Analysis of variance showed highly significant differences between pathogen pathotypes (Probability = 0.0; Variation coefficient = 10.01%). However, these results were mainly due to pathotype 314 (Table 2) which caused less reduction in hypocotyl length (40.7 mm) than the other six pathotypes (mean 29.4 mm).

There was significant correlation among some aggressiveness criteria (Table 3). Latent period was negatively correlated with sporulation density at P = 0.01 and with percentage infection at P = 0.05.

Molecular analysis

The combination of 12-EST derived markers revealed five multilocus genotypes (MLG) among seven *P. halstedii* pathotypes (Table 4). Pathotypes 100 and 710 were different for all genomic markers excepting Pha54. Furthermore,

Fig. 3. Phylogenetic tree according to Neighbour-joining analysis of 12 EST-derived markers. Figures on branches indicate bootstrap values (10.000 replicates).

pathotypes 100, 300 and 314 had the same genetic background. The Neighbour-joining tree showed that pathotype 714, 704 and 314 had an intermediary genetic position between pathotypes 100 and 710 (Fig. 3).

Discussion

Understanding the interaction between pathogen and its host plant requires knowledge of the variability of pathogenicity. With this in mind, the variability of aggressiveness and its alternation with genetic variation was studied by using seven *Plasmopara halstedii* pathotypes of several races. Differences in aggressiveness of *P. halstedii* pathotypes are indicated when pathotypes vary in the amount of damage that they cause in sunflower plants. Some aggressiveness criteria measured in the present study showed significant correlation with each other (Table 3). These

Table 4. Multilocus genotypes (MLG) characterized using 12 EST-derived genomic markers on the pathotypes of *Plasmopara halstedii*

Pathotype	EST-derived markers											
	Pha6	Pha39	Pha42	Pha43	Pha54	Pha56	Pha74	Pha79	Pha82	Pha99	Pha106	Pha120
100	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
304	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
300	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
314	1/1	2/2	1/1	2/2	1/1	2/2	2/2	3/3	2/2	1/1	2/2	1/1
704	2/2	2/2	2/2	1/1	1/1	1/1	2/2	3/3	1/1	1/1	2/2	1/1
714	1/1	2/2	1/2	1/1	1/1	1/1	1/1	3/3	2/2	1/1	2/2	1/1
710	1/1	1/1	2/2	2/2	1/1	2/2	2/2	1/1	1/1	1/1	2/2	1/1

results are not in accordance with those found by Carlisle et al. (2002) for another Oomycete *Phytophthora infestans*. They showed a strong correlation among the four criteria of aggressiveness: latent period, frequency of infection, area under the lesion expansion curve and sporulation capacity, were strongly correlated. In this study, the correlation between latent period and sporulation density ($r = -0.959$) could be explained by specialisation in aggressiveness towards tissue invasion and sporulation. The frequency of sporulated plants based on incubation period reflected the speed of appearance of symptoms on the plants (Fig. 1) (latent period), and the number of zoosporangia produced by cotyledons reflected the level of invasion of infected tissues (Fig. 2).

High percentage infection, short latent period, high sporulation density, and significant reduction in the length of the hypocotyl represent high aggressiveness (Sakr, 2009; 2010). Our results show that two criteria, latent period and sporulation density, differentiated seven pathotypes. Pathotypes 100, 300, 314 and 304 had a shorter latent period and higher sporulation density than pathotypes 710, 714 and 704. In the pathosystem *Phytophthora infestans*/potato, Montarry et al. (2006) showed that the most highly aggressive isolates had a shorter latent period and higher sporulation capacity than the less aggressive isolates. The very high percentages of infection can be explained by the ideal test conditions for the parasite (Meliala et al., 2001). Similar to our results, Tourvieille de Labrouhe et al. (2010) found that values of percentage infection varied between 98.7 and 100% for *P. halstedii* isolates. However, Carlisle et al. (2002) found that values of infection frequency for different *P. infestans* isolates, measured on a potato variety showing high level of quantitative resistance, ranged between 27 and 85%. Table 2 shows that all the pathotypes caused a large reduction in seedling size except for pathotype 314, compared to the growth of *P. halstedii*-free sunflower inbred line FU. Regarding the same pathosystem, Tourvieille de Labrouhe et al. (2010) showed that this character may be used to differentiate aggressiveness in a pathogen. Our results differ from those found by Sinclair and Griffiths (2000) for ash yellow phytoplasmas. They reported that various strains caused growth suppression ranging from slight, intermediate to severe in comparison to growth of phytoplasma-free plants. Based on important differences in the two aggressiveness criteria, latent period and sporulation density, between *P. halstedii* pathotypes (Table 2), we were able to divide them into two main groups. The first (more aggressive) group includes isolates of races 100 and 3xx, while the other group (less aggressive) is composed of isolates of races 7xx. These results are comparable with those found by Sakr (2009, 2010), who demonstrated that latent period and sporulation density

could be used to define aggressiveness in *P. halstedii* populations. The other two criteria used in the present study played a limited role in the differentiation of aggressiveness.

By using the same EST-derived markers, Delmotte et al., (2008) found that races 100, 300 and 304 had the same genetic clade as observed in our study (Fig. 3). Delmotte et al. (2008) grouped races 710, 704 and 714 together in the same genetic clade; however, this association was not identified in the present work. Either the isolates used in our study were different from the ones used by Delmotte et al. (2008), or the intrarace variance in the EST-derived marker may explain the different results reported. However, the distinctiveness of the 7xx races compared to those of races 100 or 3xx has recently been shown based on ITS sequence data (Spring et al., 2006). No correlation was detected between aggressiveness traits (Table 2) and EST genotypes. The lack of matching between aggressiveness traits and groups based on molecular markers was not surprising. Indeed, Montarry et al. (2006) did not find a clear correlation between phenotypes (aggressiveness) and genotypes based on AFLP markers for *Phytophthora infestans*. Aggressiveness is known to evolve through mutation without highly altering molecular fingerprints (Goodwin, 1997). Because most molecular markers used for fingerprinting are selectively neutral, they can be used to assess evolutionary forces other than selection (such as gene flow or genetic drift). However, regarding *Sphaeropsis sapinea*, De Wet et al. (2003) found that aggressiveness groups A, B and C were separated into three differential clades. Further studies will be necessary to investigate aggressiveness and genetic variability on a large collection of *P. halstedii* pathotypes with different races from several parts of the world in order to provide better insight into interactions between this obligate parasite and its host.

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