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Can *Pl* Resistance Gene in Sunflower Line D4 be Used to Differentiate the Pathogenicity in Four *Plasmopara halstedii* (Sunflower Downy Mildew) Races 304, 314, 704 and 714?

N. SAKR

Department of Agriculture, Syrian Atomic Energy Commission, P.O. Box 6091, Damascus, Syria
Previous address: INRA-UBP, UMR 1095, 234 Avenue du Brézet, 63100 Clermont-Ferrand, France

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In order to clarify the role of Pl_{PMI3} resistance gene in sunflower differential lines D4 for differentiation the pathogenicity in *Plasmopara halstedii* (sunflower downy mildew), analyses were carried out in two groups including four pathotypes which overcome and do not overcome Pl_{PMI3} (*Pl* gene has still not been mapped) resistance gene. Based on the reaction for the *P. halstedii* isolates to sunflower hybrids varying only in *Pl* resistance genes, there were no virulence differences for the two groups. Index of aggressiveness was calculated for pathogen isolates and revealed the presence of significant differences between isolates of races 304 and 314; however, there were no aggressiveness differences for 7xx races. Regarding the life-history trait and the genetic architecture of the pathogen: there were morphological and genetic variations for the four *P. halstedii* isolates without a correlation with pathogenic diversity. The importance of the Pl_{PMI3} resistance gene to differentiate the pathogenicity in sunflower downy mildew was discussed.

Keywords: aggressiveness, avirulence *Avr* gene, *Helianthus annuus*, virulence.

Sunflower (*Helianthus annuus* L.) is an important source of vegetable oils and it keeps gaining popularity because of its high oil percentage and quality, short duration and thermophotinsensitiveness. More than 30 diseases have been identified on sunflower (Virányi and Spring, 2011). Among these, downy mildew caused by *Plasmopara halstedii* (Farl.) Berl. and de Toni is the most destructive one (Virányi and Spring, 2011). *P. halstedii* is an obligate endoparasite that cannot be cultivated independently from its plant host. Downy mildew causes dwarfing plants and infertile capitulum which reduces productivity. Two categories of *P. halstedii* resistance exist: Qualitative resistance caused by single major *Pl* loci (Tourvieille de Labrouhe et al., 2000), and quantitative resistance which is controlled by several genes with minor effects (Tourvieille de Labrouhe et al., 2008).

The interactions between sunflower plants and *P. halstedii* on the level of pathogenicity have been the object of several studies: virulence and aggressiveness (Tourvieille de Labrouhe et al., 2000, 2010; Delmotte et al., 2008; Sakr, 2011a, 2011b, 2011c, 2012, 2014a, 2014b, 2014c, 2015a, 2015b; Ahmed et al., 2012). Virulence has been defined as

* e-mails: snachaat@hotmail.com; snachaat@gmail.com

specific disease-causing abilities and aggressiveness as non-specific disease-causing abilities (Van der Plank, 1968). It displays a gene-for-gene interaction with its host plant and shows physiological races (pathotypes) capable of infecting a variable range of sunflower genotypes. Indeed, it has been possible to identify up to 35 races, with different virulence patterns (Delmotte et al., 2008; Ahmed et al., 2012; Sakr, 2014a). To date, the genetic background for avirulence *Avr* genes in *P. halstedii* correspondent to *Pl* sunflower resistance genes has not been investigated (Virányi and Spring, 2011; Sakr, 2015a).

Genes that confer resistance to downy mildew are dominant and often form clusters. *Pl* genes are located on complex loci containing several genes tightly linked. Because of the complexity of the loci, no *Pl* gene has been cloned yet (Miller and Gulya, 1991). The *Pl* genes have been localized on four clusters in sunflower. *Pl1, Pl2, Pl6* and *Pl7* genes are clustered on LG8, *Pl5* and *Pl8* are clustered on LG13, *PlArg* is found on LG1, and two newly mapped genes (*Pl13* and *Pl14*) on LG1 independent of the *PlArg* gene (Radwan et al., 2011).

To date, genetic analyses have still not mapped *Pl* gene in sunflower differential line D4, although this line is used to identify the race in sunflower downy mildew populations. Moreover, pathogenic reaction of Pl_{PMI3} gene to avirulence *Avr* gene in *P. halstedii* races has not been studied. However, in sunflower downy mildew populations, the life-history traits: zoosporangia and sporangiophores size can be modified due to *Avr* gene in *P. halstedii* races that correspond to *Pl* genes (Sakr, 2011c, 2014a). In order to determine the importance of Pl_{PMI3} (*Pl* gene has still not been mapped) resistance gene in D4, phenotypic analyses (morphological and pathogenic) and genetic characteristics were carried out in two groups including four pathotypes which have never been documented outside of France: two isolates of 3xx races in which pathotype 304 do not overcome Pl_{PMI3} gene and 314 one can overcome, and two isolates of 7xx races in which pathotype 704 do not overcome Pl_{PMI3} gene and 714 one can overcome. Hence an attempt was made to generate information about the importance of the Pl_{PMI3} resistance gene to differentiate the pathogenicity in sunflower downy mildew.

Materials and Methods

Oomycete isolates and race identification

The four *P. halstedii* isolates used in this study were collected in France and maintained at INRA, Clermont-Ferrand (Table 1). Manipulation of this quarantine parasite respected European regulations (No 2003/DRAF/70). Pathogen isolates were isolated in 2005 from naturally infected sunflower plants. Their races identity (Table 1) was determined using the method reported by Tourvieille de Labrouhe et al. (2000): DU1767 (race 304); DU1943 (race 314); DU1734 (race 704) and DU1915 (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 20 single zoosporangium isolates. The characteriza-

Table 1

Virulence of four *Plasmopara halstedii* isolates on nine sunflower differential lines

Isolates	Race	Year isolated	Differential lines								
			D1 Ha-304 No *Pl* gene	D2 Rha-265 *Pl1*	D3 Rha-274 *Pl2*	D4 PMI3 Pl_{PM13}	D5 PM-17 *Pl5*	D6 803-1 *Pl5*	D7 HAR-4 Pl_{15}	D8 QHP1 *Pl1*/Pl_{15}	D9 Ha-335 *Pl6*
DU1943	314	2005	S	S	R	S	R	R	R	R	S
DU1767	304	2005	S	S	R	R	R	R	R	R	S
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)

tion of the race for 20 single zoosporangium isolates (Table 2) was determined using the same method adapted in the study by Tourvieille de Labrouhe et al. (2000). There were three replications for each differential line (10 plants in each replication) and the entire experiment was repeated twice for four *P. halstedii* isolates and 20 *P. halstedii* single zoosporangium isolates.

Virulence spectrum for P. halstedii *isolates and single zoosporangium isolates*

To characterize virulence spectrum in *P. halstedii* isolates and single zoosporangium 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 × L2a; H2 = L1a × L2b; H3 = L1b × L2a and H4 = L1b × L2b. Two sunflower lines were also used to analyze virulence spectrum for 19 *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*).

Measurement of aggressiveness in P. hasltedii *single zoosporangium isolates*

To characterize aggressiveness criteria: percentage infection, latent period, sporulation density and reduction of hypocotyl length for *P. halstedii* single zoosporangium isolates (Sakr, 2011a, 2011b, 2012, 2014a, 2014b, 2014c, 2015b), 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 *P. halstedii* single zoosporangium isolate was calculated as the ration of (percentage infection × sporulation density) / (latent period × dwarfing). All the pathogenic tests were carried out in growth chambers regulated at 18 hrs of light, 18 °C ± 1 and RH of 65–90%.

Morphological observations

After 13 days of infection of the sunflower inbred line 'FU', the zoosporangia and sporangiophores suspensions for 20 single zoosporangium isolates were obtained by grouping all sporulated cotyledons in a small container and adding 1 ml of physiological water for each cotyledon (9g 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 × 400) with 2 replications. Zoosporan-

Table 2

Virulence of 24 *Plasmopara halstedii* isolates and single zoosporangium isolates on four sunflower hybrids differing only in their downy mildew resistance genes

Isolates	Race	Year isolated	H1	H2	H3	H4	XRQ *Pl5*	RHA340 *Pl8*
DU1943	314	2005	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	2006	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	304	2005	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	2006	R	R	R	R	R	R
DU1767 M5	304	2006	R	R	R	R	R	R
DU1915	714	2005	S	S	S	S	R	R
DU1915 M1	714	2006	S	S	S	S	R	R
DU1915 M2	714	2006	S	S	S	S	R	R
DU1915 M3	714	2006	S	S	S	S	R	R
DU1915 M5	714	2006	S	S	S	S	R	R
DU1915 M6	714	2006	S	S	S	S	R	R
DU1734	704	2005	S	S	S	S	R	R
DU1734 M1	704	2006	S	S	S	S	R	R
DU1734 M2	704	2006	S	S	S	S	R	R
DU1734 M3	704	2006	S	S	S	S	R	R
DU1734 M7	704	2006	S	S	S	S	R	R
DU1734 M8	704	2006	S	S	S	S	R	R

gia 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 $\times 400$) with 2 replications.

DNA extraction and molecular typing

The 12 EST-derived markers were used because the other molecular markers were non-specific, insufficiently polymorphic within *P. halstedii* and no genetic structure in *P. halstedii* populations was identified by using these markers (Delmotte et al., 2008). For 20 single zoosporangium isolates tested, DNA was isolated from infected plant tissue then the 12 polymorphic EST-derived markers (Delmotte et al., 2008) were used to genotype *P. halstedii* single zoosporangium isolates. The polygenetic relations between the 20 isolates were obtained by building a Neighbour-joining (NJ) tree (Jin and Chakraborty, 1993) using Populations 1.2.28 Software (Librado and Rozas, 2009). A Bootstrap analysis was performed on 10.000 replicates.

Statistical analyses

All statistical analyses were performed using StatBox 6.7® (GimmerSoft) software. Before statistical analysis, the percentages were transformed using the Arcsines function. The values obtained were submitted to a one-way analysis of variance (ANOVA). The Newman–Keuls test (Snedecor and Gochran, 1989) was used to compare the means at $P = 0.05$.

Results

Analysis of virulence spectrum

Table 2 shows that all sunflower hybrids were resistant to isolates of two races 314 and 304, and sensitive to isolates of races 714 and 704. Moreover, the two sunflower inbred lines XRQ and RHA340 were resistant to all *P. halstedii* isolates tested.

Analysis of aggressiveness criteria
Percentage infection

Intra-isolate variability (Table 3): Few plants escaped infection, for races 3xx (11 out of 1800 plants), and for races 7xx (55 out of 1800 plants), but these very high levels of infection (98%–100%) for pathotypes 3xx, and (95%–100%) for pathotypes 7xx showed differences between single zoosporangium isolates. All pathogen isolates were uniform for the criterion "percentage of infection".

Enter-isolates variability (Table 3): The analysis of variance indicated no significant differences for 3xx pathotypes (Probability = 0.2774; F-test = 1.358), and for 7xx pathotypes (Probability = 0.3598; F-test = 0.944).

Table 3

Aggressiveness within and among pathogen isolates for 20 *Plasmopara halstedii* single zoosporangium isolates measured on the sunflower inbred line 'FU'

Single zoo-sporangium isolates	Race	Percentage infection	Latent period	Sporulation density	Hypocotyl length	Index of aggressiveness
		Mean (%)	Mean (days)	Mean (10^5 zoosporangia per cotyledon)	Mean (mm)	
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%	
		$*P = 0.2774$ ns	$*P = 0.5019$ ns	$*P = 0.5911$ ns	$*P = 0.0007$	$*P = 0.0008$
		$*F = 1.358$	$*F = 0.494$	$*F = 0.313$	$*F = 28.164$	$*F = 27.804$
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 = 2.00%	VC = 1.51%	VC = 5.50%	VC = 1.69%	
		$*P = 0.3598$ ns	$*P = 0.6262$ ns	$*P = 0.2609$ ns	$*P = 0.8880$ ns	$*P = 0.2800$ ns
		$*F = 0.944$	$*F = 0.257$	$*F = 1.463$	$*F = 0.021$	$*F = 1.342$

According to the Newman–Keuls test, means followed by the same letter are not significantly different at $P = 0.05$, ns = not significant, Probability (P), Variation Coefficient (VC), index of aggressiveness = (percentage infection × sporulation density) / (latent period × dwarfing).
Probability ($*P$) and F isolates (*F) were presented for among isolates variation

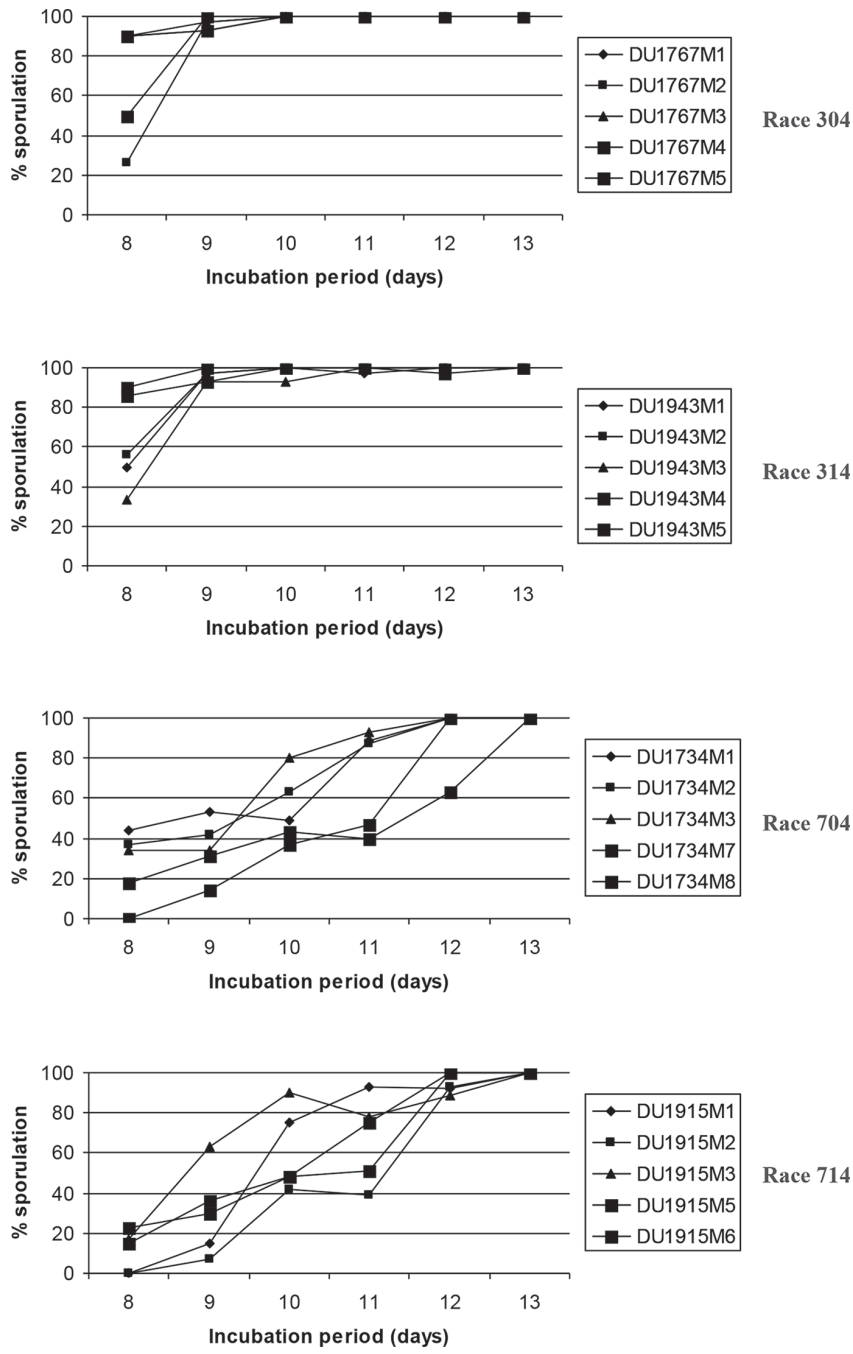

Fig. 1. Sporulation of 20 single zoosporangium *Plasmopara halstedii* isolates of five races on the sunflower inbred line 'FU', based on incubation period

Latent period

Intra-isolate variability (Table 3): The differences were highly significant for the two groups which include the four used pathotypes. Analysis of the relation between sporulation percentage based on incubation period (Fig. 1) showed differences in behaviour among *P. halstedii* single zoosporangium isolates. There were two main groups from day 8 onwards: single zoosporangium isolates of *P. halstedii* isolates DU1943 and DU1767 sporulated faster than single zoosporangium isolates of *P. halstedii* isolates DU1915 and DU1734. All infected plants with single zoosporangium isolates of races 3xx showed more than 80% sporulation 9 days after incubation, whereas pathotypes 7xx needed 11 days after incubation to reach the same level of sporulation.

Enter-isolates variability (Table 3): The analysis of variance indicated no significant differences for 3xx pathotypes (Probability = 0.5019; F-test = 0.494), and for 7xx pathotypes (Probability = 0.6262; F-test = 0.257).

Sporulation density

Intra-isolate variability (Table 3): Only single zoosporangium isolates for races 314 and 714 showed variation for sporulation density. Table 2 revealed that the single zoosporangium isolate DU1943M4 showed a significant difference, producing more than 18×10^5 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).

Figure 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* isolates DU1943 and DU1767 produced more zoosporangia than single zoosporangium isolates of pathogen isolates DU1915 and DU1734 (Fig. 2). The quantity of zoosporangia produced was at a maximum 12 days after incubation.

Enter-isolates variability (Table 3): The analysis of variance indicated no significant differences for 3xx pathotypes (Probability = 0.5911; F-test = 0.313), and for 7xx pathotypes (Probability = 0.2609; F-test = 1.463).

Reduction of hypocotyl length

Intra-isolate variability (Table 3): 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.

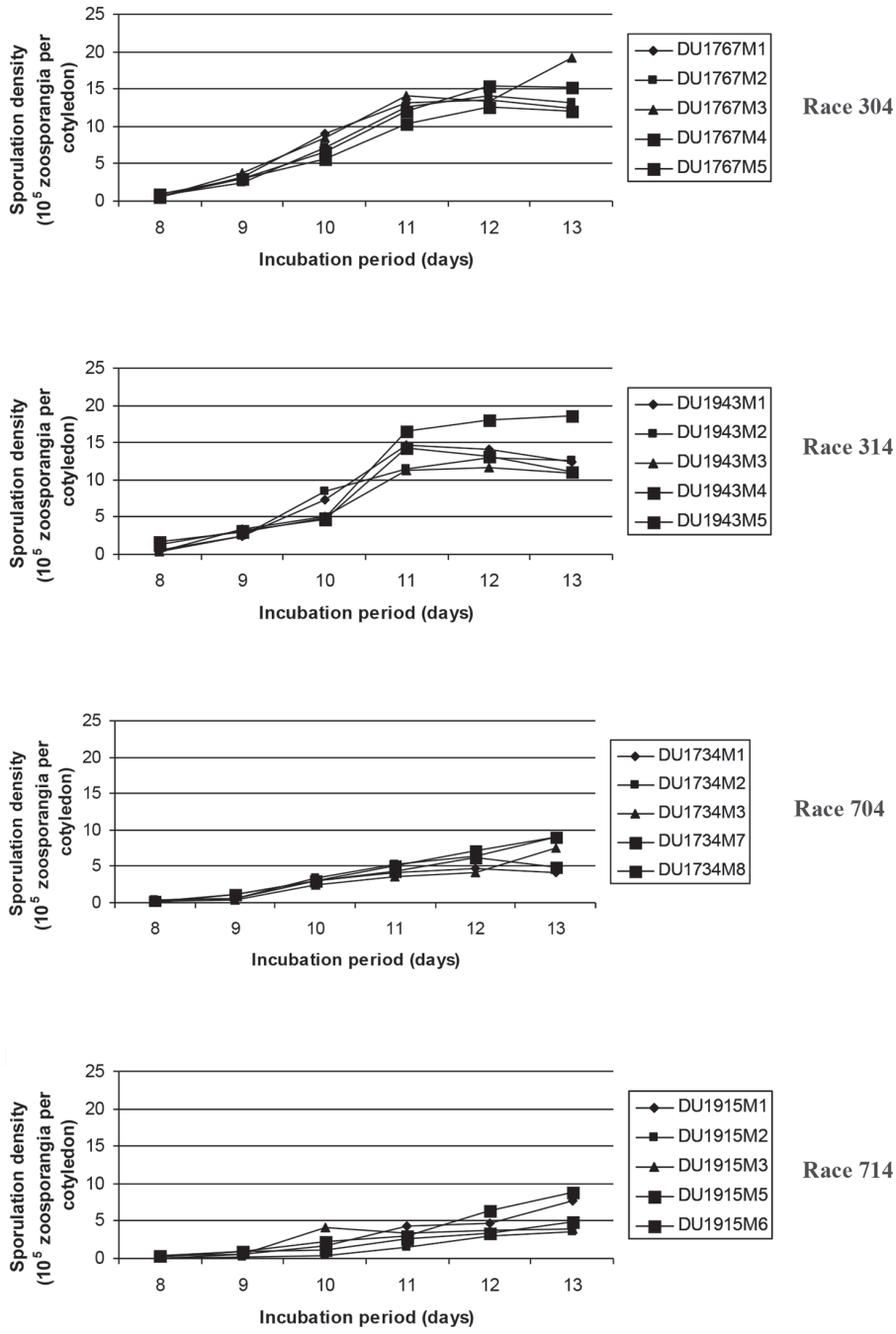

Fig. 2. Sporulation density of 20 single zoosporangium *Plasmopara halstedii* isolates of five races on sunflower inbred line 'FU', based on incubation period

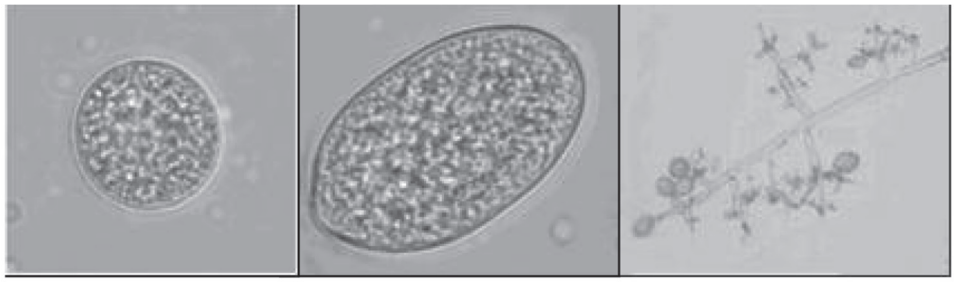

Fig. 3. *Plasmopara halstedii* zoosporangia forms and sporangiophores observed on sunflower inbred line 'FU': round (left), oval (center) and sporangiophore (right)

Enter-isolates variability (Table 3): The analysis of variance indicated significant differences for 3xx pathotypes (Probability = 0.0007; F-test = 28.164), and no significant differences for 7xx pathotypes (Probability = 0.8880; F-test = 0.021).

For 3xx pathotypes, the index of aggressiveness varied 2.0: 3.4 for DU1943M5 and 7.3 for DU1842M5 (Table 3). There were significant differences (Probability = 0.0008; F-test = 27.804) for single zoosporangium isolates of races 3xx. For 7xx pathotypes, the index of aggressiveness varied 3.0: 1.0 for DU1915M2 and 2.9 for DU1915M2 (Table 3). There were no significant differences (Probability = 0.2800; F-test = 1.342) for single zoosporangium isolates of races 7xx.

Morphology of zoosporangia and sporangiophores

The results showed that the two most observed forms of zoosporangia were oval and round (Fig. 3). All morphological data were presented in (Table 4). The proportion of oval form varied from 56% to 93% and the zoosporangia size from 302.2 to 505.2 μm^2. The proportion of oval zoosporangia varied within the races, for example for race 314 it ranged from 56% to 93%, for race 304 it ranged from 63% to 91%, for race 714 it ranged from 86% to 93% and for race 704 it ranged from 68% to 89%. Mean sporangiophore length was the highest in DU1767M3 isolate (800.0 μm). The sporangiophore length ranged between 299.0 and 800.0 μm. Mean sporangiophore width was the largest in DU1767M1 isolate. The sporangial width varied from 5.0 μm to 16.0 μm.

Molecular analysis

The combination of 12-EST derived markers revealed four multilocus genotypes (MLG) among 20 *P. halstedii* single zoosporangium isolates (Table 5). There was no *intra-race* genetic variation for all pathotypes tested. Single zoosporangium isolates of four races were similar only for three genomic markers *Pha39*, *Pha54* and *Pha79*. The Neighbour-joining tree showed that isolates DU1915 and DU1734 had an intermediary genetic position between isolates DU1767 and DU1943 (Fig. 4). Consequently, there were four

genetically-identified groups among *P. halstedii* single zoosporangium isolates tested: group of isolates of race 304, group of isolates of race 714, group of isolates of race 704 and group of isolates of race 314 (Table 5 and Fig. 4).

Discussion

There are currently 18 resistance genes in use, sought from wild *Helianthus*, some of which are believed to be tightly linked in at least two different linkage groups

Table 4

Morphological characters of zoosporangia and sporangiophores obtained on sunflower genotype FU for 20 isolates of *Plasmopara halstedii*

Isolates of *P. halstedii*	Race	% of oval zoosporangia[a]	Size of zoosporangia in μm^2 [b]	Sporangiophore length (μm)[c]	Sporangiophore Width (μm)[d]
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
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
Probability		0.0003	0.0001	0.0001	0.9853 ns
F isolates		5.163	312.86	816.325	0.358

[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), Probability (P = 0.05)

Table 5

Multilocus genotypes (MLG) characterized using 12 EST-derived genomic markers on 20 isolates of *Plasmopara halstedii*

Isolates of *P. halstedii*	EST-derived markers											
	Pha6	*Pha39*	*Pha42*	*Pha43*	*Pha54*	*Pha56*	*Pha74*	*Pha79*	*Pha82*	*Pha99*	*Pha106*	*Pha120*
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
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

The values of two figures indicate to the alleles of seven Plasmopara halstedii isolates for 12 EST-derived genomic markers. For each isolate, the race and the two alleles at each derived genomic marker were indicated

(Vear et al., 1997, 2003). Dominance in the pathogen avirulence genes is not known, but by analogy with other plant-pathogen species they are probably recessive (Stukenbrock and McDonald, 2008). However, in sunflower downy mildew, there are no studies about the genetic background for avirulence *Avr* genes correspondent to *Pl* resistance genes (Virányi and Spring, 2011; Sakr, 2015a). With bearing this in mind, analyses were carried out in two groups which include four pathotypes similar in reaction for all sunflower differential lines except for D4 carrying Pl_{PMI3} (*Pl* gene has still not mapped).

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 there were no virulence differences for the two groups of four races (304 and 314; 704 and 714). Race 314 is more virulent than race 304, and race 714 is more virulent than race 704 according to the method adapted by Tourvieille de Labrouhe et al. (2000) to identify virulence in sunflower downy mildew populations. It seems that avirulence pathogen gene corresponds to Pl_{PM13} resistance gene does not contribute in increasing the

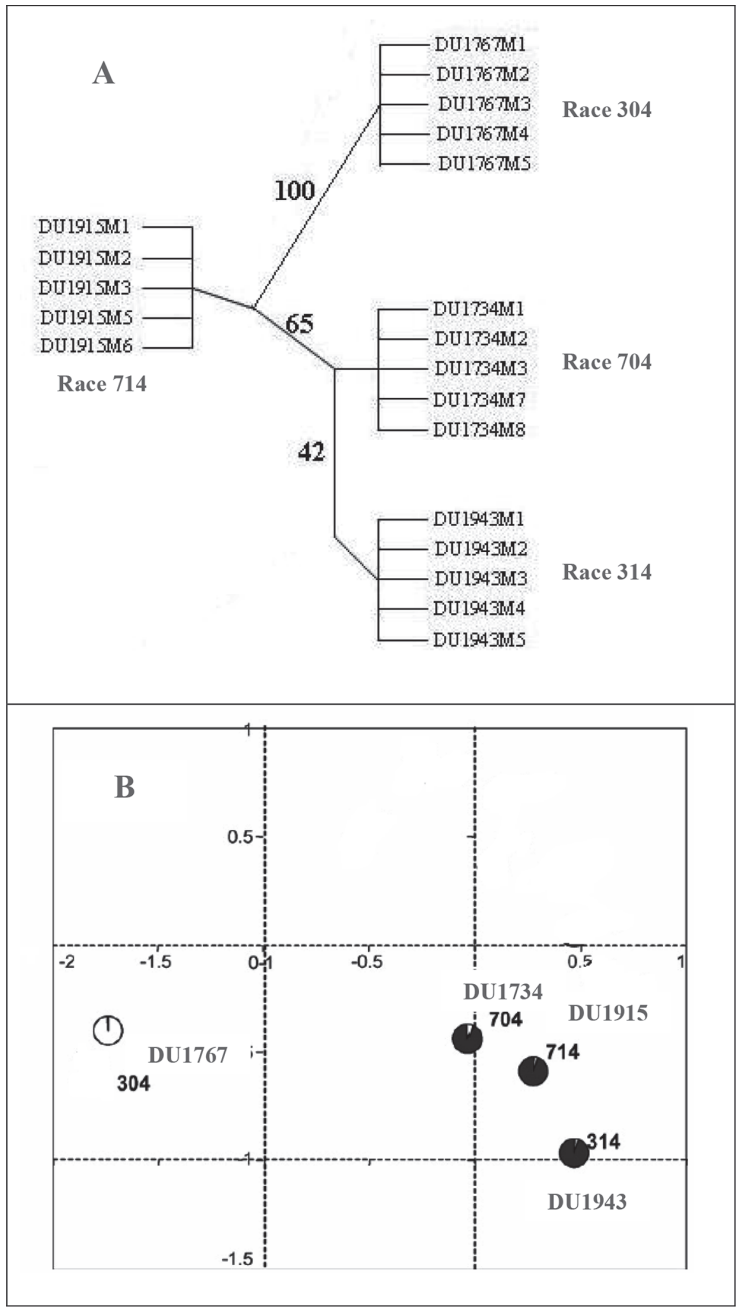

Fig. 4. A: Phylogenetic tree according to Neighbour-joining analysis of 12 EST-derived markers. Figures on branches indicate bootstrap values (10.000 replicates).
B: Multilocus genotypes of *P. halstedii* races based on 12 EST-derived markers

level of virulence in *P. halstedii*. 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).

High percentage infection, short latent period, high sporulation density, and significant reduction in the length of the hypocotyl represent high aggressiveness (Sakr, 2011a, 2011b, 2012, 2014a, 2014b, 2014c, 2015b). The frequency of sporulated plants based on incubation period reflected the speed of appearance of symptoms on the plants (Fig. 1) (latent period), and the number of zoosporangia produced by cotyledons reflected the level of invasion of infected tissues (Fig. 2). Although the index of aggressiveness can mask the type of resistance, for example, DU1943 M5 has significantly shorter latent period, but also significantly less sporulation density than DU1943 M4. However, in the current study, the index of aggressiveness was used to summarize all values for criteria on sunflower inbred line into one value to facilitate the comparison between the different *P. halstedii* single zoosporangium isolates (Sakr, 2014a). 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 3). 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.

For 3xx races, our results show that there were significant differences among pathogen isolates only for reduction of hypocotyl length. Single zoosporangium isolates of DU1943 gave the least reduction compared with single zoosporangium isolates of DU1767. The presence of significant differences among isolates of 3xx races is due to one aggressiveness criterion (dwarfing). Dwarfing is a symptom characteristic of plants systemically infected by *P. halstedii* and is explained by a decrease in the concentration of growth hormone (Iodole Acetic Acid) in infected tissue (Tourvieille de Labrouhe et al., 2000). It seems that isolate of 314 stimulates to produce smaller quantities of growth hormone than isolate of race 304. Consequently, isolate of race 304 was more aggressive than isolate of race 314. For 7xx races, our results show that there were no significant differences among pathogen isolates for all aggressiveness criteria. Two hypotheses could explain that variability was not established between pathogen isolates used in this study (Table 3). First, it seems that races 714 and 704 emerged from the evolution of race 710 regarding only the aggressiveness (Sakr, 2014c). Second, these isolates belong to 7xx races that accumulate a large number of virulence genes and might never show differences for aggressiveness on sunflower genotypes (Sakr, 2012, 2014a).

We still have little data on the evolution of life-history traits of plant pathogens and especially on the potential trade-off between fitness components that could limit their evolutionary potential (Lannou, 2012). However, a recent study has demonstrated that trade-offs do exist in plant pathogens, as found between latency period and spore production capacity in wheat brown rust (Pariaud et al., 2012). However, for another Oomycete *Plasmopara viticola*, Delmotte et al. (2014) found evidence for a trade-off between the

size and the number of sporangia produced within *P. viticola*. In the same pathosystem, Sakr (2011c) found a relationship between a life-history trait (viability of zoosporangia) and aggressiveness in *P. halstedii*. Table 4 showed that there were significant morphological differences for 20 pathogen single zoosporangium isolates in two groups including four isolates DU1943 and DU1767, DU1915 and DU1734. The proportion of zoosporangia of different forms and their sizes and the morphology of sporangiophores do not appear to be useable to differentiate 20 single zoosporangium isolates of four races 314, 304, 714 and 704 as defined by Tourvieille de Labrouhe et al. (2000). The results also showed that zoosporangia morphology did not distinguish single zoosporangium isolates according to their aggressiveness (Table 3).

There was no *intra-race* genetic variation (Table 5), but four genetically-identified groups were detected among *P. halstedii* single zoosporangium isolates of three races (Fig. 4). Delmotte et al. (2008) and Ahmed et al. (2012) grouped race 304 a genetic clade and race 314 in another cluster; however, this association was identified in the present work. Delmotte et al. (2008) and Ahmed et al. (2012) grouped races 704 and 714 together in the same genetic clade; however, this association was not identified in the present work and in data by Sakr (2011a, 2014b, 2014c). Our results underlined non correlation between EST genotypes (Table 5 and Fig. 4) and pathogenic traits (Tables 2 and 3).

Conclusion

The current study helps us to underline the role of Pl_{PMI3} (*Pl* gene has still not been mapped) in differentiation the pathogenicity in *P. halstedii*. Regarding avirulence *Avr* gene correspondent to Pl_{PMI3} gene in the two groups of four *P. halstedii* races (304 and 314; 704 and 714), it seems that *Avr* gene stimulates no virulent spectrum, no aggressiveness variation for 7xx races, and significant differences in races 304 and 314. There was phenotypic variation (morphological and genetic characteristics) for two groups of four *P. halstedii* races without a correlation with pathogenic diversity. It will be necessary to analyze avirulence *Avr* genes in on a large collection of *P. halstedii* isolates to provide a better insight into interactions between this obligate parasite and its host.

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