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Diversity in *Plasmopara halstedii*, the causal agent of sunflower downy mildew

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Abstract – Diversity of the level of morphological, pathogenic and genetic characteristics was studied in seven *Plasmopara halstedii* (sunflower downy mildew) isolates of seven races namely 100, 300, 304, 314, 710, 704 and 714. All analyses were carried out by using five single zoosporangium isolates per pathogen isolate. Morphological analyses were performed on zoosporangia for *P. halstedii* single zoosporangium isolates. Aggressiveness criteria were analysed in one sunflower inbred line showing a high level of quantitative resistance. Genetic relationships were detected between the single zoosporangium isolates using 12 EST-derived as SNPs markers. Based on the aggressiveness reaction for the *P. halstedii* single zoosporangium isolates, there were significant *intra* and *inter-race* differences for all criteria studied. Isolates of races 100 and 3xx were less virulent and more aggressiveness than isolates of races 7xx. There was no relation between morphology of zoosporangia and pathogenic characteristics for 35 single zoosporangium isolates. There was no *intra-race* genetic variation, but five genetically-identified groups were detected among pathogen isolates of all races. No correlation was detected between EST genotypes on the one hand and both pathogenic traits and morphological characteristics on the other.

EST-derived / morphology / pathogenicity / SNP markers

Résumé – **Diversité chez *Plasmopara halstedii*, l'agent du mildiou du tournesol.** La diversité au niveau des caractéristiques morphologiques, génétiques et pathogènes a été étudiée pour sept isolats de *Plasmopara halstedii* (mildiou de tournesol) des races 100, 300, 304, 314, 710, 704 et 714. Toutes les analyses ont été effectuées en utilisant cinq cultures monozoosporanges pour chaque isolat de pathogène. Les analyses morphologiques ont été réalisées sur les zoosporanges des isolats monozoosporanges de *P. halstedii*. Les critères d'agressivité ont été analysés sur une lignée de tournesol caractérisée par un niveau élevé de résistance quantitative. Les relations génétiques ont été détectées entre les isolats monozoosporanges en utilisant des 12 EST-derived as SNPs markers. Basé sur la réaction d'agressivité pour les isolats monozoosporanges de *P. halstedii*, il y avait des différences significatives *intra* et *inter-race* pour tous les critères étudiés. Les isolats de 100 et 3xx races sont moins virulents et plus agressives que les isolats de 7xx races. Il n'y avait pas de relation entre les caractéristiques morphologiques des zoosporanges et les caractéristiques pathogènes pour les 35 cultures monozoosporanges. Il n'y avait pas de variation génétique *intra-race*, mais cinq groupes génétiquement identifiées ont été détectés parmi les isolats pathogènes de toutes les races. Aucune corrélation n'a été détectée entre, d'un côté, les génotypes de EST et la pathogènéicité et caractéristiques morphologiques de l'autre.

EST-dérivé / marqueurs SNP / morphologie / pathogénécité

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INTRODUCTION

Sunflower downy mildew is a common disease in many regions where sunflowers (*Helianthus annuus* L.) are grown. The pathogen, *Plasmopara halstedii* (Farlow) Berles & de Toni, is an obligate parasite that cannot be cultivated independently from its plant host. *P. halstedii* is a homothallic parasite to Oomycetes, a group of filamentous protists closely related to algae, with a life-cycle that is made up of a single sexual generation permitting overwintering and one or perhaps two asexual generations which occur during the growing season (Spring & 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 (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).

Several studies have revealed the components of pathogenicity in *P. halstedii*: virulence (Tourvieille de Labrouhe *et al.*, 2000, 2010; Gulya, 2007; Sakr, 2011a,b) and aggressiveness (Sakr *et al.*, 2009, 2011; Tourvieille de Labrouhe *et al.*, 2010; Sakr, 2011a,b; 1012). Virulence has been defined as specific disease-causing abilities and aggressiveness as non-specific disease-causing abilities (Van der Plank, 1984). Recently, Sakr (2012) revealed the importance to scale virulence standard of *P. halstedii* in relation to sunflower differential line D3. For morphological characteristics in *P. halstedii*, Spring and Thines (2004) and Viranyi and Spring (2011) found phenotypic limited tools for analyzing this obligate parasite because most of its life cycle takes place inside sunflower plants. Using RAPD markers ISSR sequences, low levels of genetic variation and genotypic diversity have been revealed in *P. halstedii* (Roeckel-Drevet *et al.*, 1997; Intelmannand and Spring, 2002). Spring *et al.* (2006) differentiated between two *P. halstedii* populations. Moreover, Delmotte *et al.* (2008) identified three genetically different groups of isolates organized around the first three races described in France. Indeed, the interest of ITS (Spring *et al.*, 2006) and EST (Delmotte *et al.*, 2008) sequences to characterize *P. halstedii* isolates has been shown, but races can still not be defined with certainty. However, As-sadi *et al.* (2011) reported that genetic distance between four *P. halstedii* races 100, 304, 703 and 710 can be detected using SNPs discovered in CRN effector sequences. The interaction of non virulence 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).

In this study, morphological, pathogenic and genetic variation was analysed for seven *P. halstedii* isolates using single zoosporangium isolates. Four aggressiveness criteria were analyzed by using sunflower genotype not carrying any qualitative resistance *Pl* genes but showing a high level of quantitative resistance (Tourvieille de Labrouhe *et al.*, 2008). Morphological variability of zoosporangia produced by *P. halstedii* single zoosporangium isolates on the surface of cotyledons in sunflower plants infected thought roots was studied in alternation with pathogen diversity (virulence and aggressiveness). The genetic relationships between *P. halstedii* single zoosporangium isolates were also

analyzed using 12 EST-derived as SNPs markers (Giresse *et al.*, 2007) to clarify the correlation between pathogenicity traits and EST genotypes. Hence an attempt was made to generate information about the variability of several *P. halstedii* isolates belonging to seven races.

MATERIALS AND METHODS

Fungal isolates. Seven *P. halstedii* isolates used in this study were collected in France and maintained at INRA, Clermont-Ferrand. Manipulation of this quarantine parasite respected European regulations (No 2003/DRAF/70). Pathogen isolates were isolated from naturally infected sunflower plants. Their races identity (Table 1) was determined using the method reported by Tourvieille de Labrouhe *et al.* (2000): MIL 001 (race 100); DU 1842 (race 300); DU 1767 (race 304); DU 1943 (race 314); MIL 002 (race 710); DU 1734 (race 704) and DU 1915 (race 714). For each *P. halstedii* isolate, five single zoosporangium isolates were obtained according to the method described by Sakr *et al.* (2007). This study dealt with five single zoosporangium isolates per pathogen isolate, giving a total of 35 single zoosporangium isolates. The characterization of the race for 35 single zoosporangium isolates (Table 2) was determined using the same method adapted in the study by Tourvieille de Labrouhe *et al.* (2000). Since histological analyses were not carried out on plants, the pathogen's mycelium may exist inside the plants that did not show sporulation, consequently, the infection is considered to be as a latent form. Unidentified biological and genetic causes between the 35 *P. halstedii* single zoosporangium isolates may explain the presence or absence of a latent pathogen infection as it has been observed in other pathogens (Brown & Tellier, 2011). There were three replications for each differential line (10 plants in each replication) and the entire experiment was repeated twice for seven *P. halstedii* isolate and 35 *P. halstedii* single zoosporangium isolates.

Measurement of aggressiveness in *P. hasltedii* single zoosporangium isolates. To characterize aggressiveness of *P. halstedii* single zoosporangium isolates (Sakr *et al.*, 2009, 2011; Sakr, 2011a,b; 2012), one INRA inbred line FU was used. It carried no *Pl* gene, but is known to a have high level of quantitative resistance (Tourvieille de Labrouhe *et al.*, 2008). Percentage of infection was considered as successful when the seedlings showed sporulation of the pathogen on the shoot surface. Period of incubation of infected plants 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

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

Isolates	*Race*	*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*
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

S: susceptible, sporulation on cotyledons. R: resistant, no sporulation data from Tourvieille de Labrouhe *et al.* (2000), identification of virulence profiles for the seven *P. halstedii* isolates was presented by Sakr (2011a,b).

Table 2. Aggressiveness within pathogen isolate for 35 *Plasmopara halstedii* single zoosporangium isolates measured on the sunflower inbred line "FU3

Single zoosporangium isolates	*Race*	*Percentage of infection*	*Period of incubation of infected plants*	*Sporulation density*	*Hypocotyl length of healthy plants*	*Index of aggressiveness*
		Mean (%)	Mean (days)	Mean (10^5 zoosporangia per cotyledon)	Mean (mm)	
MIL001 M2	100	95.0 ab	9.09	19.68	32.4 b	6.3
MIL001 M3	100	100.0 a	9.30	12.70	28.7 c	4.8
MIL001 M4	100	95.6 ab	10.20	11.97	28.3 c	3.9
MIL001 M5	100	97.2 ab	8.93	13.16	27.9 c	5.1
MIL001 M6	100	92.1 b	8.58	14.11	36.8 a	4.1
		P = 2.8	P = 6.3 ns	P = 12.2 ns	P = 0.0	
		VC = 4.61%	VC = 5.76%	VC = 16.13%	VC = 2.05%	
DU1842 M1	300	98.3	7.93 b	16.61	29.1 b	7.1
DU1842 M2	300	98.8	8.09 b	17.20	28.4 b	7.4
DU1842 M3	300	98.9	8.83 a	18.33	29.0 b	7.1
DU1842 M4	300	99.4	7.82 b	14.03	33.6 a	5.3
DU1842 M5	300	100.0	7.80 b	17.42	26.8 a	8.3
		P = 64.7 ns	P = 0.0	P = 31.5 ns	P = 0.00001	
		VC = 2.49%	VC = 1.63%	VC = 10.63%	VC = 2.93%	
DU1943 M1	314	100.0	8.61 b	13.25 b	42.1 b	3.7
DU1943 M2	314	100.0	8.53 b	12.75 b	40.9 b	3.7
DU1943 M3	314	99.4	8.89 a	11.30 b	35.6 d	3.5
DU1943 M4	314	98.9	8.20 c	18.27 a	39.7 c	3.6
DU1943 M5	314	98.3	7.88 d	12.10 b	44.9 a	3.4
		P = 22.6 ns	P = 0.0	P = 0.0	P = 0.0	
		VC = 2.00%	VC = 1.51%	VC = 5.50%	VC = 1.69%	
DU1767 M1	304	100.0	7.94 b	13.04	35.2 a	4.7
DU1767 M2	304	98.9	8.74 a	13.60	27.9 b	5.5
DU1767 M3	304	100.0	8.01 b	16.26	27.6 b	7.4
DU1767 M4	304	100.0	8.59 a	15.31	26.2 b	6.8
DU1767 M5	304	100.0	7.97 b	13.32	27.4 b	6.1
		P = 45.0 ns	P = 0.0	P = 43.5 ns	P = 0.00008	
		VC = 1.77%	VC = 1.67%	VC = 15.02%	VC = 4.42%	
MIL002 M1	710	96.0	10.35	7.44	28.3 b	2.4
MIL002 M2	710	95.6	11.76	5.45	32.8 a	1.3
MIL002 M3	710	90.6	11.07	8.45	29.6 b	2.3
MIL002 M4	710	95.6	10.32	8.25	26.1 c	2.9
MIL002 M5	710	94.4	10.48	5.56	27.4 b	1.8
		P = 51.43 ns	P = 6.0 ns	P = 51.7 ns	P = 0.00011	
		VC = 7.06%	VC = 5.52	VC = 29.68%	VC = 4.42%	
DU1915 M1	714	95.9	10.56 b	6.20 a	28.3 b	2.0
DU1915 M2	714	95.5	11.85 a	3.33 b	28.2 ab	1.0
DU1915 M3	714	95.0	10.15 b	3.91 b	29.6 a	1.2
DU1915 M5	714	98.3	11.54 a	4.07 b	26.7 b	1.3
DU1915 M6	714	91.1	11.17 a	7.62 a	25.6 c	2.4
		P = 8.1 ns	P = 0.0	P = 0.0	P = 0.0005	
		VC = 4.69%	VC = 2.89%	VC = 3.42%	VC = 2.67%	
DU1734 M1	704	95.0	10.97 c	4.37	26.6 b	1.4
DU1734 M2	704	98.3	10.88 c	7.72	26.5 b	2.6
DU1734 M3	704	95.6	10.51 c	5.84	26.8 b	2.0
DU1734 M7	704	97.8	12.48 a	5.58	28.3 b	1.5
DU1734 M8	704	95.6	11.61 b	8.07	31.0 a	2.5
		P = 9.3 ns	P = 0.0	P = 21.5 ns	P = 0.00017	
		VC = 3.22%	VC = 2.88%	VC = 22.66%	VC = 2.68%	

The method described to measure aggressiveness in *P. halstedii* isolates was presented by Sakr (2011a,b) and Sakr *et al.* (2009, 2011), according to the Newman-Keuls test, means followed by the same letter (a, b, c) are not significantly different at P = 0.05, ns = not significant, Probability (P), Variation Coefficient (VC) index of aggressiveness = (percentage of infection × sporulation density)/(Period of incubation of infected plants × dwarfing).

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 *et al.*, 2009,2011; Sakr 2011a,b; 2012). The index of aggressiveness of *P. halstedii* single zoosporangium isolate was calculated as the ration of (percentage infection × sporulation density)/ (period of incubation of infected plants × dwarfing). 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 & Gochran, 1989) was used to compare the means at $P = 0.05$. The sample correlation coefficients (Pearson r) were calculated at $P = 0.05$ and $P = 0.01$.

Morphological observations. After 13 days of infection of the sunflower inbred line "FU", the zoosporangia suspensions were obtained by grouping all sporulated cotyledons in a small container and adding 1 ml of physiological water for each cotyledon (9g NaCl + 1L permuted water). This slowed zoosporangia maturation to facilitate observations before liberation of zoospores (Sakr *et al.*, 2007). Identification of form and measurement of size was carried out on 50 zoosporangia per treatment under a light microscope (magnification X400) with 2 replications. Zoosporangia size was calculated from an oval $\pi \times a \times b$, $a = {}^1/_2$ length, $b = {}^1/_2$ width.

DNA extraction and molecular typing. For single zoosporangium isolates tested, DNA was isolated from infected plant tissue as previously described for *Plasmopara viticola* by Delmotte *et al.* (2006). Then the 12 polymorphic EST-derived as SNPs markers (Giresse *et al.*, 2007) were used to genotype *P. halstedii* single zoosporangium isolates. The polygenetic relations between the 35 single zoosporangium isolates were obtained by building a Neighbour-joining (NJ) tree (Jin & Chakraborty, 1993) using Populations 1.2.28 Software (Langella, 1999). A Bootstrap analysis was performed on 10.000 replicates.

RESULTS

Analysis of aggressiveness criteria

Percentage of infection

Intra-isolate variability: Few plants escaped infection (191 out of 6300 plants), but these very high levels of infection (89.6% - 100%) showed differences between single zoosporangium isolates. Except for isolate MIL 001, all pathogen isolates were uniform for the criterion "percentage of infection". MIL 001 showed the greatest variability with the widest range: from 92 to 100%. MIL 002 showed no significant variability and from 90 to 96%, with the lowest means level (93%). The DU isolates showed very little variability for this characteristic (Table 2).

Inter-isolate variability: The analysis of variance indicated highly significant differences (Probability = 0.00001; Variation coefficient = 4.52%). The Newman-Keuls test showed that the pathogen isolates formed two very distinct groups. The first group containing the isolates DU 1842, DU 1767 and DU 1943 showed a higher infection level than the second group containing the isolates MIL 001, MIL 002, DU 1734 and DU 1915 (Table 3).

Period of incubation of infected plants

Intra-isolate variability: The two isolates MIL 001 and MIL 002 did not reveal significant variability. However, although the differences were highly

Table 3. Aggressiveness among seven *Plasmopara halstedii* isolates (five replications per pathogen isolate that correspond to five single zoosporangium isolates) on the sunflower inbred line "FU"

Isolates	*Percentage of infection (%)*	*Period of incubation of infected plants (days)*	*Sporulation density (10^5 zoosporangia per cotyledon)*	*Hypocotyl length of healthy plants (mm)*
MIL 001 (race 100)				
Mean	96.1 b	9.22 b	14.32 b	30.8 b
Standard deviation	2.6	0.54	2.77	3.4
DU 1842 (race 300)				
Mean	99.1 a	8.09 c	16.72 a	31.1 b
Standard deviation	0.6	0.38	1.4	2.9
DU 1943 (race 314)				
Mean	99.3 a	8.42 c	13.53 b	40.6 a
Standard deviation	0.6	0.35	2.46	3.1
DU 1767 (race 304)				
Mean	99.8 a	8.25 c	14.10 b	28.9 b
Standard deviation	0.4	0.34	1.46	3.2
MIL 002 (race 710)				
Mean	93.2 b	10.80 a	7.03 c	28.3 b
Standard deviation	2.6	0.55	1.29	2.3
DU 1915 (race 714)				
Mean	95.2 b	11.06 a	5.02 c	27.7 b
Standard deviation	2.3	0.62	1.62	1.4
DU 1734 (race 704)				
Mean	96.4 b	11.29 a	6.31 c	27.9 b
Standard deviation	1.3	0.69	1.39	1.7
Probability	0.00001	0.0	0.0	0.0
Variation Coefficient	4.52%	6.01%	21.86%	10.01%

According to the Newman-Keuls test, means followed by the same letter (a, b, c) are not significantly different at P = 0.05.

significant for the five isolates of races 300, 314, 304, 704 and 714, they were small for those with a short latent period (DU1842, DU 1943 and DU 1767). Deviations were slightly larger for the isolates with the longest incubation periods DU 1915 and DU 1734 (Table 2).

Analysis of the relations between percentage sporulation according to incubation period (Fig. 1) showed differences in behaviour between *P. halstedii* single zoosporangium isolates. There were two main groups from day 8 onwards: single zoosporangium isolates of P. halstedii isolates MIL 001, DU 1842, DU 1943 and DU 1767 sporulated faster than single zoosporangium isolates of *P. halstedii* isolates MIL 002, DU 1915 and DU 1734. All infected plants with isolates of races 100 and 3xx showed more than 80% sporulation after 9 days incubation, isolates of races 7xx needed 11 days incubation to reach the same intensity of sporulation.

Inter-isolate variability: There were highly significant differences between *P. halstedii* isolates (Probability = 0.0; Variation Coefficient = 6.01%). The Newman-Keuls test classified the isolates into three distinct groups. Those with the shortest length of latent period (< 9 days) were the isolates DU 1842, DU 1767 and DU 1943. MIL 001 was intermediate. The isolates MIL 002, DU 1734 and DU 1915 were grouped together and showed longer latent periods (>10 days) (Table 3).

Sporulation density

Intra-isolate variability: Variation among the MIL isolates is at least as great as among the ones where a significant difference is observed. However, Table 2 revealed that the single zoosporangium isolate DU1943M4 showed a significant difference, producing more than 1.8×10^6 zoosporangia per cotyledon as compared to a mean of 12×10^5 zoosporangia per cotyledon for the other four isolates. Similarly, the two single zoosporangium isolates DU1915M1 and DU1915M6 showed twice as much sporulation as compared with the other three isolates (7×10^5 zoosporangia per cotyledon compared to 4×10^5) (Table 2). Fig. 2 shows that the quantities of zoosporangia produced increased with time. There were two main groups from day 9 onwards: single zoosporangium isolates of *P. halstedii* MIL 001, DU 1842, DU 1943 and DU 1767 produced more zoosporangia than single zoosporangium isolates of *P. halstedii* isolates MIL 002, DU 1915 and DU 1734 (Fig 2). The quantity of zoosporangia produced was at a maximum 12 days after incubation.

Inter-isolate variability: There were highly significant differences between the isolates for sporulation density which varied from 5×10^5 zoosporangia per ml for DU 1915 to 16×10^5 for DU 1842 (Probability = 0.0; Variation Coefficient = 21.86%). The Newman-Keuls test classified the isolates into three very distinct groups. The isolates MIL 002, DU 1734 and DU 1915 showed the lowest sporulation density ($< 7 \times 10^5$ zoosporangia per cotyledon). Isolates MIL 001, DU 1767 and DU 1943 were intermediate. DU 1842 had the highest sporulation density (Table 3).

Reduction of hypocotyl length

Intra-isolate variability: All pathogen isolates showed variability within isolates for this criterion of aggressiveness. The length of *P. halstedii*-free sunflower inbred line FU varied between 87.7 to 92.3 mm. Diseased plants had hypocotyls with only one third the mean lengths of *P. halstedii*-free sunflower inbred line FU (30.85 ± 0.6 mm and 90.0 ± 2.3 mm respectively) whatever the single zoosporangium isolate of *P. halstedii*. The single zoosporangium isolate that caused greatest reduction in length was DU1915M6 with a mean length of 25.6 mm. Single zoosporangium isolate DU1943M5 gave the least reduction (44.9 mm). In all cases, infected plants were smaller than healthy plants (Table 2).

Inter-isolate variability: The analysis of variance with five replications per pathogen isolate corresponding to the five single zoosporangium isolates showed highly significant differences between pathogen isolates (Probability = 0.0; Variation coefficient = 10.01%). However, these results were mainly due to pathogen isolate DU 1943 which caused less reduction in hypocotyl length than the other six pathogen isolates (Table 3). The index of aggressiveness varied 8.0: 1.0 for DU1915M2 and 8.3 for DU1842M5. There were highly significant differences (Probability = 0.0001, T-test = 8.672) between the index of aggressiveness for both single zoosporangium isolates of races 100 and 3xx (5.5 ± 0.6) and single zoosporangium isolates of races 7xx (1.9 ± 0.3). There was a significant correlation among the different aggressiveness criteria (Table 4). Sporulation density was positively correlated with hypocotyl length and percentage infection, latent period was negatively correlated with other criteria at $P = 0.01$. Only hypocotyl length showed no significant correlation with percentage of infection.

Race 100
MIL001M2
MIL001M3
MIL001M4
MIL001M5
MIL001M6
Race 300
DU1842M1
DU1842M2
DU1842M3
DU1842M4
DU1842M5
Race 304
DU1767M1
DU1767M2
DU1767M3
DU1767M4
DU1767M5
Race 314
DU1943M1
DU1943M2
DU1943M3
DU1943M4
DU1943M5
Sporulation density (10^5 zoosporangia per cotyledon)
% sporulation
Incubation period (days)
0
5
10
15
20
25
20
40
60
80
100
8
9
10
11
12
13

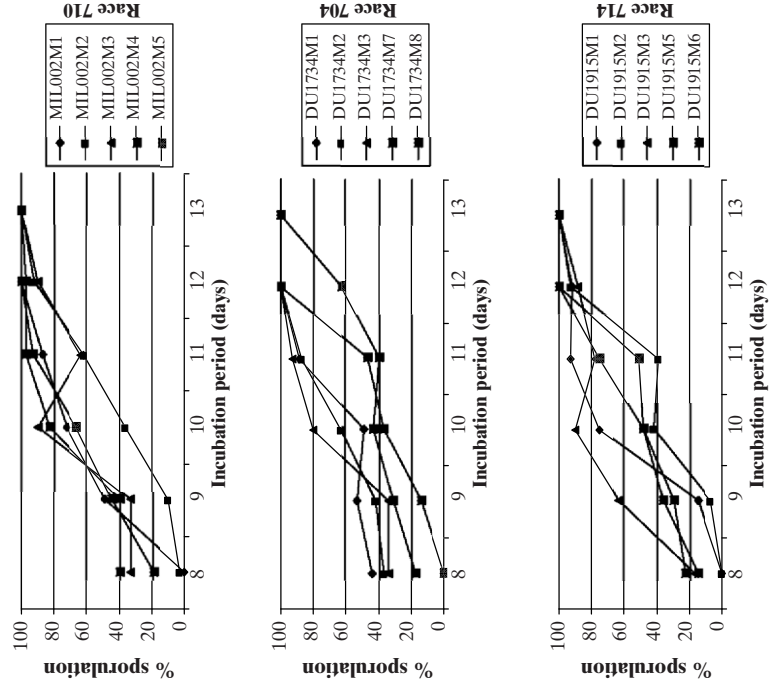

Fig. 1. Sporulation of 35 single zoosporangium *Plasmopara halstedii* isolates of seven races on the sunflower inbred line "FU", based on incubation period

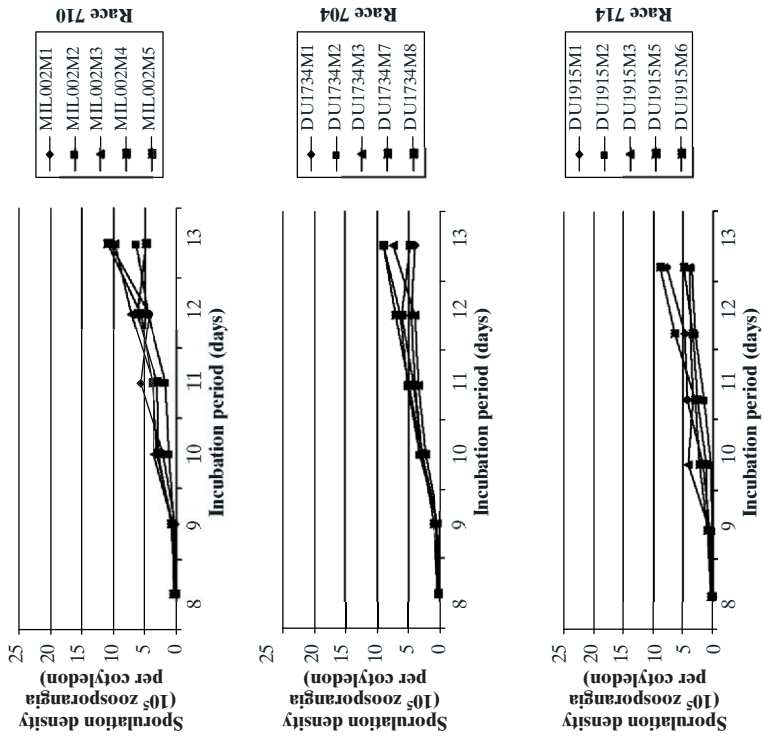

Fig. 2. Sporulation density of 35 single zoosporangium *Plasmopara halstedii* isolates of seven races on sunflower inbred line "FU", based on incubation period

Table 4. Correlation coefficients on sunflower inbred line `FU` among criteria of aggressiveness for 35 single zoosporangium isolates of *Plasmopara halstedii*

	Sporulation density	*Latent period*	*Hypocotyl length*	*Percentage of infection*
Sporulation density	1.000	–0.840**	0.366*	0.499**
Latent period		1.000	–0.473**	–0.624**
Hypocotyl length			1.000	0.222^{ns}
Percentage of infection				1.000

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

Form and size of zoosporangia

The results showed that the two forms most observed were oval and round (Fig. 3 and Table 5). The proportion of oval form varied from 37 to 94% and the zoosporangia size from 302.2 to 918.6 μm^2. The proportion of oval zoosporangia varied within the races for which five single zoosporangium were available, for example for race 100 it varied from 87% to 94% and for race 710 it ranged from 37% to 92%. Zoosporangia size also varied considerably within and between races, with no relation to form. There was thus no relationship between zoosporangia form or size and virulence characteristics as defined by Tourvieille de Labrouhe *et al.*, (2000). All aggressiveness criteria were not correlated with form or size of zoosporangia ($r = -0.039$ and $r = -0.067$ for percentage infection, $r = -0.047$ and $r = 0.150$ for period of incubation of infected plants, $r = 0.132$ and $r = -0.178$ for sporulation density and $r = 0.002$ and $r = -0.056$ for hypocotyl length).

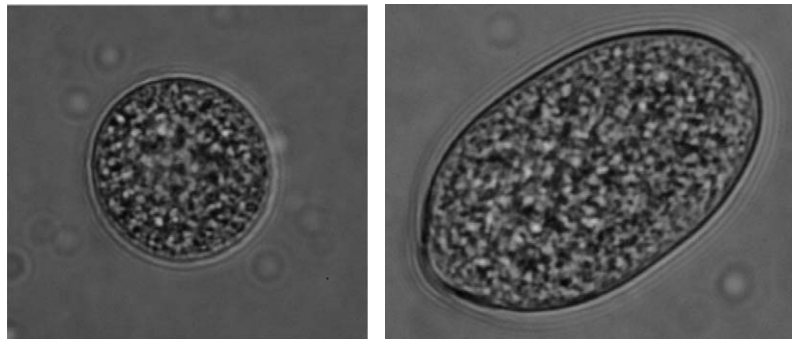

Fig. 3. *Plasmopara halstedii* zoosporangia forms observed on sunflower inbred line "FU": round (left) and oval (right)

Molecular analysis

The combination of 12-EST derived markers revealed five multilocus genotypes (MLG) among 35 *P. halstedii* single zoosporangium isolates (Table 6). There was no *intra-genetic* variation for all pathogen isolates tested. Excepting Pha54 the single zoosporangium isolates of MIL 001 and MIL 002 were different for all genomic markers. The single zoosporangium isolates MIL 001, DU 1842

Table 5. Morphological characters of zoosporangia obtained on sunflower inbred line "FU" for 35 single zoosporangium isolates of *Plasmopara halstedii*

Isolates of P.halstedii	*Race*	*% of oval zoosporangia**	*Size of zoosporangia in µm²* **
MIL001 M2	100	87	315.8
MIL001 M3	100	94	434.9
MIL001 M4	100	88	392.9
MIL001 M5	100	91	418.7
MIL001 M6	100	90	432.2
DU1842 M1	300	88	398.0
DU1842 M2	300	89	511.7
DU1842 M3	300	68	436.4
DU1842 M4	300	82	315.2
DU1842 M5	300	89	381.4
DU1943 M1	314	93	424.8
DU1943 M2	314	86	425.4
DU1943 M3	314	80	387.6
DU1943 M4	314	56	372.0
DU1943 M5	314	56	380.4
DU1767 M1	304	86	394.0
DU1767 M2	304	78	422.3
DU1767 M3	304	90	505.2
DU1767 M4	304	91	478.7
DU1767 M5	304	63	344.7
MIL002 M1	710	82	463.5
MIL002 M2	710	92	513.3
MIL002 M3	710	90	918.6
MIL002 M4	710	37	352.9
MIL002 M5	710	53	419.4
DU1915 M1	714	87	320.5
DU1915 M2	714	90	477.8
DU1915 M3	714	93	734.6
DU1915 M5	714	86	374.6
DU1915 M6	714	87	358.9
DU1734 M1	704	74	505.4
DU1734 M2	704	68	357.1
DU1734 M3	704	68	314.3
DU1734 M7	704	74	302.2
DU1734 M8	704	89	436.9
F isolates		4.524*	25.3*
Probability		0.0001	0.0001

* 50 zoosporangia per replication, ** 50 zoosporangia per replication, F-tests (* P = 0.05).

Table 6. Multilocus genotypes (MLG) characterized using 12 EST-derived genomic markers on 35 single zoosporangium isolates of *Plasmopara halstedii*

Isolate	*EST-derived markers*											
	Pha6	*Pha39*	*Pha42*	*Pha43*	*Pha54*	*Pha56*	*Pha74*	*Pha79*	*Pha82*	*Pha99*	*Pha106*	*Pha120*
MIL001 M2	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
MIL001 M3	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
MIL001 M4	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
MIL001 M5	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
MIL001 M6	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
DU1842 M1	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
DU1842 M2	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
DU1842 M3	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
DU1842 M4	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
DU1842 M5	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
DU1943 M1	1/1	2/2	1/1	2/2	1/1	2/2	2/2	3/3	2/2	1/1	2/2	1/1
DU1943 M2	1/1	2/2	1/1	2/2	1/1	2/2	2/2	3/3	2/2	1/1	2/2	1/1
DU1943 M3	1/1	2/2	1/1	2/2	1/1	2/2	2/2	3/3	2/2	1/1	2/2	1/1
DU1943 M4	1/1	2/2	1/1	2/2	1/1	2/2	2/2	3/3	2/2	1/1	2/2	1/1
DU1943 M5	1/1	2/2	1/1	2/2	1/1	2/2	2/2	3/3	2/2	1/1	2/2	1/1
DU1767 M1	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
DU1767 M2	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
DU1767 M3	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
DU1767 M4	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
DU1767 M5	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
MIL002 M1	1/1	1/1	2/2	2/2	1/1	2/2	2/2	1/1	1/1	1/1	2/2	1/1
MIL002 M2	1/1	1/1	2/2	2/2	1/1	2/2	2/2	1/1	1/1	1/1	2/2	1/1
MIL002 M3	1/1	1/1	2/2	2/2	1/1	2/2	2/2	1/1	1/1	1/1	2/2	1/1
MIL002 M4	1/1	1/1	2/2	2/2	1/1	2/2	2/2	1/1	1/1	1/1	2/2	1/1
MIL002 M5	1/1	1/1	2/2	2/2	1/1	2/2	2/2	1/1	1/1	1/1	2/2	1/1
DU1915 M1	1/1	2/2	1/2	1/1	1/1	1/1	1/1	3/3	2/2	1/1	2/2	1/1
DU1915 M2	1/1	2/2	1/2	1/1	1/1	1/1	1/1	3/3	2/2	1/1	2/2	1/1
DU1915 M3	1/1	2/2	1/2	1/1	1/1	1/1	1/1	3/3	2/2	1/1	2/2	1/1
DU1915 M5	1/1	2/2	1/2	1/1	1/1	1/1	1/1	3/3	2/2	1/1	2/2	1/1
DU1915 M6	1/1	2/2	1/2	1/1	1/1	1/1	1/1	3/3	2/2	1/1	2/2	1/1
DU1734 M1	2/2	2/2	2/2	1/1	1/1	1/1	2/2	3/3	1/1	1/1	2/2	1/1
DU1734 M2	2/2	2/2	2/2	1/1	1/1	1/1	2/2	3/3	1/1	1/1	2/2	1/1
DU1734 M3	2/2	2/2	2/2	1/1	1/1	1/1	2/2	3/3	1/1	1/1	2/2	1/1
DU1734 M7	2/2	2/2	2/2	1/1	1/1	1/1	2/2	3/3	1/1	1/1	2/2	1/1
DU1734 M8	2/2	2/2	2/2	1/1	1/1	1/1	2/2	3/3	1/1	1/1	2/2	1/1

and DU 1767 had the same genetic background. The Neighbour-joining tree showed that single zoosporangium isolates of DU 1915, DU 1734 and DU 1943 had an intermediary genetic position between single zoosporangium isolates of MIL 001 and MIL 002 (Fig. 4).

DISCUSSION

Knowledge of the interaction between a parasite and its host plant would help to understand the co-evolution of pathogen populations that use their pathogenicity to better improve their adaptation to a host, in particular processes

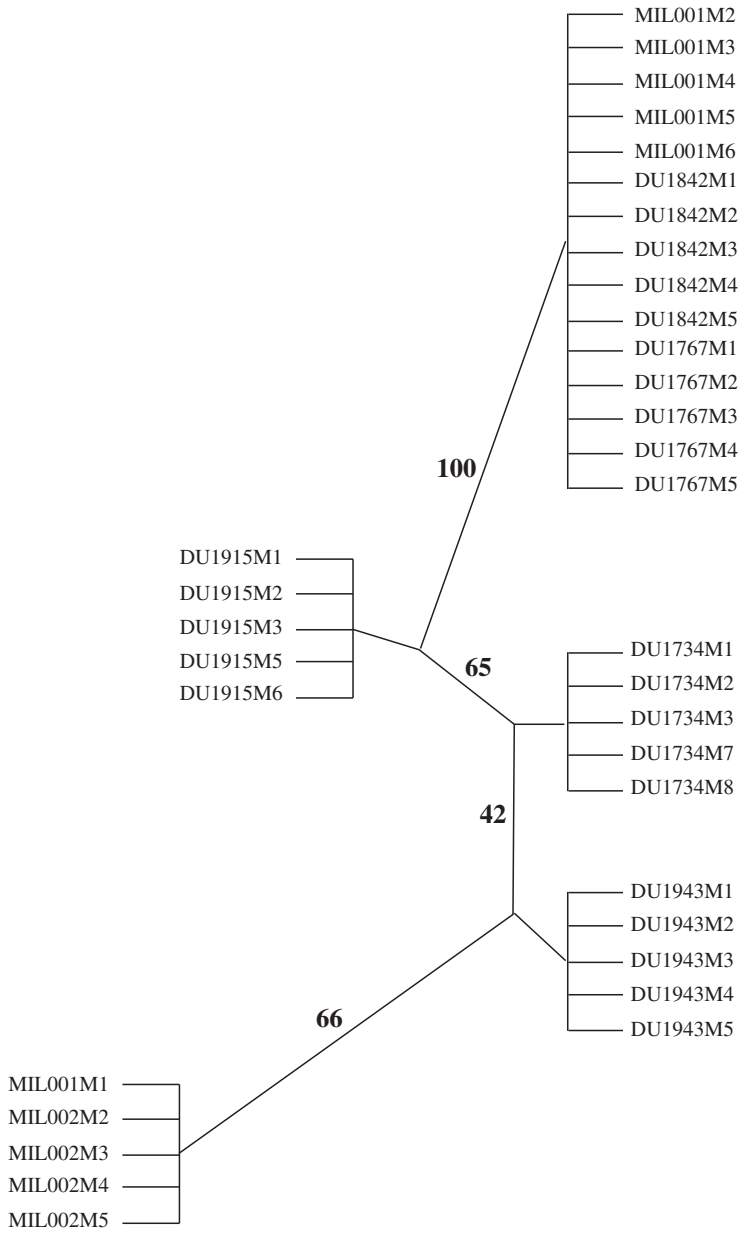

Fig. 4. Phylogenetic tree according to Neighbour-joining analysis of 12 EST-derived markers. Figures on branches indicate bootstrap values (10.000 replicates)

that generate genetic diversity (Brown & Tellier, 2011). With this in mind, morphological, pathogenic and genetic variation was studied in seven *P. halstedii* isolates of several races. Studying these aspects could help to decide on the

diagnostic value of these characters in population studies of *P. halstedii*, a species that is characterized by a high level of evolutionary potential (Sakr 2011a; 2012; Viranyi and Spring 2011). Since tools for analyzing of obligate parasite Peronosporaceae are very limited (Spring & Thines, 2004), it appears desirable to contain research for new methods.

The *P. halstedii* isolates of races 100, 3xx and 7xx were sampled from naturally infected plants in sunflower parcels. Consequently, they represent a mixture of organisms with different virulence profiles which may show the same race of phenotype. To diminish this problem, analyses were performed with single zoosporangium isolates (Sakr *et al.*, 2007). These may give rise to genetically homogeneous isolates if only a single karyon enteres the zoosporangium or when the parental mycelium is homokaryotic. Furthermore, we confirmed that single zoosporangium isolates were of the same race as the pathogen isolates (Tables 1 and 2). Our data were not revealed in the same pathogenic system in which Molinero-Ruiz *et al.*, (2002) found that some single zoosporangium *P. halstedii* isolates, obtained from one parental isolate, showed differences in the virulence profile as compared with the parental one. However, there is a possibility of genetic inhomogeneity of single zoosporangium isolates (in contrast to single zoospore isolates) because indications now exist (Spring & Zipper, 2006) that two or even more nuclei immigrate into a single zoosporangium; these nuclei may then mitotically divide there. As a result, genetically inhomogenic zoospores can develop in a single zoosporangium in a heterokaryotic mycelium.

Differences in aggressiveness of *P. halstedii* isolates are indicated when single zoosporangium isolates vary in the amount of damage that they cause in sunflower plants. The four aggressiveness criteria measured in the present study showed significant correlation with each other (Table 4). These results are comparable with those found by Chacon *et al.* (2007) for *Phytophthora infestans*, another Oomycete. They showed a strong correlation among the four criteria of aggressiveness: latent period, frequency of infection, area under the lesion expansion curve and the sporulation capacity. In this study, the correlation between hypocotyl length, period of incubation of infected plants ($r = -0.473$) and the sporulation density ($r = 0.366$) could be explained by specialisation in aggressiveness towards tissue invasion and sporulation, which is detrimental to the deregulating activity of plant auxin activity (Cohen and Sackston, 1974). The frequency of sporulation based on the incubation period reflected the speed of appearance of symptoms on the plants (Fig. 1) (period of incubation of infected plants), and the number of zoosporangia produced by cotyledons reflected the level of invasion of infected tissues (sporulation density) (Fig. 2).

Analysis of five single zoosporangium isolates of each pathogen isolate showed variability for aggressiveness criteria studied within *P. halstedii* isolate, but not for all pathogen isolates (Table 2). The difference in aggressiveness within *P. halstedii* isolates may be due to the variability in aggressiveness within a population of zoosporangia, of which a single zoosporangium isolate is only one preventative. This character (aggressiveness) can be multiplied and become dominant as observed in parental isolate variability. Moreover, Sakr *et al.* (2007) found differences in morphology between single zoosporangium isolates arising from the parental isolate of race 707. It is possible that variability between pathogen isolates is due to the origin of pathogen isolates used in this study (Table 1). These isolates belong to several races and may be found to be an effect of additional virulence genes in *P. halstedii* parental isolates as observed for other pathogens (Montarry *et al.*, 2010; De Vallavieille-Pope *et al.*, 2012).

High percentage of infection, short period of incubation of infected plants, high sporulation density, and significant reduction in the length of the hypocotyl represent high aggressiveness (Sakr *et al.*, 2009, 2011; Sakr 2011a,b; 2012). Our results show that two criteria, period of incubation of infected plants and sporulation density, differentiated pathogen isolates (Table 3). Isolates MIL 001, DU 1842, DU 1767 and DU 1943 had a shorter latent period and higher sporulation density than isolates MIL 002, DU 1734 and DU 1915. In the pathosystem *P. 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 ideal test conditions for the parasite (Meliala *et al.*, 2000). However, Chacon *et al.* (2007) found that values of infection frequency for different *P. infestans* isolates, measured on a potato variety showing a high level of quantitative resistance, ranged between 27 and 85%. Table 4 shows that all the isolates caused a large reduction in seedling size except for DU 1943, compared to the growth of *P. halstedii*-free sunflower inbred line FU. Our results differ from those found by Sinclair & 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.

Single zoosporangium isolates of races 7xx were more virulent than those of races 100 and 3xx (Tables 1 and 2) as defined by Sakr (2012). Moreover, the index of aggressiveness revealed the presence of significant differences between the single zoosporangium isolates of races 100 and 3xx and single zoosporangium isolates of races 7xx (Table 2). It seems that *P. halstedii* single zoosporangium isolates may be divided into two pathogenic groups as more virulent and less aggressive single zoosporangium isolates of races 7xx and less virulent and more aggressive single zoosporangium isolates of races 100 and 3xx. The fact about that both virulence and aggressiveness groups are identical has also been demonstrated for some pathogens (De Wet *et al.*, 2003; Bonde *et al.*, 2006; Montarry *et al.*, 2006).

The proportion of zoosporangia of different forms and their sizes (Fig. 3) do not appear to be useable to differentiate the virulent characteristics for single zoosporangium isolates identified in this study (Table 2). This is in contrast with the results of De Wet *et al.* (2003) who observed morphological differences between strains of *Sphaeropsis sapinea*, which divided them into 3 morphotypes (A, B and C) that presented differences in pathogenicity (virulence and aggressiveness). The results also showed that zoosporangia and sporangiophores morphology did not distinguish the two aggressiveness groups (Tables 2 and 3). However, for the same pathosystem, Sakr (2011b) found a relationship between another morphological character (viability of zoosporangia) and aggressiveness in *P. halstedii*. For a facultative parasitic Oomycete, *Phytophthora capsici*, Islam *et al.* (2004) did not find any relationship between groups of isolates characterized for their growth patterns on artificial medium and their aggressiveness. Other facultative parasites may show correlations between morphological aspects measured on artificial medium and aggressiveness criteria. In the pathosystem *Sclerotinia sclerotiorum* / rapeseed and mustard, Ghasolia & Asha (2007) described nine groups based on morphological characters which were divided into two series according to degree of aggressiveness.

There was no *intra-race* genetic variation (Table 6), but five genetically-identified groups were detected among *P. halstedii* isolates of all races (Fig. 4). Our results observed genetic distances between two races 100 and 710 in agreement with the conclusions of Delmotte *et al.* (2008) and As-sadi *et al.* (2011). By using the same EST-derived as SNPs markers, Delmotte *et al.* (2008) found

that races 100, 300 and 304 had the same genetic clade as observed in our study (Fig. 2). However, As-sadi *et al.* (2011) reported that certain SNPs might allow for clear differentiation between races 304 and 100, which has not been detected in our work (Fig. 2) or in previous studies (Delmotte *et al.*, 2008). 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 as SNPs 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 EST genotypes (Table 6 and Fig. 4) and both pathogenicity traits (Tables 1 and 2) and morphological characteristics (Table 5). In accordance with our results, Mahdizadeh *et al.* (2011) reported no correlation between genetic diversity based on ISSR and morphological characteristics for *Macrophomina phaseolina*. 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 pathogenicity phenotypes and genotypes based on AFLP markers for *Phytophthora infestans*. Pathogenicity 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 pathogenicity groups A, B and C were separated into three differential clades. It will be necessary to analyze the morphological, pathogenic and genetic variation in a large sampling of *P. halstedii* of different races from several parts of world to provide a better insight into interactions between this obligate parasite and its host.

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