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Pathogenic, morphological and genetic diversity in *Plasmopara halstedii*, the causal agent of sunflower downy mildew

Nachaat Sakr

Department of Agriculture, Syrian Atomic Energy Commission, Damascus, P.O. Box 6091, Syria Previous address INRA-UBP, UMR 1095, 234 Avenue du Brézet, 63100 Clermont-Ferrand, France. E-mail: snachaat@hotmail.com; snachaat@gmail.com

ABSTRACT. Pathogenic, morphological and genetic variation was studied in 35 *Plasmopara halstedii* (sunflower downy mildew) isolates of different races. Virulence spectrum was analyzed in sunflower hybrids carrying effective *Pl*genes. Aggressiveness was analyzed in one sunflower inbred line showing a high level of quantitative resistance. There were differences in virulence spectrum for pathogen isolates. Index of aggressiveness was calculated for each isolate and two groups were revealed as more aggressive isolates of 100 and 3xx races, and less aggressive isolates of 7xx races. Significant morphological differences were found in zoosporangia and sporangiophores morphology. Genetic relationships were detected between the pathogen isolates using 12 EST-derived markers. Five multilocus genotypes (MLG) were identified among 35 *P. halstedii* isolates. Our results did not show a correlation between pathogen variation and both morphological and genetic characteristics.

Keywords: aggressiveness, EST-derived markers, virulence, zoosporangia, sporangiophores.

Diversidade patogênica e morfológica em *Plasmopara halstedii*, o agente causador de míldio do girassol

RESUMO. Foi analisada a variação patogênica, morfológica e genética de isolados de 35 *Plasmopara halstedii* (míldio do girassol) de diferentes genótipos. Investigou-se efetivamente o espetro de virulência em híbridos de girassol com genes de *Pl*. A agressividade foi analisada num genótipo de girassol, o qual mostrou alto nível de resistência quantitativa. Houve diferenças no espetro de virulência para os isolados patogênicos. O índice de agressividade foi determinado para cada isolado e dois isolados mais agressivos de 100 e 3xx genótipos e isolados menos agressivos de 7xx genótipos foram identificados. Foram encontradas diferenças morfológicas significantes em zoosporangia e na morfologia de esporangióforos. Detectou-se o relacionamento genético entre os isolados do agente patogênico pelos marcadores 12-EST-derivados. Cinco genótipos multilocos foram identificados entre os 35 isolados de *P. halstedii*. Os resultados não evidenciaram uma correlação entre a variação do agente patogênico e as características morfológicas e genéticas.

Palavras-chave: agressividade, marcadores EST-derivados, virulência, zoosporangia, esporangióforos.

Introduction

Sunflower (*Helianthus annuus* L.) is one of the major oilseed crops cultivated worldwide. The biotrophic oomycete *Plasmopara halstedii* (Farl.) Berl. and de Toni is a soil-, seed- and wind-borne pathogen causing downy mildew in sunflower, which can survive up to 10 years in the soil in the form of oospores (VIRANYI; SPRING, 2011). Downy mildew is a common sunflower disease responsible for significant yield loss and can be controlled by fungicides and cultivation of resistant hybrids. *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). 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 that *P. halstedii* is characterized by a high level of evolutionary potential (SAKR, 2011b, 2012; VIRANYI; SPRING, 2011), there have been several studies on virulence (DELMOTTE et al., 2008; SAKR, 2011b, 2012; TOURVIEILLE DE LABROUHE et al., 2000, 2010) and more recently on

aggressiveness (SAKR, 2011a, b and c, 2012; SAKR et al., 2011). Virulence has been defined as specific disease-causing abilities and aggressiveness as non-specific disease-causing abilities (VAN DER PLANK, 1968). For morphological characteristics in *P. halstedii*, Spring and Thines (2004) and Viranyi and Spring (2011) found phenotypic limited markers 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* (INTELMANN; SPRING, 2002).

Applying molecular techniques such as PCR method enable us to detect the pathogen even there are no symptoms within host plants (IOOS et al., 2007). 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; SAKR, 2011a) sequences to characterize *P. halstedii* isolates has been showed, but races can not be defined with certainty. However, As-Sadi et al. (2011) reported that genetic distance between four *P. halstedii* races can be detected using SNPs discovered in CRN effector sequences.

In the context of sustainable agriculture, management of durable genetic resistance and minimization of selective pressure on pathogens are key objectives. Discovering the pathogenic, morphological and genetic variation is crucial for research on *P. halstedii* populations of several races. In order to clarify the interaction among the pathogenic, morphological and genetic variations, the objectives of this study were (i) to analyze the variability of virulence and aggressiveness of *P. halstedii* isolates in sunflower plants showing different levels of qualitative and quantitative resistance (ii) to determine the extent of variation on the zoosporangia and sporangiophores morphology; and (iii) to analyze the genetic relationships between 35 isolates by using 12 EST-derived markers.

Material and methods

Fungal isolates

The 35 *P. halstedii* isolates used in this study were collected in France and maintained at INRA, Clermont–Ferrand following European regulations (No 2003/DRAF/70) in respect to this quarantine parasite. Pathogen isolates were sampled from naturally infected sunflower plants. Their races (Table 1) and the characterization of the virulence for 35 isolates (Table 2) were carried out following the protocol developed by Tourvieille de Labrouhe et al. (2000).

The *P. halstedii* isolates were multiplied on the same sunflower genotype in the same conditions, such that any original intra-isolate variability would be maintained to the same extent for the seven isolates. In addition, the isolates are multiplied using their zoospores, which could be a continued source of new variability, even for originally single zoosporangium isolates.

Virulence spectrum for *P. halstedii* isolates

To characterize virulence spectrum in *P. halstedii* isolates, four quasi-isogenic hybrids differing only in their downy mildew resistance genes were used, obtained from crosses of 2 forms (TOURVIEILLE DE LABROUHE et al., 2010): L1a, carrying resistance gene *Pl2*; L1b, carrying resistance genes *Pl2* and *Pl8*; L2a, carrying no known resistance gene; L2b, carrying resistance gene *Pl6*. The four hybrids were produced as follows: H1= L1a x L2a; H2= L1a x L2b; H3= L1b x L2a and H4= L1b x L2b. Two sunflower lines were also used to analyze virulence spectrum for 50 *P. hasltedii* isolates: XRQ (INRA, resistant to all French pathotypes except pathotype 334, carrying *Pl5*) and RHA340 (USDA, resistant to all known pathotypes, carrying *Pl8*). For race identification and virulence spectrum tests, there were three replications for each sunflower variety (10 plants in each replication) and the entire experiment was repeated twice for 35 *P. halstedii* isolates.

Measurement of aggressiveness in *P. hasltedii* isolates

To characterize the two aggressiveness criteria latent period and sporulation density for *P. halstedii* isolates (SAKR, 2011a, b and c, 2012; SAKR et al., 2011), 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). The index of aggressiveness of *P. halstedii* isolates was calculated as the ration of sporulation density / latent period. The index of aggressiveness of the *P. halstedii* isolate was used to summarize all values for two criteria on sunflower inbred lines `FU` in one value to facilitate the comparison between the different *P. halstedii* isolates (SAKR, 2011b). 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. All the pathogenic tests were carried out in growth chambers regulated at 18h 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; COCHRAN, 1989) was used to compare the means at $p = 0.05$. The sample correlation coefficients (Pearson r) were calculated at $p = 0.05$ and $p = 0.01$.

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

Race	Differential lines								
	D1 Ha-304 Without *Pl* gene	D2 Rha-265 *Pl1*	D3 Rha-274 *Pl2*	D4 PMI3 *Pl(?)*	D5 PM-17 *Pl(?)*	D6 803-1 *Pl(?)*	D7 HAR-4 *Pl(?)*	D8 QHP1 *Pl(?)*	D9 Ha-335 *Pl6*
100	S	R	R	R	R	R	R	R	R
300	S	S	R	R	R	R	R	R	R
314	S	S	R	S	R	R	R	R	S
304	S	S	R	R	R	R	R	R	S
710	S	S	S	S	R	R	R	R	R
714	S	S	S	S	R	R	R	R	S
704	S	S	S	R	R	R	R	R	S

S: susceptible, sporulation on cotyledons. R: resistant, no sporulation. *Pl(?)* = carrying no known resistance gene (TOURVIEILLE DE LABROUHE et al., 2000).

Table 2. List and virulence of 35 *Plasmopara halstedii* isolates on four sunflower hybrids differing only in their downy mildew resistance genes.

Isolates	race	Year isolates	H1	H2	H3	H4	XRQ *Pl5*	RHA340 *Pl8*
MIL001 M2	100	2006	R	R	R	R	R	R
MIL001 M3	100	2006	R	R	R	R	R	R
MIL001 M4	100	2006	R	R	R	R	R	R
MIL001 M5	100	2006	R	R	R	R	R	R
MIL001 M6	100	2006	R	R	R	R	R	R
DU1842 M1	300	2006	R	R	R	R	R	R
DU1842 M2	300	2005	R	R	R	R	R	R
DU1842 M3	300	2006	R	R	R	R	R	R
DU1842 M4	300	2006	R	R	R	R	R	R
DU1842 M5	300	2006	R	R	R	R	R	R
DU1943 M1	314	2006	R	R	R	R	R	R
DU1943 M2	314	2006	R	R	R	R	R	R
DU1943 M3	314	2005	R	R	R	R	R	R
DU1943 M4	314	2006	R	R	R	R	R	R
DU1943 M5	314	2006	R	R	R	R	R	R
DU1767 M1	304	2006	R	R	R	R	R	R
DU1767 M2	304	2006	R	R	R	R	R	R
DU1767 M3	304	2006	R	R	R	R	R	R
DU1767 M4	304	2005	R	R	R	R	R	R
DU1767 M5	304	2006	R	R	R	R	R	R
MIL002 M1	710	2006	S	R	R	R	R	R
MIL002 M2	710	2006	S	R	R	R	R	R
MIL002 M3	710	2006	S	R	R	R	R	R
MIL002 M4	710	2006	S	R	R	R	R	R
MIL002 M5	710	2006	S	R	R	R	R	R
DU1915 M1	714	2005	S	S	S	S	R	R
DU1915 M2	714	2005	S	S	S	S	R	R
DU1915 M3	714	2005	S	S	S	S	R	R
DU1915 M5	714	2005	S	S	S	S	R	R
DU1915 M6	714	2005	S	S		S	R	R
DU1734 M1	704	2005	S	S	S	S	R	R
DU1734 M2	704	2005	S	S	S	S	R	R
DU1734 M3	704	2005	S	S	S	S	R	R
DU1734 M7	704	2005	S	S	S	S	R	R
DU1734 M8	704	2005	S	S	S	S	R	R

Morphological observations

After 13 days of infection of the sunflower inbred line `FU`, the zoosporangia and sporangiophores suspensions were obtained by grouping all sporulated cotyledons in a small container and adding 1 mL of physiological water for each cotyledon (9 g NaCl + 1 L sterilized 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 = ½ length, b = ½ width. Furthermore, sporangiophore dimensions were observed by measuring 50 fresh sporangiophores in physiological water under a light microscope (magnification X400) with 2 replications.

DNA extraction and molecular typing

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 *P. halstedii* isolates. The polygenetic relations between the 50 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

Characterization of pathogenicity for *P. halstedii* isolates

Based on the reaction for the *P. halstedii* isolates to four sunflower hybrids H1 to H4 varying only in their *Pl* genes, there were differences in virulence spectrum in *P. halstedii* isolates (Table 2). All sunflower hybrids were resistant to isolates of races 100, 300, 304 and 314, and sensitive to isolates of races 714 and 704. H1 was only sensitive to isolates of race 710. Moreover, the two sunflower inbred lines XRQ and RHA340 were resistant to all *P. halstedii* isolates tested.

The differences in behaviour among *P. halstedii* isolates were revealed by analysis between sporulation percentages based on incubation period (Figure 1). There were two main groups from day 8 onwards: isolates of races 100, 300, 304 and 314 sporulated faster than isolates of races 710, 704 and 714. All infected plants with races 100 and 3xx showed more than 80% sporulation 9 days after incubation, whereas isolates 7xx needed 11 days after incubation to reach the same level of sporulation.

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

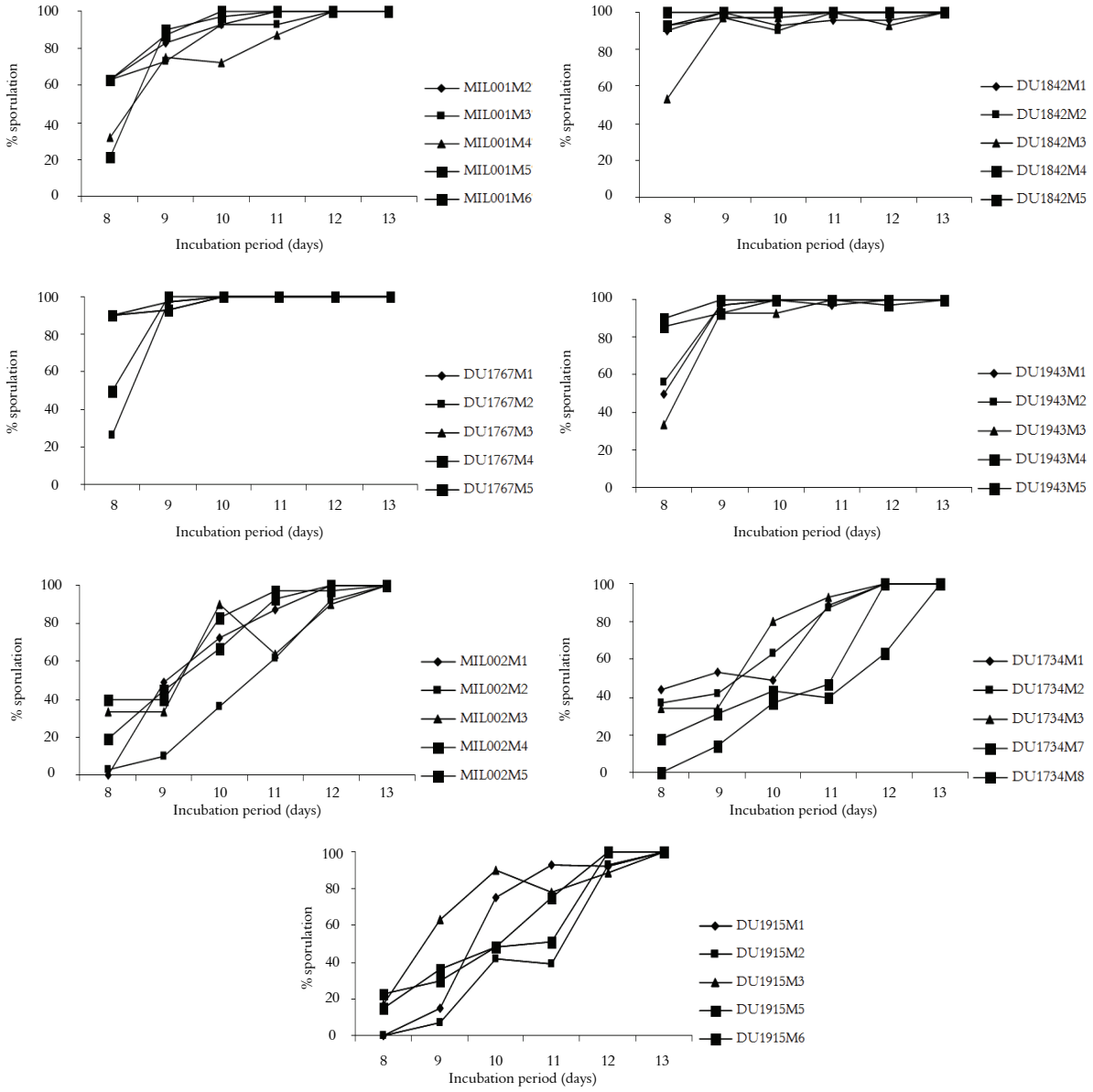

Figure 1. Sporulation of 35 *Plasmopara halstedii* isolates of seven races on the sunflower inbred line `FU`, based on incubation period.

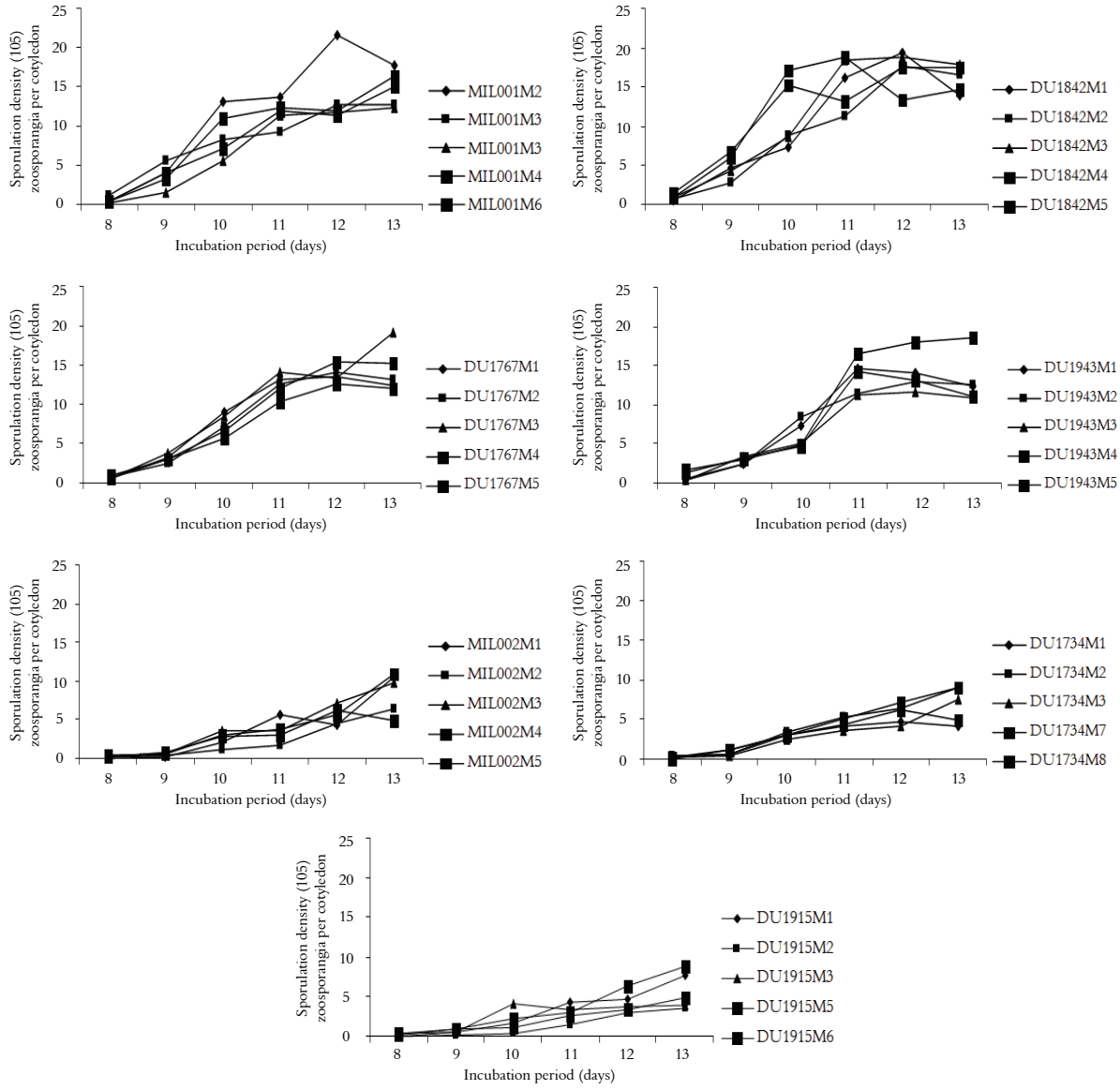

Figure 2. Sporulation density of *Plasmopara halstedii* isolates of seven races on sunflower inbred line `FU`, based on incubation period.

Comparison between the two aggressiveness criteria of 35 *P. halstedii* isolates was presented in Table 3. There were significant differences between *P. halstedii* isolates regarding the latent period (F = 55.47) and sporulation density (F = 12.39). The latent period ranged from 7.8 days for isolates DU1842 M4 and DU1842 M5 to 12.5 days for isolate DU 1734 M87. Sporulation density varied five folds: 3.91×10^5 zoosporangia were produced by cotyledons for isolate DU 1915 M5 and 19.6×10^5 for isolate MIL 001 M2. The index of aggressiveness varied seven folds: 0.3 for DU1915 M3 and 2.2 for MIL 001 M2. There were highly significant differences (Probability = 0.0055, T-test = 4.006) between the index of aggressiveness for both isolates of races 100 and 3xx (1.7±0.2) and isolates of races 7xx (0.5±0.1). There was a highly significant negative correlation (P = 0.01) between latent period and sporulation density: r = -0.847.

Morphology of zoosporangia and sporangiophores

The results showed that the two most observed forms of zoosporangia were oval and round (Figure 3). The proportion of oval form varied from 37 to 92% and the zoosporangia size from 315.2 to 918.6 μm^2. The highest mean sporangiophore length was in DU 1767 M3 (800.0 µm). The sporangiophore length ranged between 325.9 and 800.0 µm. The largest mean sporangiophore width was in DU 1767 M1. The sporangial width varied from 4.3 to 16.0 µm. There was thus no relationship between morphology of zoosporangia and sporangiophores and virulent characteristics for isolates identified in

Table 3. Aggressiveness criteria on sunflower inbred line `FU` for 35 isolates of *Plasmopara halstedii*.

Isolates	Race	Latent period[b] mean (days)	Sporulation density[c] mean (10^5 zoosporangia per cotyledon)	Index of aggressiveness
MIL001 M2	100	9.1	19.68	2.2
MIL001 M3	100	9.3	12.70	1.4
MIL001 M4	100	10.2	11.97	1.2
MIL001 M5	100	8.9	13.16	1.5
MIL001 M6	100	8.6	14.11	1.6
DU1842 M1	300	7.9	16.61	2.1
DU1842 M2	300	8.1	17.20	2.1
DU1842 M3	300	8.8	18.33	2.1
DU1842 M4	300	7.8	14.03	1.8
DU1842 M5	300	7.8	17.42	2.2
DU1943 M1	314	8.6	13.25	1.5
DU1943 M2	314	8.5	12.75	1.5
DU1943 M3	314	8.9	11.30	1.3
DU1943 M4	314	8.2	18.27	2.2
DU1943 M5	314	7.9	12.10	1.5
DU1767 M1	304	7.9	13.04	1.7
DU1767 M2	304	8.7	13.60	1.6
DU1767 M3	304	8.0	16.26	2.0
DU1767 M4	304	8.6	15.31	1.8
DU1767 M5	304	8.0	13.32	1.7
MIL002 M1	710	10.4	7.44	0.7
MIL002 M2	710	11.8	5.45	0.5
MIL002 M3	710	11.1	8.45	0.8
MIL002 M4	710	10.3	8.25	0.8
MIL002 M5	710	10.5	5.56	0.5
DU1915 M1	714	10.6	6.20	0.6
DU1915 M2	714	11.9	3.33	0.3
DU1915 M3	714	10.2	3.91	0.4
DU1915 M5	714	11.5	4.07	0.4
DU1915 M6	714	11.2	7.62	0.7
DU1734 M1	704	11.0	4.37	0.4
DU1734 M2	704	10.9	7.72	0.7
DU1734 M3	704	10.5	5.84	0.6
DU1734 M7	704	12.5	5.58	0.4
DU1734 M8	704	11.6	8.07	0.7
F isolates		55.47**	12.39**	
LSD		0.53	357	

[a]10 plants per replication, b 18 counts per replication, index of aggressiveness =sporulation density / latent period, F-tests (**p = 0.01), least significant differences LSD (p = 0.05).

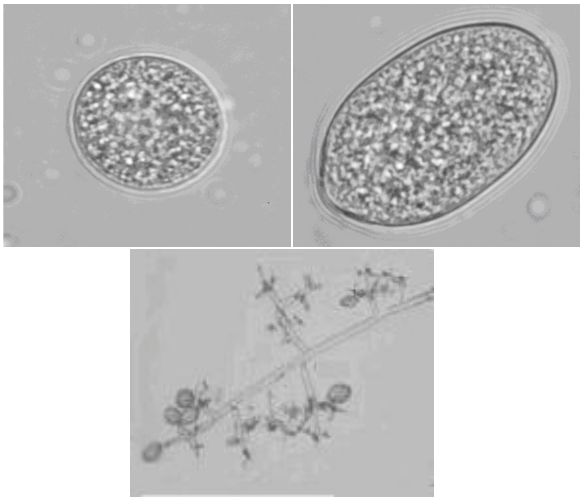

Figure 3. *Plasmopara halstedii* zoosporangia forms and sporangiophores observed on sunflower inbred line `FU`: round (left), oval (center) and sporangiophore (right).

this study (Tables 2 and 4). All aggressiveness criteria were not correlated with both form and size of zoosporangia (r = 0.009 and r = 0.184 for latent period, and r = -0.202 and r = -0.312 for sporulation density), and length or width of sporangiophore (r = 0.071 and r = -0.220 for latent period, and r = 0.161 and r = 0.232 for sporulation density). Consequently, there was no relationship between morphological characteristics of zoosporangia and sporangiophores and aggressiveness groups identified in the present study (Tables 3 and 4).

Table 4. Morphological characters of zoosporangia and sporangiophores obtained on sunflower genotype `FU` for 35 isolates of *Plasmopara halstedii*.

Isolates	Race	% of oval zoosporangia[a]	Size of zoosporangia in μm^2[b]	Sporangiophore length (μm)[c]	Sporangiophore Width (μm)[d]
MIL001 M2	100	87	315.8	325.9	12.3
MIL001 M3	100	94	434.9	550.2	15.1
MIL001 M4	100	88	392.9	715.9	10.9
MIL001 M5	100	91	418.7	660.2	8.7
MIL001 M6	100	90	432.2	489.3	6.9
DU1842 M1	300	88	398.0	333.8	5.1
DU1842 M2	300	89	511.7	568.2	4.3
DU1842 M3	300	68	436.4	663.6	7.7
DU1842 M4	300	82	315.2	785.2	14.8
DU1842 M5	300	89	381.4	559.3	12.3
DU1943 M1	314	93	424.8	663.5	9.7
DU1943 M2	314	86	425.4	489.3	6.3
DU1943 M3	314	80	387.6	356.2	8.2
DU1943 M4	314	56	372.0	689.3	10.9
DU1943 M5	314	56	380.4	299.0	14.3
DU1767 M1	304	86	394.0	478.6	16.0
DU1767 M2	304	78	422.3	559.6	8.3
DU1767 M3	304	90	505.2	800.0	6.5
DU1767 M4	304	91	478.7	456.3	8.1
DU1767 M5	304	63	344.7	765.3	9.6
MIL002 M1	710	82	463.5	459.3	7.8
MIL002 M2	710	92	513.3	569.3	6.6
MIL002 M3	710	90	918.6	553.6	8.9
MIL002 M4	710	37	352.9	440.4	12.0
MIL002 M5	710	53	419.4	554.3	11.9
DU1915 M1	714	90	477.8	700.9	8.5
DU1915 M2	714	90	477.8	456.3	7.6
DU1915 M3	714	93	734.6	552.1	6.2
DU1915 M5	714	86	374.6	456.3	5.0
DU1915 M6	714	87	358.9	558.2	6.8
DU1734 M1	704	74	505.4	335.6	7.4
DU1734 M2	704	68	357.1	663.3	6.8
DU1734 M3	704	68	314.3	712.3	8.1
DU1734 M7	704	74	302.2	322.2	9.6
DU1734 M8	704	89	436.9	455.9	6.0
F isolates		7.52**	8.40**	7.69**	4.89**
LSD		13.2	110.3	101.3	9.63

[a]50 zoosporangia per replication, [b]50 zoosporangia per replication, [c]50 sporangiophores per replication, [d]50 sporangiophores per replication, F-tests (**p=0.01), least significant differences LSD (p= 0.05).

Molecular analysis

The combination of 12-EST derived markers revealed five MLG among 35 *P. halstedii* isolates (Table 5). Isolates of races 100 and 710 were different for all genomic markers excepting Pha54. Furthermore, isolates of races 100, 300 and 304 had the same genetic background. The Neighbour-joining tree showed that isolates of races 704, 704 and 314 had an intermediary genetic position between isolates of races 100 and 710 (Figure 4).

Table 5. Multilocus genotypes (MLG) characterized using 12 EST-derived genomic markers on 35 isolates of *Plasmopara halstedii*.

Isolates	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

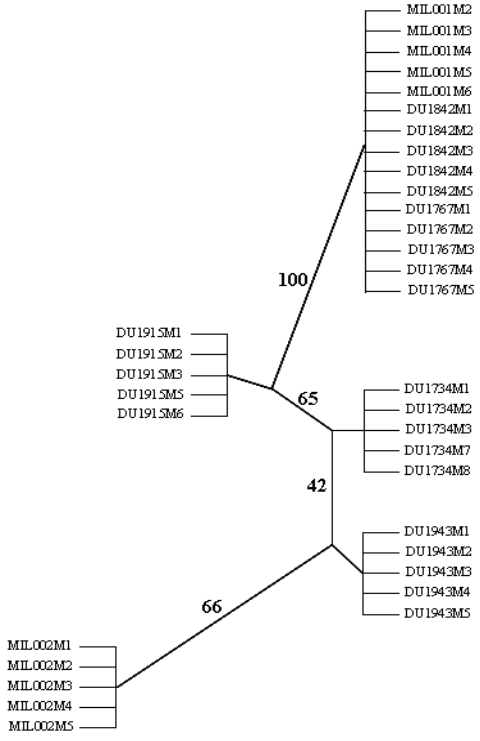

Figure 4. Phylogenetic tree according to Neighbour-joining analysis of 12 EST-derived markers. Figures on branches indicate bootstrap values (10.000 replicates).

Discussion

The aim of this present study was to analyze the pathogen itself. Naturally, any biological property of *Plasmopara halstedii* is related to pathogenesis as this living is an obligatory biotrophic one. However, the phenotypic properties expressed in abiotrophic stages can be factors of selection of zoospores (this is an obligatory biotroph and the motile phase is also obligatoric for this parasite) or cystospores, but this kind of selection is not necessarily related directly to pathogenicity of *P. halstedii* (SPRING; THINES, 2004; VIRANYI; SPRING, 2011). The virulence and aggressiveness manifest in relations to host, and it is a question of evaluation method as well as of economic requirements (SAKR, 2011b, 2012).

Sunflower hybrids H1 to H4, differing only in their downy mildew resistance genes, were used to analyze the virulence spectrum in *P. halstedii* isolates. Table 2 demonstrates that all sunflower hybrids were resistant to isolates of races 100, 300, 314 and 304, as those sunflower hybrids carrying effective *Pl* genes were resistant to races 100, 300, 304 and 314. On the other hand, all sunflower hybrids were sensitive to the isolates of races 714 and 704. The races 704 and 714 can overcome *Pl2* and *Pl6* present

in H1 and H2 as observed from its behavior on sunflower differential lines carrying the same genes (Table 1).

The two sunflower hybrids H3 and H4 came from L1b, which may carry *Pl8*. Since *Pl8* confers resistance to all known races and *Pl2* and *Pl8* segregate independently in L1b (TOURVIEILLE DE LABROUHE et al., 2010), it was not possible to determine whether L1b carried either resistance gene, *Pl2* or only *Pl8*. Moreover, in virulence seedling tests to isolates of races 714 and 704, certain sunflower plants (1-3) per replication for H3 and H4 produced no sporulation on cotyledons and leaves. However, H3 and H4 generated effective resistance to races 714 and 704 in field conditions (TOURVIEILLE DE LABROUHE et al., 2010). The sunflower hybrid H1 was only sensitive to isolates of the race 710 because it carries *Pl2* sensitive against race 710, and the effective *Pl* genes in other sunflower hybrids are resistant against race 710. Our results confirmed that the two sunflower lines XRQ and RHA340 were resistant to all *P. halstedii* isolates used in this study because they carry effective *Pl* genes against all races tested in the present study. This type of resistance may be controlled by non-TIR-NBS-LRR (Toll/interleukin-1 receptor (TIR) nucleotide-binding site leucine-rich repeat class) which clustered and linked to the *Pl5/Pl8* locus for resistance to downy mildew in sunflower (RADWAN et al., 2003).

Our results showed that the high level of quantitative resistance in the inbred line FU made it possible to detect significant differences between *P. halstedii* isolates (Table 3). These results are in accordance with our previous analysis on the comportment of FU in the growth chamber (SAKR, 2011a; SAKR et al., 2011). Thus our observation suggests that in inbred line FU, the development of the pathogen was slowed, and may be due to resistance mechanisms expressed by accumulation of QTL in sunflower cultivars (VEAR et al., 2008) or presence of resistance-inducing chemicals which improve resistance in sunflower genotypes to downy mildew and increase enzyme activities (KÖRÖSI et al., 2011). Differences in aggressiveness of *P. halstedii* isolates are indicated when isolates vary in the degree of damage that they can cause in sunflower plants (SAKR, 2011a, b and c, 2012; SAKR et al., 2011).

The two criteria of aggressiveness measured in the present study were strongly correlated ($r = -0.847$) when all *P. halstedii* isolates were involved in the analysis. These results correspond to those reported by Chacon et al. (2007) for another oomycete, *Phytophthora infestans*. They showed that the two measures of aggressiveness, latent period and sporulation capacity, were strongly correlated. In our study, the correlation between latent period and sporulation density could be explained by specialisation in aggressiveness towards tissue invasion and sporulation. The changes in frequency of sporulating plants during the incubation period reflect the manifestation of disease syndrome on the plants (Figure 1) while the number of zoosporangia produced on cotyledons reflects the fitness of pathogen in the infected tissues (Figure 2).

Short latent period and high sporulation density represent high aggressiveness (SAKR 2011a, b and c; 2012; SAKR et al., 2011). According to significant differences in aggressiveness criteria between *P. halstedii* isolates (Table 3), we can divide the isolates into two main groups. The first (more aggressive) includes isolates of races 100 and 3xx, while the other group (less aggressive) is composed of isolates of races 7xx. Our results are comparable with those found by Sakr (2011a). In the pathosystem of *P. infestans* / *Solanum tuberosum*, Montarry et al. (2010) showed that the most highly aggressive isolates had a shorter latent period and higher sporulation capacity than the less aggressive isolates. It is possible that the variation of aggressiveness between *P. halstedii* pathotypes is due to be an effect of additional virulence genes in aggressiveness as observed for other pathogens (DE VALLAVIEILLE-POPE et al., 2012; MONTARRY et al., 2010).

The proportion of zoosporangia of different forms and their sizes and the morphology of sporangiophores (Figure 3) do not appear to be useable to differentiate the virulent characteristics for isolates identified in this study (Tables 2 and 4). The results also showed that zoosporangia and sporangiophores morphology did not distinguish the two aggressiveness groups (Tables 3 and 4). However, for the same pathosystem, Sakr (2011c) found a relationship between another morphological character (viability of zoosporangia) and aggressiveness in *P. halstedii*. In accordance with our results, Islam et al. (2004) did not find any relationship between groups of isolates characterized for their morphological patterns of *Phytophthora capsici* and their pathogenicity traits. 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).

Because the effectiveness of the major resistance genes has recently been overcome and fourteen different races have been described (DELMOTTE

et al., 2008; TOURVIEILLE DE LABROUHE et al., 2000), our understanding of the recurrent breakdown of sunflower major resistance genes could be improved by new findings concerning the key processes governing the evolution of *P. halstedii* populations. Our results observed genetic distances between two races 100 and 710 in agreement with the conclusions of Delmotte et al. (2008), As-Sadi et al. (2011) and Sakr (2011a). By using the same EST-derived markers, Delmotte et al. (2008) and Sakr (2011a) found that races 100, 300 and 304 had the same genetic clade as observed in our study. 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 (Figure 4) and previously studies (DELMOTTE et al., 2008; SAKR, 2011a). 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 and data presented by Sakr (2011a). The isolates used in our study were different from thoses used by Delmotte et al. (2008), and this may explain the different results reported. Absence of genetic variation among isolates of the same race (Table 5) for the seven pathotypes (Tables 1 and 2) may be due to that molecular markers used in the present study were non-specific and insufficiently polymorphic within *P. halstedii* to detect differences among pathogen isolates of the same race. But the distinctiveness of the 7xx races compared to those of 100 or 3xx has recently been shown on the basis of ITS sequences data (SPRING et al., 2006). No correlation was detected between EST genotypes (Table 5 and Figure 4) and both pathogenicity traits (Tables 2 and 3) and morphological characteristics (Table 4). Indeed, for *P. halstedii*, Sakr (2011a) found no correlation between aggressiveness traits and EST genotypes. Moreover, our results are in accordance with those reported by Mahdizadeh et al. (2011) for *Macrophomina phaseolina*, who reported no correlation between genetic diversity based on ISSR and morphological characteristics. Also these results are comparable with those found by Montarry et al. (2006) for *P. infestans* by using AFLP genotypes for pathogenicity traits.

Conclusion

Even though a big step has been made towards understanding the complex interaction of *P. halstedii* and sunflower, as well as the mechanisms of pathogeneicity evolution, a number of questions have still remained unanswered. However, this paper presents fundamental data about significant differences among seven *P. halstedii* races. The most important tolls which distinguish the single zoosporangium isolates for seven races were aggressiveness and virulence criteria. The genetic relationships revealed five identified groups and morphological criteria did not distinguish between all races in the present study. To improve the knowledge regarding the pathology of sunflower downy mildew populations, it will be necessary to identify the morphological, pathogenic and genetic groups in on a large collection of *P. halstedii* isolates with different races from several parts of world.

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