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ANALYZING THE INFLUENCE OF QUALITATIVE RESISTANCE SELECTION PRESSURE ON VARIATION OF AGGRESSIVENESS IN *PLASMOPARA HALSTEDII*

Nachaat Sakr[1]*, *Denis Tourvieille de Labrouhe*[2], *Pascal Walser*[2], *Mireille Ducher*[3] *Francois Delmotte*[4], *Jeanne Tourvieille*[3], *Felicity Vear*[2]

[1] Syrian Atomic Energy Commission, Damascus, P.O. Box 6091, Syria
[2] INRA-UBP, UMR 1095, 234 Avenue du Brézet, 63100 Clermont-Ferrand, France
[3] UBP-UHR 1095, 24 Avenue des Landais, 63/77, Aubiére, France
[4] INRA-UHR 1065, La Grande Ferrade, BP81, 33883 Villenave d'Ornon Ceder, France

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Abstract: Variation of aggressiveness in populations of race 710 of *Plasmopara halstedii* [(Farl.) Berl. et Toni] (sunflower downy mildew) was measured under different strategies of qualitative resistance selection pressure: mixture, alternation and monoculture of major resistance genes in comparison with a population under no selection pressure. Two sunflower lines showing different levels of quantitative resistance were used to measure four aggressiveness criteria: percentage infection, latent period, sporulation density and reduction of hypocotyl length. *P. halstedii* strains multiplied under varietal mixtures presented the greatest sporulation densities and shortest hypocotyl lengths, those multiplied under alternation presented a reduced latent period and shorter hypocotyl lengths compared with those not influenced by selection pressure. There were no significant differences between populations multiplied under monoculture of resistance genes and those under no selection pressure. These changes appear as being linked to the number of infected plants present. The results suggested that the method of *Pl* gene management affects aggressiveness because it determines the number of susceptible plants harbored by the parasite.

Key words: alternation, mixture, monoculture, pathogenicity, *Pl* gene

INTRODUCTION

Selective effect on pathogenicity due to host resistance is an important aspect of plant-pathogen interactions, which can be divided into two parts: virulence (specific disease-causing abilities) and aggressiveness (non-specific disease-causing abilities) according to van der Plank (1968). There have been many reports concerning increase of virulence in relation to host resistance in pathogens of economically important crops (McDonald and Linde 2002). Similarly, Gandon and Michalakis (2000) predicted that increased levels of quantitative host resistance may select for increased aggressiveness of parasites, leading to increased crop losses. Cowger and Mundt (2002) showed that wheat cultivars with good quantitative resistance selected more aggressive isolates of *Mycosphaerella graminicola*. However, this is not always true, Sullivan *et al.* (2005) reported that tobacco cultivars with high levels of quantitative resistance did not select for more aggressive isolates of *Phytophthora parasitica* var. *nicotiana*e. Also, Flier *et al.* (2007) showed that, following large-scale introduction of more resistant potato varieties in organic production systems in Europe, there was no shift towards increased levels of aggressiveness of *Phytophthora infestans* populations.

Sunflower downy mildew is caused by *Plasmopara halstedii* [(Farl.) Berl. et Toni], an invasive species where sunflower (*Helianthus annuus* L.) is grown. It is an obligate endoparasite that cannot be cultivated independently from its plant host. *P. halstedii* is a homothallic oomycete, whose cycle is made up of a single sexual generation permitting overwintering and one or perhaps two asexual generations which occur during the growing season. The disease affects young plants when the water content of the soil is high and maximum temperature is between 15 and 18°C.

P. halstedii shows physiological races capable of infecting a variable range of sunflower genotypes. The nomenclature of these races is based on the reaction of a series of differential lines (Tourvieille de Labrouhe *et al.* 2000). To date, there are at least 35 races in different parts of the world (Gulya 2007). Disease resistance in sunflowers to *P. halstedii* can be classified into one of two categories. The first is qualitative resistance which is conferred by the major *Pl* genes and tends to produce a disease-free plant (Tourvieille de Labrouhe *et al.* 2000). The second is quantitative resistance which is controlled by minor genes and tends to impact the rate of disease development (rate reducing) rather than producing a disease-free plant (Tourvieille de Labrouhe *et al.* 2008). For qualitative resistance

*Corresponding address:
snachaat@hotmail.com

selection pressure, Tourvieille de Labrouhe *et al.* (2010) showed that whatever the method of management (mixture, alternation, and monoculture) of *Pl* genes, their selection pressure led to appearance of new virulence.

This paper reports studies of levels of aggressiveness in three populations of *P. halstedii*, race 710, obtained under different strategies of qualitative resistance selection pressure: mixture, alternation and monoculture, in comparison with a population obtained in the absence of any effective *Pl* gene.

MATERIALS AND METHODS

Sunflower genotypes

Four quasi-isogenic hybrids were used, obtained from crosses of 2 forms each of two inbred lines:

– L1a: carrying resistance gene *Pl2*, resistant to race 100 and susceptible to race 710,
– L1b: carrying resistance genes *Pl2* and *Pl8*, resistant to races 100 and 710,
– L2a: carrying no known resistance gene,
– L2b: carrying resistance gene *Pl6*, resistant to races 100 and 710.

The four hybrids were produced as follows: H1 = L1a x L2a, H2 = L1a x L2b, H3 = L1b x L2a and H4 = L1b x L2b.

P. halstedii strains present in the soil were trapped with a sunflower hybrid (Airelle), carrying no downy mildew resistant gene.

Experimental protocol

The protocol was developed by Tourvieille de Labrouhe *et al.* (2010) to determine durability of resistance. Four plots constituted by netting cages were maintained with climate conditions favorable for the expression of the disease. Plot P1 was planted in four consecutive years with H1 (no effective resistance against race 710). Plot P2 was planted all years with an equal mixture of the four hybrids. Plot P3 was planted in first year with H1, then successively with H2, H3 and H4. Plot P4 was planted with H2, resistant to race 710, in all 4 years.

***P. halstedii* strains of race 710**

After 4 years, *P. halstedii* strains were collected from soil according to the method described by Tourvieille de Labrouhe *et al*. (2010) and their virulence profile characterized by the method of Tourvieille de Labrouhe *et al.* (2000). For plots 1, 2 and 3, four strains were analyzed and for P4, 3 strains. However, the total numbers of infected plants differed between plots (from 737 for plot P1 to 82 for plot P4). There was a continued reduction in percentage of *P. halstedii* samples of race 710 especially in the absence of susceptible sunflower genotypes in plots P3 and P4. Nevertheless, this race was present in soil samples taken in 2005 from all parts (Tourvieille de Labrouhe *et al*. 2010).

Measurements of aggressiveness

To characterize aggressiveness criteria of *P. halstedii* strains, two INRA inbred lines carrying no *Pl* gene and known to have different levels of quantitative resistance (Vear *et al*. 2007; Tourvieille de Labrouhe *et al*. 2008) were studied: FU, which has greater resistance in the field and BT, rather susceptible in the field. However, these criteria were used by Sakr (2009a, b) to analyze aggressiveness of *P. halstedii* strains.

Percentage infection

Infection is considered as successful when the seedlings show sporulation on the shoot surface. Observations were made 13 days after infection and expressed as the percentage of seedlings showing sporulation, whatever the plant parts concerned and the amount of sporulation observed. For each strain 60 plants were measured and the experiment was repeated 3 times.

Latent period

This was defined as the number of days of incubation necessary to obtain sporulating pathogen on 80% of the plants. For measurement, 6 treatments were carried out: sixty infected germinating seeds were planted in 6 pots (10 seeds per pot). Each day, after incubation for 7 to 12 days, one pot was covered with a polythene bag. After 24 hours at 100% RH (relative humidity), the number of seedlings in the pot showing sporulation on the shoot was noted. These observations were combined to give the number of plants expressing symptoms after 13 days of incubation, which enabled calculation of the percentage of infected plants expressing symptoms for each incubation period compared with the total after 13 days. The latent period was read from a quadratic regression curve considering percent plants sporulating, including the intermediate points and the first day when 100% sporulation was obtained. For each strain, the experiment was repeated 3 times.

Sporulation density

This was defined as the number of zoosporangia produced by one cotyledon. For measurement, the cotyledons of seedlings showing sporulation after a given incubation period, were grouped together in a small container. One ml of physiological sporulation (9 g NaCl/1l permuted water) per cotyledon were added and vigorously shaken before counting (18 observations per sample) under an optical microscope with a Malassez cell. The statistical analysis was performed concerning concentrations measured after 12 and 13 days of incubation, which corresponded to the maximum quantity of spores produced by a cotyledon.

Reduction of hypocotyl length

Corresponding to the distance from the stem base to cotyledon was measured after 13 days incubation on diseased plants that showed sporulation on the shoot. It was compared with the length measured on the same but uninfected genotype grown in identical conditions. Data were expressed in percentage of length of healthy plants. For each strain 10 plants were measured, the experiment was repeated 3 times.

Statistical analyses

Statistical analyses of the aggressiveness data were performed using StatBox 6.7® (GrimmerSoft) software, France. Before statistical analysis, the percentages were

transformed using the Arcsines function. A complete randomized design with two factors (*P. halstedii* strain and sunflower genotype) and 3 replications was used for analysis of percentage infection, latent period and reduction of hypocotyl length. A randomized complete block design with two factors (*P. halstedii* strain and sunflower genotype) and 2 blocks corresponding to two incubation periods was used for analysis of sporulation density. The Newman-Keuls test (Snedecor and Gochran 1989) was used to compare the means at p = 0.05. To compare each characteristic in the different plots, the means of each strain were used as replications in one-way analyses of variance (ANOVA). The Newman-Keuls test (Snedecor and Gochran 1989) was used to compare the means at p = 0.05.

RESULTS

Comparison of aggressiveness of 15 strains of race 710 on inbred lines FU and BT

The two sunflower lines gave a significantly different response (Table 1). Mean percentage infection on sunflower inbred line BT (100%) was significantly greater than on sunflower inbred line FU (99.3%). Mean latent period on sunflower inbred BT (8.1 days) was significantly shorter than on sunflower inbred FU (9.0 days). Mean Sporulation density (10^5 zoosporangia per cotyledon) on sunflower inbred BT (9.63 x 10^5) was significantly greater than on sunflower inbred FU (7.88 x 10^5). Mean reduced hypocotyl length on sunflower inbred BT (33%) was significantly less than on sunflower inbred FU (40.1%). Based on these data, the inbred line BT showed a higher percentage infection, a higher sporulation density, a shorter latent period and less reduced hypocotyl length than FU. The 15 strains appeared as being homogeneous for percentage infection and latent period except sporeulation density and hypocotyl length (Table 1). There was no significant interaction between parasite strains and host for reduction of hypocotyl length.

Comparison of strain aggressiveness in each plot

Percentage infection showed high values whatever the plot (more than 99%). However, there were significant differences between plots for other aggressiveness criteria. The values of latent period ranged between 8.41 to 8.83 days, the values of sporulation density varied between 7.32x10^5 to 10.89x10^5 zoosporangia per cotyledon and the values of percentage for infected hypocotyl length compared with the length of healthy plants ranged between 35.10 to 40.00%. Plot P4 was not distinct from P1 whereas P2 presented greater mean sporulation density and reduction in hypocotyl length, and P3 showed a shorter latent period and greater reduction in hypocotyl length (Table 2).

Table 1. The Comparison of aggressiveness of 15 *P. halstedii* strains of race 710 on sunflower inbred lines FU and BT

	Line effect			Strain effect			Interaction
	BT	FU	significant	minimum	maximum	significant	significant
Percentage infection	100%	99.3%	s	98.6%	100%	ns	s
Sporulation density (zoosporangia per cotyledon)	9.63 10^5	7.88 10^5	s	6.77 10^5	12.64 10^5	s	s
Latent period (days)	8.1	9.0	s	8.3	8.9	ns	s
Hypocotyl length (% of length of healthy plants)	33.0%	40.1%	s	31.1%	40.3%	s	ns

Test of Newman Keuls; ns – not significant; s – significant at p = 0.05

Table 2. The comparison of means observed for *Plasmopara halstedii* strains of race 710 from each plot compared with P1 (no effective *Pl* gene)

	% infection		Latent period [days]		Sporulation density [10^5 zoosporangia per cotyledon]		Hypocotyl length [% of length of healthy plants]	
	mean	reference	mean	reference	mean	reference	mean	reference
P1 (reference)	99.65		8.83		8.15		40.00	
P2	99.84	ns	8.55	ns	10.89	s	35.11	s
P3	99.86	ns	8.41	s	8.30	ns	35.10	s
P4	99.09	ns	8.71	ns	7.32	ns	38.03	ns

Test of Newman Keuls; ns – not significant; s – significant at p = 0.05

DISCUSSION

Understanding the interaction between pathogen and its host plant requires knowledge of the variability of pathogenicity. With this in mind, the variability of aggressiveness and its alternation with different strategies of qualitative resistance selection pressure was studied by using 15 *P. halstedii* strains of race 710. However, the presence of strains of race 710 in plots not grown with a susceptible genotype for three (P3) or four years (P4) trapped by a susceptible genotype in soil tests may be explained by the maintenance of the inoculum in the soil and/or hybrid seed impurities as isolates sampled in 2005 (Tourvieille de Labrouhe *et al.* 2010). With the first hypothesis, the evolution of parasitic populations may depend on characters linked to fitness but it is independent of aggressiveness, such as their capacity to survive for a long time as oospores. With the second hypothesis, the level of susceptible seed impurities would be the important factor which intervenes in the evolution of parasitic populations.

Study of the reaction of two inbred lines to 15 *P. halstedii* strains underlined their differences in behavior. The very good resistance of inbred line FU observed in the field was confirmed by the measurements of aggressiveness criteria described by Sakr (2009a, b). These results showed that there is an interaction between *P. halstedii* and host plant. However, the sunflower inbred line FU, which shows greater resistance in the field, enabled better characterization the components of aggressiveness in controlled conditions in comparison with sunflower inbred line BT, rather susceptible in the field. For the 15 strains analyzed, only sporulation density varied (from 1 to 2), overall, the *P. halstedii* strains appeared to be quite homogeneous (Table 1).

Comparison of parasite populations isolated from the 4 plots showed that strains of race 710 from plot P4 (monoculture of *Pl*6) were not different from the population isolated from P1, with no efficient *Pl* gene (Table 2). This could be explained on one hand by selection of strains which survive in the soil, independently from the factors of aggressiveness measured, or, on the other hand, by a weak level of parasitic multiplication linked to a small number of plants susceptible to race 710, thus giving incomplete expression of parasitic diversity. This second hypothesis appears most likely because the number of plants infected with race 710 was always very low in plot P4. Plot P2 was grown with a mixture of different hybrid forms, giving 25% of plants susceptible to race 710, one third of which contributed to parasitic multiplication. Compared with plot P1, and with few infected plants, it is reasonable to suggest that isolates with a high sporulation capacity could have been favored and may have caused the secondary infections shown by 20% of infested plants in this plot between 2001 and 2004 (Tourvieille de Labrouhe *et al.* 2010). These secondary infections contributed to the stock of inoculum which may explain why strains isolated from this plot showed a significantly higher sporulation density. In plot P3 (alternation), the abundant downy mildew population created in the first year, from more than 230 diseased plants, was confronted with new resistance genes every year but race 710 remained in 2005, although at a lower level than in the other three plots (Tourvieille de Labrouhe *et al.* 2010). This population evolved towards increased aggressiveness as measured by latent period. Compared with plot P4, it had a wider genetic base. Differences in aggressiveness, as compared with plot P1 (Table 2), were weak but significant for latent period, suggesting that, from a similar number of infected plants, different aggressiveness factors could be selected if the number of infected plants is small. The two plots that significantly differed for either latent period or sporulation density (*i.e.*, P3 and P2) also differed for hypocotyl length.

It is commonly admitted that quantitative resistance applies selection pressure on parasitic populations, which may lead to more aggressive strains. An example was maize resistance against *Cochliobolus heterostrophus* (Kolmer and Leonard 1986). In contrast, many authors reported that the use of qualitative resistance did not lead to modifications in aggressiveness. Sullivan *et al.* (2005) showed that qualitative resistance in tobacco did not exert a selective effect on aggressiveness of *Phytophthora parasitica* var. *nicotiana*e. In the pathosystem *Venturia inaequalis*/apple, Parisi *et al.* (2004) found that virulent strains taken from cultivars carrying vertical resistance genes were highly aggressive. Since the four sunflower hybrids in the present study were isogenic except for their *Pl* genes, it is probable to consider that selection pressure was mainly applied on criteria linked to virulence (Tourvieille de Labrouhe *et al.* 2010). The results obtained showed positive effects of certain modes of *Pl* gene management on aggressiveness factors. This effect no doubt depends more on the number of susceptible plants than on direct selection pressure of monogenic resistances. It could be suggested that management of *Pl* genes which reduce the number of susceptible plants, limits selection pressure for more aggressive strains, but increases the risk of appearance of new virulence. In contrast, management modes which lead to a higher number of diseased plants (mixtures and alternation), may slow down the appearance of new virulence (Tourvieille de Labrouhe *et al.* 2010), but could favor more aggressive strains. This conclusion must be taken into account in the choice of methods to obtain durable control of sunflower downy mildew with both qualitative and quantitative resistance.

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POLISH SUMMARY

WPŁYW SELEKCYJNEJ PRESJI ODPORNOŚCI JAKOŚCIOWEJ NA AGRESYWNOŚĆ GRZYBA *PLASMOPARA HALSTEDII*

Przedmiotem badań była zmiana agresywności w populacjach 710 rasy grzyba *Plasmopara halstedii* (mączniak rzekomy słonecznika) w zależności od zróżnicowania selekcyjnej presji odporności jakościowej. Następujące czynniki wzięto pod uwagę: mieszaninę, przemienność oraz monokulturę głównych genów odporności. Dla celów porównawczych włączono populacje patogena nie poddane działaniu presji selekcyjnej. Dwie linie hodowlane słonecznika o różnym poziomie odporności jakościowej wykorzystano do pomiaru 4 kryteriów agresywności patogena: procent porażenia, okres latencji, obfitość zarodnikowania oraz skrócenie hipokotylu siewek roślin słonecznika. Izolaty grzyba *P. halstedii* rozmnażane w warunkach presji mieszaniny genów odporności wykazywały najwyższą gęstość zarodnikowania, a porażone siewki słonecznika posiadały najkrótszy hipokotyl. Izolaty grzyba rozmnażane w warunkach przemienności (alternation) genów odporności charakteryzowały się skróconym okresem latencji i powodowały też skrócenie hipokotylu siewek słonecznika. Nie stwierdzono istotnych różnic pomiędzy populacjami rozmnażającymi się pod wpływem działania pojedynczych genów odporności a populacjami nie poddanymi presji selekcyjnej. Różnice dotyczyły liczby porażanych roślin. Uzyskane wyniki badań dowodzą, że metoda operowania genami *Pl* wpływała na agresywność patogena, a tym samym wzrost liczby podatnych roślin.