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Pl2 resistance gene differentiates the pathogenicity in four *Plasmopara halstedii* (sunflower downy mildew) races 304, 704, 314 and 714

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Abstract: Sakr, N. 2015. *Pl2* resistance gene differentiates the pathogenicity in four *Plasmopara halstedii* (sunflower downy mildew) races 304, 704, 314 and 714. *Bot. Complut*. 39: 19-29.

In order to clarify the role of *Pl2* resistance gene in differentiation the pathogenicity in *Plasmopara halstedii* (sunflower downy mildew), analyses were carried out in four pathotypes: isolates of races 304 and 314 that do not overcome *Pl2* gene, and isolates of races 704 and 714 that can overcome *Pl2* gene. Based on the reaction for the *P. halstedii* isolates to sunflower hybrids varying only in *Pl* resistance genes, isolates of races 704 and 714 were more virulent than isolates of races 304 and 314. Index of aggressiveness was calculated for pathogen isolates and revealed the presence of significant differences between isolates of races 304 and 314 (more aggressive) and isolates of races 704 and 714 (less aggressive). There were morphological and genetic variations for the four *P. halstedii* isolates without a correlation with pathogenic diversity. The importance of the *Pl2* resistance gene to differentiate the pathogenicity in sunflower downy mildew was discussed.

Key words: aggressiveness, avirulence *Avr* gene, *Helianthus annuus*, virulence.

Resumen: Sakr, N. 2015. El gen de la resistencia *Pl2* diferencia la patogenicidad en las razas 304, 704, 314 y 714 de *Plasmopara halstedii* (mildiu del girasol). *Bot. Complut*. 39: 19-29.

Con objeto de clarificar la acción del gen de la resistencia *Pl2* en la diferenciación de la patogenicidad en *Plasmopara halstedii* (mildiu del girasol) se han realizado análisis en cuatro patotipos: aislados de las razas 304 y 314 que no superan el gen *Pl2*, y aislados de las razas 704 y 714 que pueden superar al gen *Pl2*. Basados en la reacción de los aislados de *P. halstedii* sobre los híbridos de girasol que solo varían en la resistencia a los genes *Pl*, los aislados de las razas 704 y 714 fueron más virulentos que los aislados de las razas 304 y 314. Se ha calculado un índice de agresividad para los aislados patógenos de las razas 304 y 314 (más agresivos) y los aislados 704 y 714 (menos agresivos). Se han encontrado diferencias morfológicas y variaciones genéticas para los cuatro aislados de *P. halstedii* pero sin correlación con la diversidad patogénica. Se discute la importancia de la resistencia del gen *Pl2* para diferenciar la patogenicidad en el mildiu del girasol.

Palabras clave: agresividad, gen de avirulencia *Avr*, *Helianthus annuus*, virulencia.

INTRODUCTION

Downy mildew is one of the major diseases for sunflower cultivation (*Helianthus annuus* L.). The causal agent is *Plasmopara halstedii*, an obligate biotroph oomycete from the *Peronosporaceae* family, diploid, homothallic, and can reproduce sexually and asexually. This disease is found in most parts of the world where the crop is cultivated and where a co-evolution between *H. annuus* and *P. halstedii* has taken place. Downy mildew causes dwarfing plants and infertile capitulum which reduces productivity (Viranyi & Spring 2011). 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).

P. halstedii is characterized by a high level of evolutionary potential expressed by high virulence, aggressiveness and a great potential in developing new races (Tourvieille de Labrouhe *et al*. 2000, 2010; Delmotte *et al.* 2008, Sakr 2011, 2012, 2014; Ahmed *et al.* 2012). Virulence has been defined as 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 (pathoty-

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pes) capable of infecting a variable range of sunflower genotypes. It must be pointed out that all these races are characterized upon the phenotypes (resistant or susceptible) of a set of differential sunflower lines carrying different *Pl* genes (Tourvieille de Labrouhe *et al.* 2000). Indeed, it has been possible to identify up to 35 races, with different virulence patterns (Delmotte *et al.* 2008, Ahmed *et al.* 2012, Sakr 2014). Fourteen different reference races of this pathogen have now been characterized in France, nine of which emerged in the last ten years. Eight of the races (100, 703, 710, 300, 700, 707, 717, and 730) are also found in other countries, but the six endemic races (304, 307, 314, 334, 704, and 714) provide evidence for evolution *in situ* (Delmotte *et al.* 2008, Ahmed *et al.* 2012, Sakr 2014). To date, the genetic background for avirulence *Avr* genes in *P. halstedii* correspondent to *Pl* sunflower resistance genes has not been investigated (Viranyi & Spring 2011).

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 & 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). Concerning the importance of *Pl* genes, *Pl2* gene has been the object of several studies. Mouzeyar *et al.* (1993) observed in histological studies, that incompatible interaction *H. annuus/P. halstedii* is due to a hypersensitive reaction for *Pl2*. Vear *et al.* (1997) mapped *Pl2* and found that its locus is located in LG8. Furthermore, *Pl2* resistance gene is mainly used in breeding material (Vear *et al.* 2003). Recently, Sakr (2012) reported that sunflower differential line D3 (RHA-274) carrying *Pl2* gene is important for studying virulence cost in *P. halstedii*. In this study, phenotypic analyses (morphological, pathogenic and genetic characteristics) were carried out in four pathotypes which have never been documented outside of France: two isolates of races 304 and 314 that do not overcome *Pl2* gene, and isolates of races 704 and 714 that can. Hence an attempt was made to generate information about the importance of the *Pl2* 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): DU 1767 (race 304); DU 1943 (race 314); 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 20 single zoosporangium isolates. The characterization 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

Table 1

Virulence of four *Plasmopara halstedii* isolates on nine sunflower differential lines. **S**: susceptible, sporulation on cotyledons. **R**: resistant, no sporulation. ***Pl*(?)**: *Pl* gene not identified, data from Tourvieille de Labrouhe *et al.* (2000)

Isolates	Race	Year Isolated	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*
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

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 isolates	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

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 agressiveness in *P. halstedii* single zoosporangium isolates. To characterize aggressiveness criteria: percentage infection, latent period, sporulation density and reduction of hypocotyl length for *P. halstedii* single zoosporangium isolate (Sakr 2011, 2012, 2014), 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* 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 18hrs 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 + 1L 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. Zoosporangia size was calculated from an oval $\pi \times a \times b$, $a = {}^1/_2$ length, $b = {}^1/_2$ 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. The 12 EST- derived markers were sequenced by Giresse *et al*. (2007). In the current study, these markers were used because the other molecu-

lar markers were non-specific, insufficiently polymorphic within *P. halstedii*, and no genetic structure in *P. halstedii* populations was identified by using these markers (Giresse *et al*. 2007). GenBank accession numbers for 12 EST-derived genomic markers (Giresse *et al*. 2007) were presented as following: *Pha6* CB174585, *Pha39* CB174648, *Pha42* CB174650, *Pha43* CB174680, *Pha54* CB174708, *Pha56* CB174714, *Pha74* CB174642, *Pha79* CB174692, *Pha82* CB174573, *Pha99* CB174703, *Pha106* CB174676, and *Pha120* CB174660. For 20 single zoosporangium isolates tested, DNA was isolated from infected plant tissues, the 12 polymorphic EST-derived markers were used to genotype *P. halstedii* single zoosporangium isolates. The polygenetic relations between the 15 isolates were obtained by building a Neighbour-Joining (NJ) tree (Jin & Chakraborty 1993) using Populations 1.2.28 Software (Librado & Rozas 2009). A Bootstrap analysis was performed on 10.000 replicates.

Statistical analysis. All statistical analyses were performed using StatBox 6.7® (GimmerSoft) software. Before statistical analysis, the percentages were transformed using the Arcsines function. A normality test showed that the transformed variables were normaly distributed, so 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 $P = 0.05$.

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 (89 out of 3750 plants) but these very high levels of infection (95%-100%) showed differences between single zoosporangium isolates. All pathogen isolates were uniform for the criterion «percentage infection». *Enter-isolates* variability (Table 4): the analysis of variance indicated highly significant differences ($p = 0.0005$; F-test = 10.230). The Newman-Keuls test showed that the pathogen isolates formed two very distinct groups. The first group containing the isolates DU 1767 and DU 1943 showed a higher infection level than the second group containing the isolates DU 1734 and DU 1915.

Latent period. *Intra-isolate* variability (Table 3): although the differences were highly significant for the four isolates of races 314, 304, 704 and 714, they were small for those with a short latent period (DU 1943 and DU 1767). Deviations were slightly larger for the isolates with the longest incubation periods DU 1915 and DU 1734. Analysis of the relation between sporulation percentages 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 zoospo-

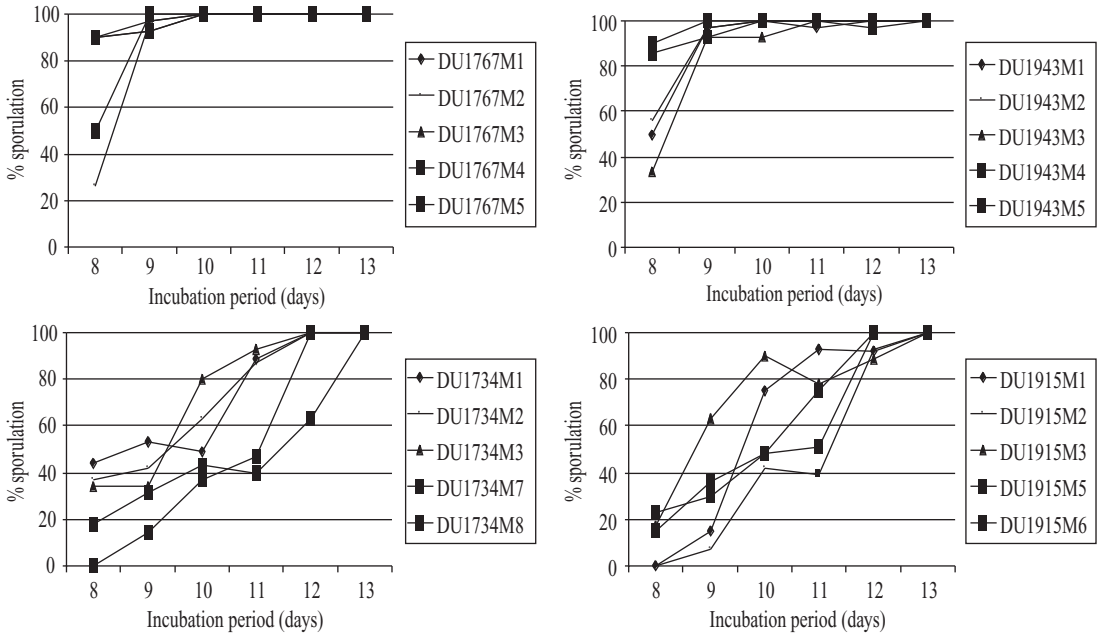

Fig. 1— Sporulation of 20 single zoosporangium *Plasmopara halstedii* isolates of four races on the sunflower inbred line 'FU', based on incubation period.

Table 3
Aggressiveness within pathogen isolate for 20 *Plasmopara halstedii* single zoosporangium isolates measured on the sunflower inbred line 'FU'. According to the Newman-Keuls test, means followed by the same letter are not significantly different at $P = 0.05$, ns = not significant, ***P***: probability, **VC**: variation coefficient. Index of aggressiveness = (percentage infection × sporulation density) / (latent period × dwarfing) (Sakr 2011, 2012, 2014)

Zoosporangium isolates	Race	Percentaje infection Mean (%)	Latent period Mean (days)	Sporulation density Mean (10^5 zoosporangia cotyledon)	Hypocotyl length Mean (mm)	Index of aggressiveness
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%	
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%	

rangium isolates of *P. halstedii* isolates DU 1943 and DU 1767 sporulated faster than single zoosporangium isolates of *P. halstedii* isolates DU 1915 and DU 1734. 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 4): there were highly significant differences between *P. halstedii* isolates ($p = 0.0001$; F-test = 38.928). The Newman-Keuls test classified the isolates into two distinct groups. Those with the shortest length of latent period (< 9 days) were the isolates DU 1767 and DU 1943. The isolates DU 1734 and DU 1915 were grouped together and showed longer latent periods (> 10 days).

Sporulation density. *Intra-isolate* variability (Table 3): only single zoosporangium isolates for races 314 and

Table 4

Mean and standard deviation of aggressiveness among four *Plasmopara halstedii* isolates (five replications per pathogen isolate that correspond to five single zoosporangium isolates) on the sunflower inbred line 'FU'. According to the Newman-Keuls test, means followed by the same letter are not significantly different at $P = 0.05$), index of aggressiveness = (percentage infection × sporulation density) / (latent period × dwarfing) (Sakr 2011, 2012, 2014)

Isolates	Percentage infection (%)	Latent period (days)	Sporulation density (10^5 zoosporangia per cotyledon)	Hypocotyl length (mm)	Idex of aggressiveness
DU 1943 (race 314)	99.3 a ± 0.6	8.42 b ± 0.35	13.53 a ± 2.46	40.6 a ± 3.1	3.58
DU 1767 (race 304)	99.8 a ± 0.4	8.25 b ± 0.34	14.10 a ± 1.46	28.9 b ± 3.2	6.1
DU 1915 (race 714)	95.2 b ± 2.3	11.06 a ± 0.62	5.02 b ± 1.62	27.7 b ± 1.4	1.58
DU 1734 (race 704)	96.4 b ± 1.3	11.29 a ± 0.69	6.31 b ± 1.39	27.9 b ± 1.7	2.0
Probability	0.0005	0.0001	0.0001	0.001	0.0001
F isolates	10.230	38.928	30.332	25.683	46.566

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). 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* isolates DU 1943 and DU 1767 produced more zoosporangia than single zoosporangium isolates of pathogen isolates DU 1915 and DU 1734 (Fig. 2). The quantity of zoosporangia produced was at a maximum 12 days after incubation. *Enter-isolates* variability (Table 4): there were large differences between the isolates; sporulation density varied from 5×10^5 zoosporangia per ml for DU 1915 to 14×10^5 for DU 1842. Differences were highly significant ($p = 0.0$; F-test = 30.332). The Newman-Keuls test classified the isolates into two very distinct groups. The

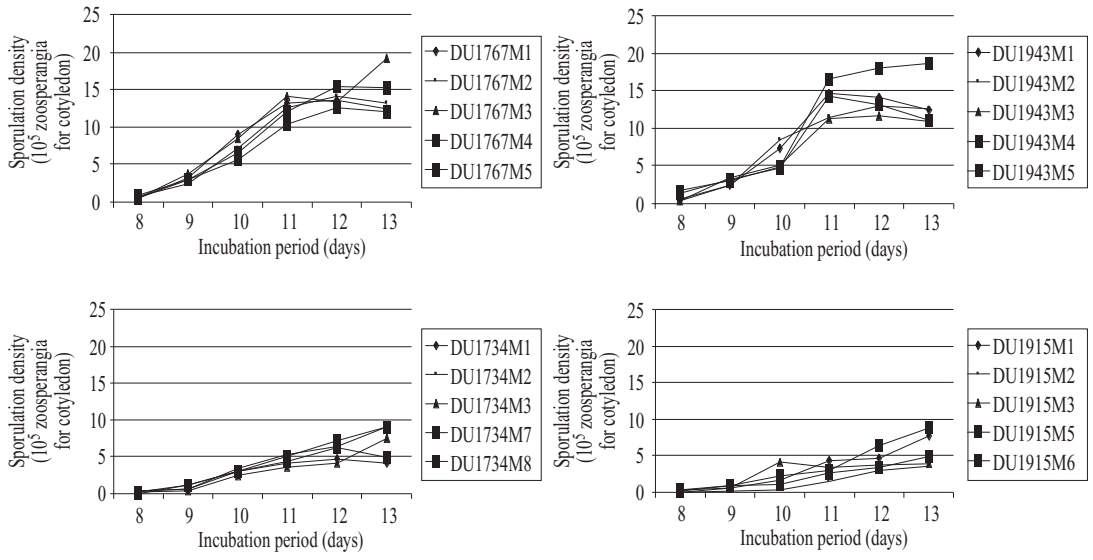

Fig. 2— Sporulation density of 20 single zoosporangium *Plasmopara halstedii* isolates of four races on sunflower inbred line 'FU', based on incubation period.

isolates DU 1734 and DU 1915 showed the lowest sporulation density (< 7×10^5 zoosporangia per cotyledon). Isolates DU 1767 and DU 1943 showed the highest sporulation density (< 13×10^5 zoosporangia per cotyledon).

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. Plants diseased 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. *Enter-isolates* variability (Table 4): The analysis of variance with five replications per pathogen isolate corresponding to the five single zoosporangium isolates showed highly significant differences between pathogen isolates ($p = 0.001$; F-test = 25.683). However, these results were mainly due to pathogen isolate DU 1943 which caused less reduction in hypocotyl length than the other three pathogen isolates.

The index of aggressiveness varied 7.0: 1.0 for DU1915M5 and 6.8 for DU1767M4 (Table 3). There were significant differences ($p = 0.0001$, F-test = 46.566) for single zoosporangium isolates of four races (Table 4). Isolates DU 1767 (race 304) and DU 1943 (race 314) were more aggressive with an index of 5.9 and 3.58 respectively, than isolates DU 1734 (race 704) and DU 1915 (race 714) with an index of 2 and 1.58 respectively.

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 5. 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 İm). The sporangiophore length ranged between 299.0 and 800.0 İm. 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 haplotypes (MLH) among 20 *P. halstedii* single zoosporangium isolates (Table 6). 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 DU 1915 and DU 1734 had an intermediary genetic position between isolates DU 1767 and DU 1943 (Fig. 4).

DISCUSSION

For the first time, Flor (1971) propagated the gene-for-gene resistance concept and observing specificity between single host resistance *(R)* genes and single pathogen avirulence (*Avr*) genes. However, in sunflower downy mildew, there are no studies about the genetic background for avirulence *Avr* genes correspondent to *Pl* resistance genes (Viranyi & Spring 2011). Since tools for analyzing pathogenicity of obligate parasite *Plasmopara halstedii* are very limited, it appears desirable to use sunflower resistance genes to better analyze the differentiation of virulence and aggressiveness. With bearing this in mind, two isolates of races (304, 704) and two isolates of races (314 and 714) similar in reaction for all sunflower differential lines except for D3 carrying *Pl2* gene were used.

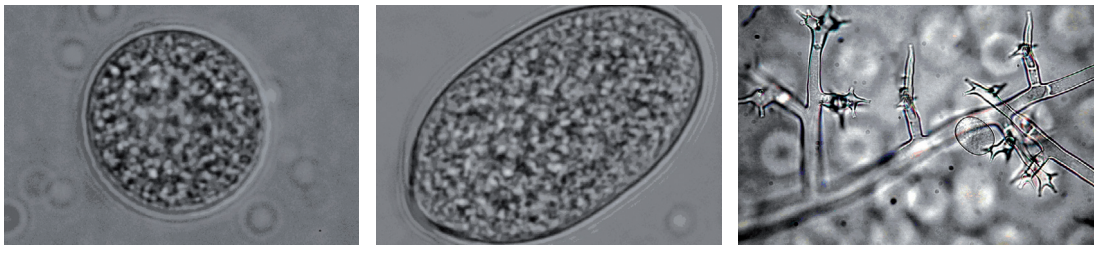

Fig. 3– *Plasmopara halstedii* zoosporangia forms and sporangiophores observed on sunflower inbred line 'FU': round (left), oval (center) and sporangiophore (right).

Table 5
Morphological characters of zoosporangia and sporangiophores obtained on sunflower genotype FU for 20 isolates of *Plasmopara halstedii.* [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 ($P = 0.05$)

Isolates	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

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 single zoosporangium isolates and isolates of races 314 and 304, as those sunflower hybrids carrying effective *Pl* genes were resistant to the two races 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). 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 (Table 2) confirmed that isolates of races 714 and 704 were more virulent than isolates of races 314 and 304. In accordance with our data, Sakr (2011, 2012, 2014) reported that isolates of races 7xx more virulent than isolates of races 3xx using sunflower differential line D3 (carries *Pl2* gene) for virulence reaction.

Table 6
Multilocus haplotypes (MLH) characterized using 12 EST-derived genomic markers on 20 isolates of *Plasmopara halstedii*

Isolates	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

High percentage infection, short latent period, high sporulation density, and significant reduction in the length of the hypocotyl represent high aggressiveness (Sakr 2011, 2012, 2014). 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). 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. Our results show that there were significant differences among pathogen isolates for all aggressiveness criteria. Indeed, the index of aggressiveness revealed the presence of significant differences between the isolates of races 304 and 314 and isolates of races 704 and 714 (Table 4). These results are comparable with those found by Sakr (2011, 2012, 2014), who reported that isolates of races 3xx more aggressive than isolates of races 7xx. Our results (Tables 2 and 4) confirmed that isolates of races 714 and 704 were more virulent and less aggressive than isolates of races 314 and 304.

Table 5 showed that there were significant morphological differences for 20 pathogen single zoosporangium isolates of four isolates DU 1943 and DU 1767, DU 1915 and DU 1734. 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 three races 300, 314 and 304 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 6), but four genetically-identified groups were detected among 20 *P. halstedii* single zoosporangium isolates of four races: 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 6, Fig. 4). However, the present association was not identified in the previous works (Del-

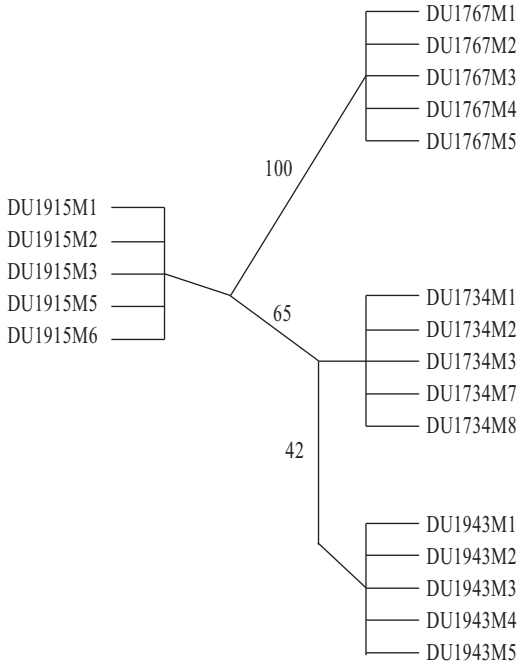

Fig. 4— Phylogenetic tree according to Neighbour-Joining analysis of 12 EST-derived markers. Figures on branches indicate bootstrap values (10.000 replicates). The 20 *Plasmopara halstedii* single zoosporangium isolates used in this study belong to races: DU 1767 (race 304); DU 1943 (race 314); DU 1734 (race 704) and DU 1915 (race 714).

motte *et al.* 2008, Ahmed *et al.* 2012). Indeed, Delmotte *et al.* (2008) and Ahmed *et al.* (2012) grouped race 304 in a genetic clade and race 314 in another cluster. Also, Delmotte *et al.* (2008) and Ahmed *et al.* (2012) grouped races 704 and 714 together in the same genetic clade. Our results underlined non correlation between EST genotypes (Table 5, Fig. 4) and pathogenic traits (Tables 2, 4).

The current study helps us to underline the role of *Pl2* gene in differentiation the pathogenicity in *P. halstedii*. Races 704 and 714 that accumulate a large number of virulence genes might never be the most aggressive on sunflower genotypes as compared with non virulent pathotypes 304 and 314. Regarding avirulence *Avr* gene correspondent to *Pl2* gene in *P. halstedii*, it seems that *Avr* gene stimulates less virulent spectrum and more aggressive characteristics in races 304 and 314 than races 704 and 714. There was phenotypic variation (morphological and genetic characteristics) for the 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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BIBLIOGRAPHY

Ahmed, S.; Delmotte, F. & Tourvieille de Labrouhe, D. 2012. Emerging virulence arising from hybridisation facilitated by multiple introductions of the sunflower downy mildew pathogen *Plasmopara halstedii*. *Fungal Genet. Biol.* 49(10): 847-855.

Delmotte, F.; Giresse, X.; Richard-Cervera, S.; M'Baya, J.; Vear, F.; Tourvieille, J.; Walser, P. & Tourvieille de Labrouhe, D. 2008. Single nucleotide polymorphisms reveal multiple introductions into France of *Plasmopara halstedii*, the plant pathogen causing sunflower downy mildew. *Infect. Genet. Evol.* 8(5): 534-540.

Flor, H. H. 1971. Current status of the gene-for-gene concept. *Annu. Rev. Phytopathol.* 9: 275-296.

Giresse, X.; Tourvieille de Labrouhe, D.; Richard-Cervera, S.; Delmotte, F. 2007. Twelve polymorphic EST-derived markers for *Plasmopara halstedii*, the causal agent of sunflower downy mildew. *Mol. Ecol. Notes* 7(6): 1363-1365.

Jin, L. & Chakraborty, R. 1993. Estimation of genetic distance and coefficient of gene diversity from single-probe multilocus DNA fingerprinting data. *Mol. Biol. Evol.* 11(1): 1112-1114.

Librado, P. & Rozas, J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25(11): 1451-1452.

Miller, J. F. & Gulya, T. J. 1991. Inheritance of resistance to race 4 of downy mildew derived from interspecific crosses in sunflower. *Crop Sci.* 31(1): 40-43.

Mouzeyar, S.; Tourvieille de Labrouhe, D. & Vear, F. 1993. Histopathological studies of resistance of sunflower (*Helianthus annuus* L.) to downy mildew (*Plasmopara halstedii* Farl. Berlese et de Toni). *J. Phytopathol.* 139(4): 289-297.

Radwan, O.; Bouzidi, M. F. & Mouzeyar, S. 2011. Molecular characterization of two types of resistance in sunflower to *Plasmopara halstedii*, the causal agent of downy mildew. *Phytopathology* 101(8): 970-979.

Radwan, O.; Bouzidi, M. F.; Vear, F.; Phillipon, J.; Tourvieille de Labrouhe, D.; Nicolas, P. & Mouzeyar, S. 2003. Identification of non TIR-NBS-LRR markers linked to the *PL5*/*PL8* locus for resistance to downy mildew in sunflower. *Theor. Appl. Genet.* 106(8): 1438-1446.

Sakr, N. 2011. Evolution of pathogenicity in *Plasmopara halstedii* (sunflower downy mildew). *Arch. Phytopathol. Plant Protect.* 44(15): 1432-1436.

Sakr, N. 2012. Virulence cost in *Plasmopara halstedii* (sunflower downy mildew). *Arch. Phytopathol. Plant Protect.* 45(6): 646–651.

Sakr, N. 2014: Evolution of new *Plasmopara halstedii* races under the selection pressure with resistant sunflower plants: A review. *Hellenic Plant Protect. J.* 7(1): 1-13.

Sakr, N.; Ducher, M.; Tourvieille, J.; Walser, P. & Tourvieille de Labrouhe, D. 2007. A new method to obtain monozoosporangial isolates of sunflower downy mildew (*Plasmopara halstedii*). *Cryptogamie, Mycol.* 28(2): 123-131.

Snedecor, G. W. & Gochran, W. G. 1989. *Statistical Methods*. The Iowa State University Press, Iowa.

Tourvieille de Labrouhe, D.; Bordat, A.; Tourvieille, J.; Mestries, E.; Walser, P.; Sakr, N.; Ducher, M.; Delmotte, F. & Vear, F. 2010. Impact of major gene resistance management for sunflower on fitness of *Plasmopara halstedii* (downy mildew) populations. *Oleagineux, Corps Gras, Lipides* 17(1): 56-64.

Tourvieille de Labrouhe, D.; Pilorge, E.; Nicolas, P. & Vear, F. 2000. *Le mildiou du tournesol*. CETIOM-INRA Publiactions, Versailles.

Tourvieille de Labrouhe, D.; Serre, F.; Roche, S.; Walser, P. & Vear, F. 2008. Quantitative resistance to downy mildew (*Plasmopara halstedii*) in sunflower (*Helianthus annuus*). *Euphytica* 164(2): 433-444.

Van der Plank, J. E. 1968. *Disease resistance in plants*. Academic Press, New York and London.

Vear, F.; Gentzbittel, L.; Phillipon, J.; Mouzeyar, S.; Mestries, E.; Roeckel-Drevet, P.; Tourvieille de Labrouhe, D. & Nicolas, P. 1997. The genetics of resistance to five races of downy mildew (*Plasmopara halstedii*) in sunflower (*Helianthus annuus* L.). *Theor. Appl. Genet.* 95(4): 584-589.

Vear, F.; Tourvieille de Labrouhe, D. & Miller, J. F. 2003. Inheritance of the wide-range downy mildew resistance in the sunflower line RHA 419. *Hellia* 26(39): 19-24.

Viranyi, F. & Spring, O. 2011. Advances in sunflower downy mildew research. *Eur. J. Plant Pathol.* 129(2): 207-220.