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DETERMINING THE RELATIONSHIP BETWEEN VIRULENCE AND AGGRESSIVENESS IN *PLASMOPARA HALSTEDII* BY USING MOLECULAR MARKERS

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ABSTRACT

The relationship between virulence and aggressiveness was analyzed in seven *Plasmopara halstedii* (sunflower downy mildew) isolates including five progeny isolates of races 300, 304, 314, 704 and 714 arising from two parental isolates of races 100 and 710. Genetic relationships were detected between the seven isolates using 12 EST-derived markers. Aggressiveness criteria were analysed in one sunflower inbred line showing a high level of quantitative resistance. There were significant differences between *P. halstedii* isolates for all aggressiveness criteria. The isolates of races 714, 704 and 314 had an intermediary genetic position between the two parental isolates of races 100 and 710. The three isolates of races 100, 300 and 304 were localized in the same genetic clade. Pathogenicity of progeny isolates as compared with parental ones (relationship between virulence and aggressiveness) seems to be positive, negative or uncorrelated. For solving the specificity of these cases, relationship between virulence and aggressiveness among the isolates of races 100, 300 and 304 localized in the same genetic clade was positive. The hypothesis explaining these cases are discussed.

Keywords: EST-derived markers, obligate parasite, qualitative resistance

1. INTRODUCTION

Knowledge of the relations between virulence and aggressiveness would help to understand dynamics of parasitic populations that use their pathogenicity to improve adaptation to their environment (Leach et al. 2001). Virulence is a driving force in host-pathogen co-evolution since it enables pathogens to overcome qualitative resistance genes *R*. Aggressiveness enables the pathogen to develop within the host plant (Van der Plank, 1968). Van der Plank (1968) indicated that virulence (vertical pathogenicity) and aggressiveness (horizontal pathogenicity) are often negatively correlated in the case for *Phytophthora parasitica* var. *nicotianae* on tobacco (Sullivan et al. 2005) and *Leptoshaeria maculans* on oilseed rape (Huang et al. 2006). Damgaard et al. (1999) suggested the absence of difference of aggressiveness between a virulent strain and nonvirulent strain in a theoretical model. Moreover, the two components of pathogenicity are positively correlated in the case of obligate parasite *Phakospora pachyrhizi* on soybean (Bonde et al. 2006). Virulence (qualitative pathogenicity) has been defined as specific disease-causing abilities and aggressiveness (quantitative pathogenicity) as non-specific disease-causing abilities (Van der Plank 1968).

For *Plasmopara halstedii*, no studies on the relationship between virulence and aggressiveness have been reported. *P. halstedii* (sunflower downy mildew) is an obligate endoparasite that cannot be cultivated independently from its plant host. *P. halstedii* is a homothallic oomycete, whose cycle is made up of a single sexual generation permitting overwintering and one or perhaps two asexual generations which occur during the growing season (Spring and Zipper 2006). *P. halstedii* displays a gene-for-gene interaction with its host plant and shows physiological races (pathotypes) capable of infecting a variable range of sunflower genotypes. The nomenclature of these races is based on the reaction of a series of differential lines (Tourvieille de Labrouhe et al. 2000). To date, there are at least 35 races in different parts of the world according to Gulya (2007).

Disease resistance in sunflowers to *P. halstedii* can be placed in one of two categories, the first is qualitative resistance (Tourvieille de Labrouhe et al. 2000) and the second is quantitative resistance (Tourvieille de Labrouhe et al. 2008).

In this study, aggressiveness was analysed for seven *P. halstedii* isolates: race 100 present in France since 1966, race 710 introduced from the USA during the 1980s (Tourvieille de Labrouhe et al. 2000) and 5 progeny isolates of races 300, 304, 700, 704 and 714 which appeared recently in plots where the first two races were present (Tourvieille de Labrouhe et al. 2010). Four aggressiveness criteria were evaluated: percentage infection, latent period, sporulation density and reduction of hypocotyl length (Sakr 2009,Sakr et al. 2011). These criteria were analysed by using a sunflower inbred line not carrying any qualitative resistance *Pl* gene but showing a high level of quantitative resistance (Tourvieille de Labrouhe et al. 2008). In this paper, our target was (i) to study the relationship between the two components of pathogenicity of progeny isolates as compared with parental ones and (ii) to analyze the specificity of this relationship using 12 EST-derived markers. Hence an attempt was made to generate information about the possibility of using the molecular markers to determine the relationship between virulence and aggressiveness in *P. halstedii*.

2. MATERIALS AND METHODS

2.1 FUNGAL ISOLATES

The *P. halstedii* isolates used in this study were collected in France and maintained at INRA, Clermont–Ferrand. Manipulation of this quarantine pathogen followed European regulations (No 2003/DRAF/70). Isolate MIL 001 (race 100) was sampled in 1966 and isolate MIL 002 (race710) in 1988. The progeny isolates originated from an initial mixture of pathotypes 100 and 710 (Tourvieille de Labrouhe et al. 2010) and their races (Table 1) were determined using the method reported by Tourvieille de Labrouhe et al. (2000): isolate DU

1842 (race 300); isolate DU 1767 (race 304); isolate 1943 (race 314); isolate 1734 (race 704) and isolate 1915 (race 714).

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

Isolates	Race	Year isolated	Differential lines								
			D1	D2	D3	D4	D5	D6	D7	D8	D9
			Ha-304	Rha-265	Rha-274	PMI3	PM-17	803-1	HAR-4	QHP1	Ha-335
MIL001	100	1960	S	R	R	R	R	R	R	R	R
DU1842	300	2005	S	S	R	R	R	R	R	R	R
DU1943	314	2005	S	S	R	S	R	R	R	R	S
DU1767	304	2005	S	S	R	R	R	R	R	R	S
MIL002	710	1988	S	S	S	S	R	R	R	R	R
DU1915	714	2005	S	S	S	S	R	R	R	R	S
DU1734	704	2005	S	S	S	R	R	R	R	R	S

R = resistant = incompatible interaction; S = susceptible = compatible interaction ; data from Tourvieille de Labrouhe et al. (2000), identification of virulence for seven P. halstedii isolates was presented by Sakr (2009).

2.2 MEASUREMENT OF AGGRESSIVENESS IN *P. HASLTEDII* PATHOTYPES

To characterize aggressiveness of *P. halstedii* isolates, 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). The index of aggressiveness of the *P. halstedii* isolate was calculated as the ratio of (Percentage infection × sporulation density) / (latent period × reduction of hypocotyl length). Percentage infection was considered as successful when the seedlings showed sporulation of the pathogen on the shoot surface. Latent period was defined as the number of days of incubation necessary to obtain 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) corresponds 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, Sakr et al. 2011). All the pathogenic tests were carried out in growth chambers regulated at 18h 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).

2.3 DNA EXTRACTION AND MOLECULAR TYPING

For each isolate, DNA was isolated from infected plant tissue as previously described for *Plasmopara viticola* by Delmotte et al. (2006). Then the 12 polymorphic EST-derived markers (Giresse et al. 2007) were used to genotype the *P. halstedii* isolates. The polygenetic relations between the seven isolates were obtained by building a Neighbour-joining (NJ) tree (Jin and Chakraborty 1993) using Populations 1.2.28 Software (Langella 1999). A Bootstrap analysis was performed on 10.000 replicates.

3. RESULTS

3.1 ANALYSIS OF AGGRESSIVENESS CRITERIA

There were significant differences between *P. halstedii* isolates for all aggressiveness criteria (Table 2). Percentage infection ranged between 93.2% for isolate MIL 002 and 99.8% for isolate DU 1767. Latent period ranged between 8.09 days for isolate DU 1842 and 11.29 days for isolate DU 1734. Sporulation density varied three fold: 5.02×10^5 zoosporangia were produced by cotyledons for isolate DU 1915 and 16.72×10^5 for isolate DU 1842. Hypocotyl length varied from 27.5 mm for isolate DU1915 to 40.7 mm for isolate DU1943. Results shown in Table 2 indicate that the progeny isolate DU 1842 was the most aggressive among the isolates tested with an index of aggressiveness of 6.3; followed by progeny isolate DU 1767 with an index of aggressiveness of 5.9; parental isolate MIL 001 with an index of aggressiveness of 4.9, and progeny isolate DU 1734 with an index of aggressiveness of 4.5. The parental

isolate MIL 002 and the two progeny isolates DU 1734 and DU 1915 were the least aggressive, recording the mean value for index of aggressiveness of 1.9.

Table 2: Aggressiveness between Plasmopara halstedii parental and progeny isolates measured on the sunflower inbred line FU

Isolates	Percentage infection (%)[a]	Latent period (days) [b]	Sporulation density (10^5 zoosporangia per cotyledon) [c]	Hypocotyl length (mm) [d]	Index of aggressiveness
Parental isolate MIL 001 (race 100)	96.0	9.22	14.32	30.7	4.9
Progeny isolate DU 1842 (race 300)	99.1	8.09	16.72	32.4	6.3
Progeny isolate DU 1943 (race 314)	99.3	8.42	13.53	40.7	4.5
Progeny isolate DU 1767 (race 304)	99.8	8.25	14.10	29.0	5.9
Parental isolate MIL 002 (race 710)	93.2	10.80	7.03	28.7	2.1
Progeny isolate DU 1915 (race 714)	95.2	11.06	5.02	27.5	1.6
Progeny isolate DU 1734 (race 704)	96.4	11.29	6.31	27.9	1.9
	P= 0.00001 VC= 4.52%	P= 0.0 VC=6.01%	P= 0.0 VC=21.86%	P= 0.0 VC=10.01%	

[a] number replications = 3, 60 plants per replication, [b] number replications = 3, 10 plants per replication, [c] number replication = 2, 18 counts per replication, [d] number replication =3, 10 plants per replication (Sakr 2009, Sakr et al. 2011). Probability (P), Variation Coefficient (VC), index of aggressiveness = (percentage infection × sporulation density) / (latent period × reduction of hypocotyl length).

3.2 MOLECULAR ANALYSIS

The combination of 12-EST derived markers revealed five multilocus genotypes (MLG) among seven *P. halstedii* isolates (Table 3). Parental isolates MIL 001 and MIL 002 were different for all genomic markers excepting Pha54. Furthermore, isolates MIL 001, DU 1842 and DU 1767 shared the same genetic background. The Neighbour-joining tree showed that isolates DU 1915, DU 1734 and DU 1943 had an intermediary genetic position between parental isolates MIL 001 and MIL 002 (Figure 1).

Table 3: Multilocus genotypes (MLG) characterized using 12 EST-derived genomic markers on the isolates of Plasmopara halstedii

Isolates	EST-derived markers											
	Pha6	*Pha39*	*Pha42*	*Pha43*	*Pha54*	*Pha56*	*Pha74*	*Pha79*	*Pha82*	*Pha99*	*Pha106*	*Pha120*
MIL 001	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
DU 1767	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
DU 1842	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
DU 1943	1/1	2/2	1/1	2/2	1/1	2/2	2/2	3/3	2/2	1/1	2/2	1/1
DU 1734	2/2	2/2	2/2	1/1	1/1	1/1	2/2	3/3	1/1	1/1	2/2	1/1
DU 1915	1/1	2/2	1/2	1/1	1/1	1/1	1/1	3/3	2/2	1/1	2/2	1/1
MIL 002	1/1	1/1	2/2	2/2	1/1	2/2	2/2	1/1	1/1	1/1	2/2	1/1

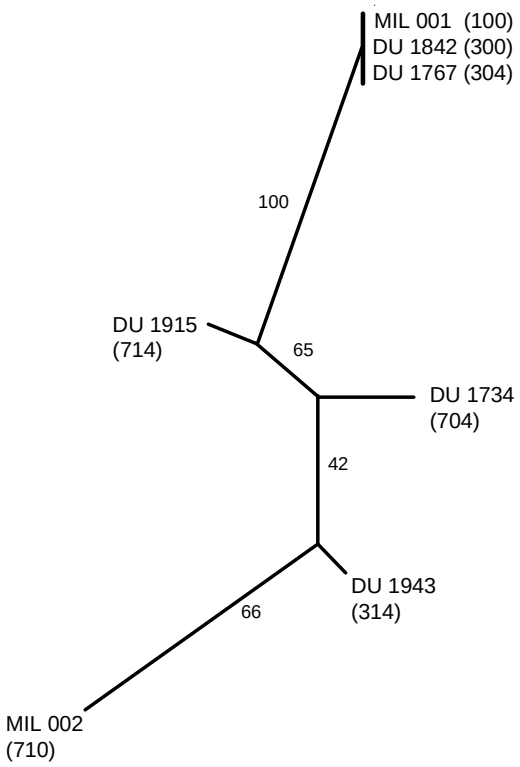

Figure 1: Phylogenetic tree according to Neighbour-joining analysis of 12 EST-derived markers. Figures on branches indicate bootstrap values (10.000 replicates)

4. DISCUSSION

Comprehension of the interaction between *Plasmopara halstedii* and its host plant *Helianthus annuus* requires knowledge of the variability of pathogenicity. With this in mind, the relationship between virulence and aggressiveness was studied by using 5 progeny isolates of races 300, 304, 314, 704 and 714 arising from two parental ones of races 100 and 710. Using molecular markers helps to underline the specificity of the relationship between the two components of

pathogenicity in *P. halstedii* which is characterized by a high level of evolutionary potential (Sakr 2011).

High percentage infection, short latent period, high sporulation density and, significant reduction in the length of the hypocotyl represent high aggressiveness (Sakr 2009, Sakr et al. 2011). Several relationship cases may be obtained when pathogenicity of progeny isolates is compared with parental ones. The two progeny isolates DU 1842 and DU 1767 were more virulent and aggressive than parental isolate MIL 001, and the correlation was positive. For other Oomycete, *Phytophthora infestans* on potato, Miller et al. (1998) found that new populations were more virulent and aggressive than the old populations. However, the three progeny isolates DU 1943, DU 1734 and DU 1915 were more virulent and less aggressive than MIL 001; the progeny isolate DU 1842 was less virulent and more aggressive than parental isolate MIL 002, and the relation was negative. In similar experimental conditions, Murakami et al. (2007) found that variant strains were more virulent and less aggressive than parental strains in pathosystem *Magnaporthe* oryza / wheat and rice. Moreover, the progeny isolate DU 1915 was more virulent and did not show differences of aggressiveness as compared with parental MIL 002, and the relation was uncorrelated.

There are three types of relationship between virulence and aggressiveness in *P. halstedii* and two hypotheses could explain them. First, it seems that virulence and aggressiveness are independent, and the coincidence makes this relation positive, negative or uncorrelated in *P. halstedii*. Second, the isolates used in this study belong to several races and may be found to be an effect of additional virulence genes in *P. halstedii* isolates for variation of aggressiveness as observed for other pathogens (Leach et al. 2001). This makes it possible to have the three types of relationship between virulence and aggressiveness.

The combination of 12-EST derived markers revealed five multilocus genotypes (MLG) between seven *P. halstedii* pathotypes (Table 3). The two isolates MIL 001 and MIL 002 were different for all genomic markers excepting Pha54. The Neighbour-joining tree showed that isolates DU 1915, DU 1734 and DU 1943 had an intermediary genetic position between the two parental pathotypes of races 100 and 710 (Figure 1). But the distinctiveness of the 7xx races compared to those of 100 or 3xx has recently been shown on the basis of ITS sequence data (Spring et al. 2006). For the three isolates MIL 001, DU 1842 and DU 1767 localized in the same genetic clade, the relation between the two components of pathogenicity was positive. Our results provide evidence suggesting that a clonal lineage (one multilocus genotype) can include several races (Table 3). This suggests that mutations in a clonal lineage may lead to the emergence of new races in the same genetic background: this is the case for races 100, 300 and 304 (Figure 1). Two non-exclusive mechanisms may be responsible for this phenotypic variability in avirulence determinants. The first of these mechanisms is recombination within the lineage via the homothallic fusion of gametangia. This hypothesis is certainly the most likely, given the high selfing rate of sunflower downy mildew inferred from our analysis. On the other hand, genetic recombination via parasexual events may have generated this variability as it has been shown recently that mitotic recombination between different isolates of *P. halstedii* may be involved in the generation of new phenotypes within a population (Spring and Zipper 2006). In this case, mitotic recombination within a genetic lineage would be required, and no such phenomenon has ever been described. The importance of mutation events in race evolution has already been highlighted in several Oomycetes plant pathogens such as *Bremia lactucae* (Lebeda and Petrzelova 2004) and *Phytophtora infestans* (Andrivon 1994). Although surprising at first sight, the greater importance of mutation rather than of recombination in populations able to outcross may be related to the large number of clonal cycles of fungal

multiplication during epidemics. The combination of large populations, due to asexual reproduction of the pathogen, with strong selective pressures induced by resistance genes in the plant is likely to favor the emergence of new virulent races. To better understand the dynamics of *P. halstedii* populations, it will be necessary to analyse the relationship between the two components of pathogenicity in a large collection of isolates with different races from several parts of the world.

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