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Evolution of Two Components of Pathogenicity in *Plasmopara halstedii* (Downy Mildew) under Sunflower Qualitative Resistance Selection Pressure

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Evolution of pathogenicity, morphological, and genetic traits were analyzed in a local *Plasmopara halstedii* (downy mildew) population (including two parental and five progeny isolates) multiplied under sunflower qualitative resistance selection pressure for five years. The two components of pathogenicity developed in response to *Pl* resistance genes selection pressure. The emergence of new virulence in *P. halstedii* progeny isolates carrying several levels of aggressiveness was an important consequence of selection pressure. However, appearance of new virulence did get along with evolution of aggressiveness in progeny isolates as compared with parental ones. For progeny *P. halstedii* isolates, an increase in pathogen virulence had direct consequences on its aggressiveness. There was no influence of selection pressure on morphological traits, but an effect was observed on evolution of genetic architecture. However, arrangement of genetic traits did get along with evolution of pathogenicity. It is clear that qualitative resistance selection pressure plays an important role in the evolution of sunflower downy mildew population.

Keywords: aggressiveness, *Helianthus annuus*, *Pl* gene, virulence.

Understating the evolution dynamics of pathogen populations in response to resistance selection pressure is an important aspect of host–pathogen interactions (Stukenbrock and McDonald, 2008; Mundt, 2014), which can be divided into two parts: virulence (ability to infect varieties with particular qualitative resistance genes) and aggressiveness (the amount of disease it causes on varieties it is able to infect) according to Van der Plank (1968). In plant–pathogen interactions, the selection pressure exercised by qualitative resistance on parasitic populations might lead to the appearance of new virulent isolates, as observed for the pathosystem of *Phytophthora infestans* and *Solanum tuberosum* (Grunwald and Flier, 2005). On the other hand, Cowger and Mundt (2002) showed that wheat cultivars with good quantitative resistance selected more aggressive isolates of *Mycosphaerella graminicola.*

The biotrophic oomycete *Plasmopara halstedii* (Farl.) Berl. and de Toni is an invasive species where sunflower (*Helianthus annuus* L.) is grown. *P. halstedii* displays a

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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). Indeed, it has been possible to identify up to 35 races, with different virulence patterns (Ahmed et al., 2012). Disease resistance in sunflowers to *P. halstedii* can be placed in one of two categories, the first is qualitative resistance (Tourvieille de Labrouhe et al., 2000), and the second is quantitative resistance (Tourvieille de Labrouhe et al., 2008).

P. halstedii is characterized by a high level of evolutionary potential expressed by high virulence, aggressiveness and a great potential in developing new races (Delmotte et al., 2008; Tourvieille de Labrouhe et al., 2010; Ahmed et al., 2012; Sakr, 2012, 2013, 2014a, 2014b, 2014c, 2014d, 2014e, 2015a, 2015b). Even thought some studies aimed to analyze the influence of qualitative sunflower selection pressure on virulence in both experimental and filed conditions (Delmotte et al., 2008; Tourvieille de Labrouhe et al., 2010; Ahmed et al., 2012; Sakr, 2014a, 2014b, 2014e, 2015b), a number of questions regarding its influence on evolution of aggressiveness (Sakr, 2014b, 2014e), morphological (Sakr, 2014c), and genetic characteristics (Sakr, 2012, 2013, 2014d, 2014e, 2015a, 2015b) have still remained unanswered. In this study, phenotypic analyses (morphological and pathogenic) and genetic characteristics were analysed for seven *P. halstedii* pathotypes: race 100 present in France since the 1966, race 710 introduced from the USA during the 1980s (Tourvieille de Labrouhe et al., 2000, 2010), and five progeny pathotypes of races 300, 304, 700, 704 and 714 originated from an initial mixture of pathotypes 100 and 710 (Tourvieille de Labrouhe et al., 2010; Sakr, 2014a, 2014b, 2014e, 2015b). Hence an attempt was made to generate information about the evolution of pathogenicity, morphological and genetic traits in a local parasitic population multiplied under qualitative sunflower resistance selection pressure.

Materials and Methods

Oomycete isolates and race identification

All isolates of *P. halstedii* used in this study were collected in France and maintained at INRA, Clermont–Ferrand. Manipulation of this quarantined pathogen followed European regulations (No 2003/DRAF/70). Isolate MIL 001 (race 100) was sampled in 1966 and isolate MIL 002 (race 710) in 1988. This collection of seven *P. halstedii* isolates included two parental ones of races 100 and 710, and five progeny isolates originated from an initial mixture of pathotypes 100 and 710. Progeny isolates were multiplied for five years in sunflower cages isolated from the exterior environment (Tourvieille de Labrouhe et al., 2010; Sakr, 2014a, 2014b, 2014e, 2015b).

The sunflower lines co-existed with *P. halstedii* carrying several *Pl* effective qualitative genes *Pl2*, *Pl6*, and *Pl8* (Tourvieille de Labrouhe et al., 2010; Sakr, 2014a, 2014b, 2014e, 2015b). The sunflower line carrying *Pl2* is sensitive to parental isolate of race 710, the other sunflower lines carrying *Pl6* and *Pl8* are resistant to parental one of race 710, and all the sunflower lines are resistant to parental one of race 100. The *P. halstedii*

progeny isolates present in the soil were trapped from sunflower parcels with a sunflower line, carrying no downy mildew resistant gene (Tourvieille de Labrouhe et al., 2010; Sakr, 2014a, 2014b, 2014e, 2015b).

These five progeny isolates were characterized for their virulence patterns by method reported by Tourvieille de Labrouhe et al. (2000): isolate DU 1842 (race 300); isolate DU 1767 (race 304); isolate 1943 (race 314); isolate 1734 (race 704) and isolate 1915 (race 714). This study dealt with five single zoosporangium isolates per parental and progeny isolate (Sakr et al., 2007), giving a total of 35 single zoosporangium isolates. The characterization of the race for 35 single zoosporangium isolates (Table 2) was determined using the same method adapted in the study by Tourvieille de Labrouhe et al. (2000).

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

To characterize aggressiveness criteria: percentage infection, latent period, sporulation density and reduction of hypocotyl length for 35 *P. halstedii* single zoosporangium isolates (Sakr, 2012, 2013, 2014a, 2014b, 2014c, 2014d, 2014e, 2015a), 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). The index of aggressiveness of the *P. halstedii* isolate was used to summarize all values for four criteria on sunflower inbred lines 'FU' in one value to facilitate the comparison between the different *P. halstedii* isolates (Sakr, 2014b). All the pathogenic tests were carried out in growth chambers regulated at 18 h 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 35 single zoosporangium isolates 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 × 400) 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 × 400) with 2 replications.

DNA extraction and molecular typing

For 35 single zoosporangium isolates tested, DNA was isolated from infected plant tissue as previously described for *Plasmopara viticola* by Delmotte et al. (2008). The 12 polymorphic EST (Expressed Sequence Tag)-derived markers (Delmotte et al., 2008) were then used to genotype *P. halstedii* isolates. The polygenetic relations between the

35 single zoosporangium isolates were obtained by building a Neighbour-joining (NJ) tree (Jin and Chakraborty, 1993) using DnaSP v5 Software (Librado and Rozas, 2009). A Bootstrap analysis was performed on 10,000 replicates.

Results

Characterization of virulence for P. halstedii *parental and progeny isolates*

Tables 1 and 2 showed that two parental isolates 100 and 710 had two different profiles of virulence reaction to nine sunflower differential lines. Parental isolate and single zoosporangium isolates of parental pathotype 710 were more virulent than parental isolate and single zoosporangium isolates of parental pathotype 100 (Tables 1, 2).

The selection pressure for five years applied on the mixture of two *P. halstedii* parental pathotypes 100 and 710 led a new virulence to appear in the progeny pathotypes: 300, 304, 314, 704 and 714 (Tables 1 and 2). In this study, the new virulence means the appearance of several levels of virulence in progeny pathotypes as compared with virulence profile in parental ones.

Characterization of the highest and the least virulent *P. halstedii* races of could be assessed in response to *Pl2* resistance gene in sunflower differential line D3 (Sakr, 2012, 2013, 2014a, 2014b, 2014e). Based on the virulence reaction for the seven *P. halstedii* pathotypes to D3, we can divide them into two groups. The highest virulent '7xx races' contains the isolates of parental pathotype 710, and two progeny ones of races 704 and

Table 1

Virulence of seven *Plasmopara halstedii* parental and progeny isolates on nine sunflower differential lines

Differential lines	*Plasmopara halstedii* isolates (race and year)						
	MIL001	DU1842	DU1943	DU1767	MIL002	DU1915	DU1734
	100	300	314	304	710	714	704
	1960	2005	2005	2005	1988	2005	2005
D1 – Ha-304 No *Pl* gene	S	S	S	S	S	S	S
D2 – Rha-265 (*Pl1*)	R	S	S	S	S	S	S
D3 – Rha-274 (*Pl2*)	R	R	R	R	S	S	S
D4 – PMI3 (*Plpmi3*)	R	R	S	R	S	S	R
D5 – PM-17 (*Pl5*)	R	R	R	R	R	R	R
D6 – 803-1 (*Pl15*)	R	R	R	R	R	R	R
D7 – HAR-4 (*Pl15*)	R	R	R	R	R	R	R
D8 – QHP1 ($Pl1/Pl_{15}$)	R	R	R	R	R	R	R
D9 – Ha-335 (*Pl6*)	R	R	S	S	R	S	S

S: susceptible = sporulation on cotyledons; R: resistant = no sporulation; data from Tourvieille de Labrouhe et al. (2000)

Table 2

Virulence of 35 single zoosporangium for seven *Plasmopara halstedii* parental and progeny isolates on nine sunflower differential lines

Single zoo-sporangium isolates	Race	Year isolates	D1 Ha-304 No *Pl* gene	D2 Rha-265 *Pl1*	D3 Rha-274 *Pl2*	D4 PMI3 *Plpmi3*	D5 PM-17 *Pl5*	D6 803-1 *Pl5*	D7 HAR-4 Pl_{15}	D8 QHP1 $Pl1/Pl_{15}$	D9 Ha-335 *Pl6*
MIL001 M2, MIL001 M3, MIL001 M4, MIL001 M5, MIL001 M6	100	2006	S	R	R	R	R	R	R	R	R
DU1842 M1, DU1842 M2, DU1842 M3, DU1842 M4, DU1842 M5	300	2006	S	S	R	R	R	R	R	R	R
DU1943 M1, DU1943 M2, DU1943 M3, DU1943 M4, DU1943 M5	314	2006	S	S	R	S	R	R	R	R	S
DU1767 M1, DU1767 M2, DU1767 M3, DU1767 M4, DU1767 M5	304	2006	S	S	R	R	R	R	R	R	S
MIL002 M1, MIL002 M2, MIL002 M3, MIL002 M4, MIL002 M5	710	2006	S	S	S	S	R	R	R	R	R
DU1915 M1, DU1915 M2, DU1915 M3, DU1915 M5, DU1915 M6	714	2005	S	S	S	S	R	R	R	R	S
DU1734 M1, DU1734 M2, DU1734 M3, DU1734 M7, DU1734 M8	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)

714. While the least virulent '100 and 3xx races' includes the isolates of parental pathotype 100, and three progeny isolates of races 300, 304 and 314.

Characterization of aggressiveness for P. halstedii *parental and progeny isolates*

There were significant differences for all aggressiveness criteria among single zoosporangium isolates in parental and progeny pathotptes (Figs 1A, 1B, 1C and 1D). Percentage infection (Fig. 1A) ranged between 94.40% for isolate MIL002 and 99.78% for isolate DU1767 (F-value = 21.07, P-value = 0.0001). Latent period (Fig. 1B) ranged between 8.08 days for isolate DU1842 and 11.30 days for isolate DU1734 (F-value = 30.78, P-value = 0.0001). Sporulation density (Fig. 1C) varied threefold: 5.03×10^5 zoosporangia were produced by cotyledons for isolate DU 1915 and 16.72×10^5 for isolate DU1842 (F-value = 77.70, P-value = 0.0001). Hypocotyl length (Fig. 1D) varied from 27.68 mm for isolate DU1915 to 40.64 mm for isolate DU1943 (F-value = 39.90, P-value = 0.0001).

There were significant differences for index of aggressiveness (F-value = 53.194, P-value = 0.0001) in progeny and parental isolates. The index of aggressiveness (Fig. 1E) varied fourfold: 1.58 for DU1915 and 7.05 for DU1842. Results shown in Fig. 1 indicate that the progeny isolate of race 300 was the most aggressive among the isolates tested with an index of aggressiveness of 7.05; followed by progeny isolate of race 304 with an index of aggressiveness of 6.09; parental isolate of race 100 with an index of aggressiveness of 4.86, and progeny isolate of race 314 with an index of aggressiveness of 4.5. The parental isolate of race 710 and the two progeny isolates of races 704 and 714 were the least aggressive, recording the mean value for index of aggressiveness of 1.9. Consequently, single zoosporangium isolates of parental pathotype 100 were more aggressiveness that parental isolate and single zoosporangium isolates of parental pathotype 710 (Fig. 1E). Single zoosporangium isolates of progeny isolates of races 300, 304 and 314 were more aggressiveness that single zoosporangium isolates of progeny isolates of races 704 and 714 (Fig. 1E).

Morphology of zoosporangia and sporangiophores for P. halstedii *parental and progeny isolates*

The results showed that the two most observed forms of zoosporangia were oval and round (Fig. 2). There were significant differences for all morphological criteria among single zoosporangium isolates in parental and progeny pathotptes (Figs 3A, 3B, 3C and 3D). The proportion of oval form (Fig. 3A) varied from 70.80 to 90% (F-value = 5.79, P-value = 0.0001). The zoosporangia size (Fig. 3B) ranged from 383.18 to 533.54 μm^2 (F-value = 4.04, P-value = 0.0012). The sporangiophore length (Fig. 3C) varied between 497.86 μm and 611.96 μm (F-value = 1.453, P-value = 0.2023). The sporangial width (Fig. 3D) ranged from 6.82 μm to 10.78 μm (F-value = 3.38, P-value = 0.005). There was thus no relationship between morphology of zoosporangia and sporangiophores (Figs 3A, 3B, 3C and 3D) and virulent and aggressiveness characteristics for parental and progeny isolates (Table 2 and Figs 1A, 1B, 1C and 1D).

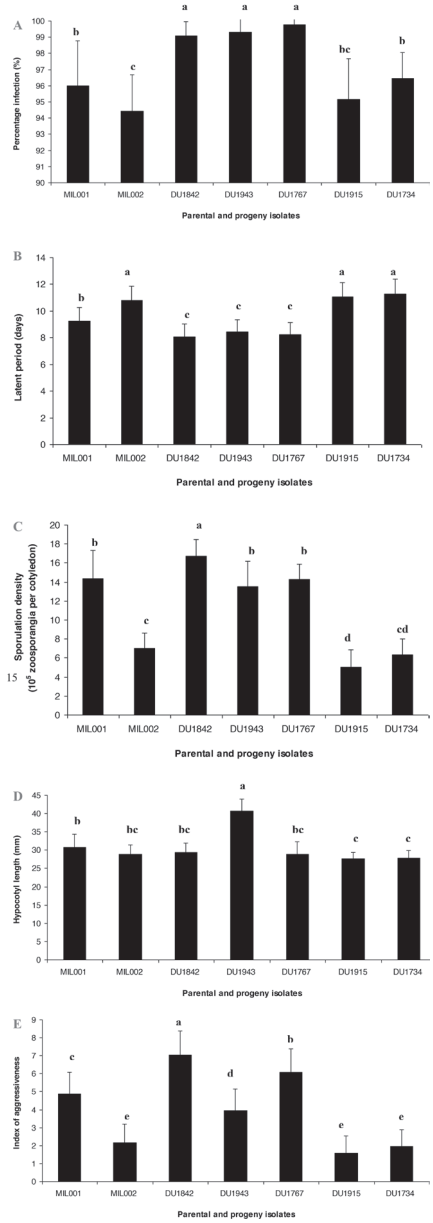

Fig. 1. Aggressiveness criteria on sunflower inbred line FU for seven parental and progeny *Plasmopara halstedii* isolates; Fig. 1A percentage infection, Fig. 1B latent period, Fig. 1C sporulation density, Fig. 1D reduction of hypocotyl length, and Fig. 1E index of aggressiveness. Data show the mean value of five single zoosporangium *Plasmopara halstedii* isolates per each parental and progeny pathotype, identical letters do not differ from each other at the significance level of $P < 0.05$ according to a Newman–Keul test

Molecular analysis for P. halstedii *parental and progeny isolates*

The combination of 12-EST derived markers revealed five MLG among 35 *P. halstedii* single zoosporangium isolates (Figs 4A and 4B). There was no *intra-race* genetic variation for all parental and progeny pathotypes tested (Fig. 4A). Single zoosporangium isolates of parental isolates of races 100 and 710 had two different genetic background (Fig. 4B). Furthermore, single zoosporangium isolates of parental pathotypes 100 and two progeny races 300 and 304 had the same genetic background (Fig. 4B). The Neighbour-joining tree showed that single zoosporangium isolates of progeny pathotyes 704, 704 and 314 had an intermediary genetic position between two parental isolates of races 100 and 710 (Fig. 4B).

Discussion

Comprehension of the interaction between pathogen and its host plant requires knowledge of the variability of pathogenicity. In the current study, evidence for race emergence in *P. halstedii* was demonstrated by infecting an experimental plot with races 100 and 710. After 5 years of selective pressure exerted by sunflower *Pl* resistance genes, five other races (300, 304, 314, 704 and 714) that had not been present at the start of the study, were reported (Tourvieille de Labrouhe et al., 2010; Sakr, 2014a, 2014b, 2014e, 2015b). Furthermore, in the absence of selection pressure, Sakr et al. (2011) and Sakr (2014b) stated that *P. halstedii* did not evolve its virulence. This rapid evolution of virulence in *P. halstedii* was revealed in France, exerted by intensive *Pl* genes resistance selection pressure. Fourteen different races of this pathogen have now been characterized, nine of which emerged in the last ten years (Delmotte et al., 2008; Sakr, 2014b).

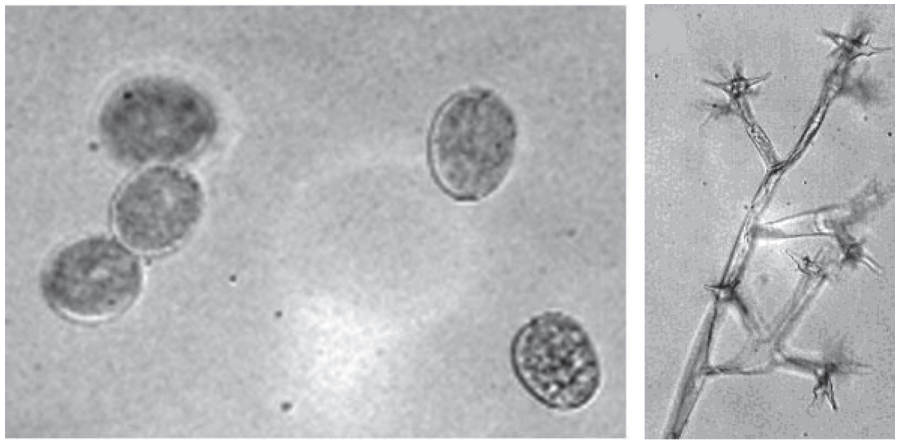

Fig. 2. *Plasmopara halstedii* zoosporangia forms and sporangiophores observed on sunflower inbred line 'FU': round and oval forms (left), and sporangiophore (right)

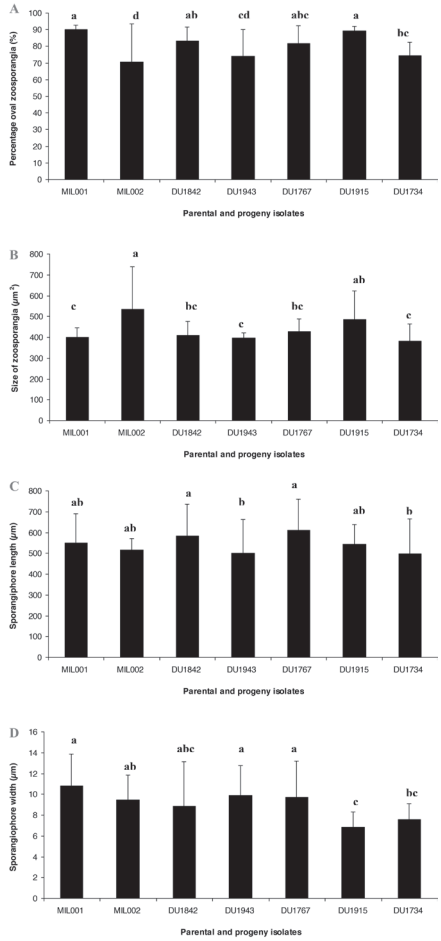

Fig. 3. Morphological characters of zoosporangia and sporangiophores obtained on sunflower genotype FU for seven parental and progeny *Plasmopara halstedii* isolates; Fig. 3A percentage oval zoosporangia, Fig. 3B size of zoosporangia, Fig. 3C sporangiophore length, and Fig. 3D sporangiophore width. Data show the mean value of five single zoosporangium *Plasmopara halstedii* isolates per each parental and progeny pathotype, identical letters do not differ from each other at the significance level of $P < 0.05$ according to a Newman–Keul test

High percentage infection, short-latent period, high sporulation density, and significant reduction in the length of the hypocotyl represent high aggressiveness (Sakr, 2012, 2013, 2014a, 2014b, 2014c, 2014d, 2014e, 2015a). Single zoosporangium isolates of parental isolate 100 and progeny isolates of races 300, 304 and 314 were more aggressiveness that single zoosporangium isolates of parental isolate 710 and progeny isolates of races 704 and 714 (Fig. 1E). Two hypotheses could explain that established aggressiveness variability in pathogen isolates used in this study (Table 2). First, it is possible that this variation is due to the origin of pathogen isolates used in this study (Tables 1 and 2).

These isolates belong to several races and may be found to be an effect of additional avirulence genes in *P. halstedii* isolates as observed for another oomycete *P. infestans* (Montarry et al., 2010). Second, isolates belong to 7xx races that accumulate a large number of avirulence genes and show the lowest levels of aggressiveness, compared with isolates belong to 100 and 3xx races that accumulate a small number of avirulence genes and show the highest levels of aggressiveness (Sakr, 2012, 2013, 2014a, 2014b, 2014d, 2014e, 2015b). For progeny isolates, appearance of the highest virulent isolates of 7xx races compared with the least virulent isolates of 3xx races in response to *Pl* genes resistance selection pressure is costly due to trade-offs with aggressiveness. This phenomenon has been observed in the context of pathogen adaptation to host's qualitative and monogenic resistances (Stukenbrock and McDonald, 2008; Mundt, 2014).

The two components of pathogenicity developed in response to sunflower qualitative selection pressure (Tables 1 and 2, Figs 1A, 1B, 1C and 1D). However, appearance of new virulence did get along with evolution of aggressiveness in progeny isolates as compared with parental ones. For example, progeny isolate of race 300 was more virulent and aggressiveness than parental one 100. On the other hand, this progeny isolate of race 300 was less virulent and more aggressive than parental one 710. For other Oomycete, *P. infestans* on potato, Montarry et al. (2010) found that new populations were more virulent and aggressiveness than the old populations. Moreover, progeny isolates of races 314, 704 and 714 were more virulent and less aggressiveness than parental one of race 100. In simmilary experimental conditions, Murakami et al. (2006) found that progeny isolates were more virulent and less aggressive than parental ones in pathosystem *Magnaporthe* oryza / wheat and rice. However, progeny isolate of race 714 was more virulent than parental one 710, and the two isolates had the same aggressiveness level; this association was identified in *Puccinia triticina* on wheat (Robert et al., 2002).

Our data showed that morphological characteristics (Figs 3A, 3B, 3C and 3D) did not distinguish the two parental isolates according to their virulence and aggressiveness on the hand, and the parental and progeny isolates on the other hand (Tables 1 and 2, Figs 1A, 1B, 1C and 1D). The studied morphological criteria did not evolve in progeny isolates as compared with parental ones (Figs 3A, 3B, 3C and 3D). There was an influence of qualitative resistance selection pressure on evolution of genetic architecture (Figs 4A and 4B). However, arrangement of genetic traits (Figs 4A and 4B) did get along with evolution of pathogenicity (Tables 1 and 2, Fig. 1E). Tables 1 and 2 demonstrated that progeny pathotypes 300 and 304 were more virulent than parental one of race 100, which has not been detected in genetic analyses (Figs 4A and 4B): the three isolates had the same genetic background. Tables 1 and 2 and Fig. 2 showed that isolates of pathotypes 100 and 3xx were more aggressiveness and less virulent than less aggressiveness and more virulent isolates of pathotypes 7xx, this association was not identified in genetic analyses (Figs 4A and 4B). Our results are in accordance with those found by Montarry et al. (2010) in *P. infestans* / potato pathosystem.

Regarding the emergence of new virulence in *P. halstedii* as shown in Tables 1 and 2, two genetic mechanisms could explain it (Sakr, 2014a, 2014e, 2015b). First, recombination events between genetically differentiated parental isolates of races 100 and 710 may have led to the emergence of new races: races 314, 704 and 714 are intermediate

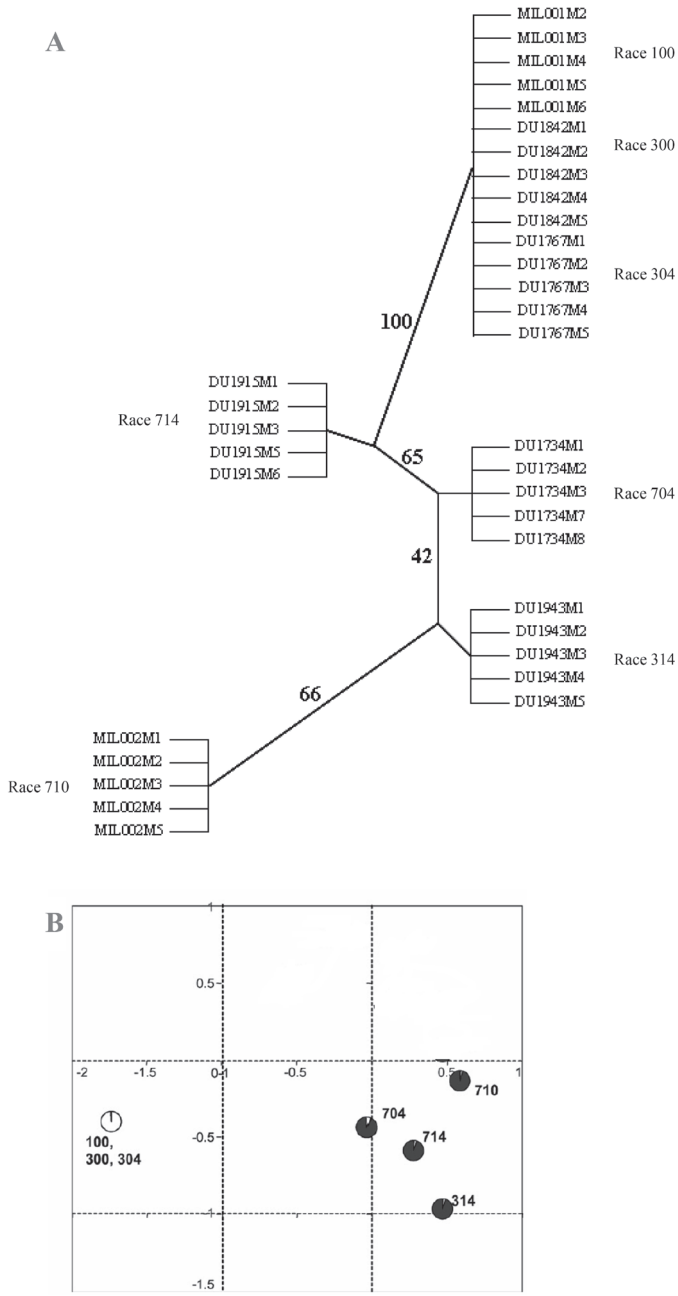

Fig. 4. Phylogenetic tree according to Neighbour-joining analysis of 12 EST-derived markers (Fig. 4A). Figures on branches indicate bootstrap values (10,000 replicates). Multilocus genotypes of *P. halstedii* races based on 12 EST-derived markers (Fig. 4B)

between races 100 and 710 (Figs 4A and 4B). In the same pathosystem, Ahmed et al. (2012) suggested that multiple introductions have aided the establishment of *P. halstedii* in France, and noted that recombination facilitated by these introductions is driving the emergence of new and endemic races in response to host resistance. Second, mutations in a clonal lineage may lead to the emergence of new races in the same genetic background: this is the cases for races 100, 300 and 304 (Figs 4A and 4B). Though it has long been suggested that compensatory mutations are crucial to the evolution of strains of virulent plant, recent understanding of the molecular basis of virulence in plant pathogens suggests that the process could in fact be determined, at least in part, by a ''reshuffling'' of effectors with differing impacts on pathogen virulence (Mundt, 2014).

Conclusion

The importance of qualitative resistance selection pressure in better adaptation of *Plasmopara halstedii* populations was revealed. Data presented in this work suggested that emergence of new virulence in *P. halstedii* progeny isolates carrying several levels of aggressiveness was an important consequence of *Pl* genes resistance selection pressure. For progeny isolates, an increase in pathogen virulence had direct consequences on its aggressiveness. There was no influence of selection pressure on morphological traits, but an effect was observed on evolution of genetic architecture. However, arrangement of EST genotypes did get along with evolution of pathogenicity. It is evident that the qualitative resistance selection pressure plays an important role in the evolution of sunflower downy mildew population. The changes on the level of pathogenicity may help to a better adaptation of *P. halstedii* in the presence of intensive use of *Pl* genes qualitative resistance.

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