Relationship Between Increased Virulence and the Aggressiveness Traits of *Melampsora medusae*

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ABSTRACT

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Five radiation-induced, mono-uredium derived mutants of *Melampsora medusae* were compared with the wild-type race 5A (from which they were derived) for certain traits of aggressiveness (period to flecking, formation of first uredium, production of 50% of the uredia; number of uredia and urediospores produced per unit leaf area; and number of uredia produced per day) on leaf disks of both a resistant (on which the mutants are virulent) and susceptible cultivar of poplar (on which the mutants can arise). The cultivar, the mutant line, and their interaction were significant contributors to variation in the traits of aggressiveness. On the susceptible cultivar, the wild type was more aggressive (incubation period, numbers of uredia, and spore production) than the mutants; hence, the traits of aggressiveness, other than the latent period, were negatively correlated with the increase in virulence. Although there were quantitative differences among the mutants for traits of aggressiveness on the susceptible cultivar, when ranked for these traits the mutants formed a cluster distinctly removed from the wild type. Hence, with these isolates, the range in aggressiveness appears to be related to the virulence makeup of the genotype. The delayed initiation and slower progress of disease indicated the "slow-rusting" nature of the resistant cultivar. It is concluded that virulent races are relatively less fit to survive on the susceptible cultivar, while their fitness on resistant cultivars depends on the background genotypes of the host.

Additional key words: avirulence, cost of virulence, host-pathogen interaction, leaf rust, mutation, parasitic fitness, resistance.

Although mutations to virulence occur at low frequency in fungal plant pathogens, the large size of the fungal population ensures that mutant alleles arise continuously (3). For example, in wheat leaf rust, Parlevliet and Zadoks (10) estimated that around 1,000 new virulent pathogen mutants arise per locus per day per hectare. Leonard (6) concluded that relative fitness of pathotypes depends on the interactions of the cost of virulence, effectiveness of resistance, and advantage of the virulent race on hosts with corresponding genes for resistance. Similar theoretical studies have been conducted to examine the aspects of fitness of new virulent mutant genotypes arising in a population of avirulent plant pathogens (7,12). However, precise comparative studies of aggressiveness in wild-type and mutant isolates on susceptible and resistant host cultivars are lacking. Information from such studies is helpful in understanding pathogen evolution, in predicting population shifts, and in planning an effective strategy of disease management through host resistance.

Mutants virulent on *Populus deltoides* Marsh. 'T-173' (a resistant cultivar) were induced by gamma irradiation in race 5A of *Melampsora medusae* Thum. which is avirulent on this cultivar (13). The qualitative resistance of this cultivar to race 5A appears to be controlled by a single dominant gene (*unpublished*). The five mutant lines and the wild type are virulent on *Populus* × *euramericana* (Dode) Guinier cultivar 'I-488,' a universal suscept. The mutant lines were also virulent on cultivar T-173.

The objectives of this study were to compare the aggressiveness traits of the five mutants with those of the wild type on the susceptible cultivar I-488 and to examine the relative resistance of cultivar T-173 to the virulent mutants.

MATERIALS AND METHODS

The mutants, designated M1, M2, M3, M4, and M5, were induced in race 5A by 200, 600, 100, 100, and 100 Gray (Gy) doses of gamma irradiation, respectively, from a Co^{60} source; they were random selections from many such mutants virulent on cultivar

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T-173 (13). The mutants and the wild-type race 5A were multiplied initially on detached leaves of cultivar I-488, supported on gibberellic acid (10 mg/L), for five cycles to produce sufficient urediospores for the study. Race 5A had undergone about 15 cycles on I-488 before the induction of the mutants.

Cultivar I-488 is a selection from Italy and cultivar T-173 is a selection from a Texas provenance. Leaves of uniform age (about 3 mo) (16) were harvested from plants of both cultivars, raised in a rust-free glass house (20 ± 3 C, 16-hr photoperiod), and disks (1.70 cm^2) were punched from surface-sterilized leaves of each cultivar. For each isolate, 5 mg of freshly harvested, dried urediospores were deposited on 15 leaf disks of each cultivar and on five coverglasses (1.32 cm^2) placed randomly on a wet cardboard template in the base of a spore settling tower (16). The spore tower was sterilized (sprayed with 90% ethanol and blown dry) between inoculations. Uniformity of deposition within and between inoculations and germinative potential (>95%) was checked on the coverglasses. Such uniformity in the inoculation permitted precise comparison of aggressiveness of the isolates and of the resistance/susceptibility of the cultivars. Inoculated leaf disks were placed on plastic foam soaked with gibberellic acid solution (10 mg/L), sealed in 14-cm-diameter glass petri dishes, and incubated for up to 21 days in controlled growth cabinets at 16 ± 1 C, 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ light intensity and with 16-hr light and 8-hr dark periods (16).

Assessment of aggressiveness. In this study, "aggressiveness" is the quantitative measure of traits of disease expression by an isolate on cultivar(s) within a monocycle and does not imply lack of specificity (c.f., Vanderplank [18]).

The traits of aggressiveness, measured directly in the disease monocycle, were: incubation period (days) to flecking (*IPF*); flecks, localized chlorotic areas, the initial symptoms of disease; latent period (days) to eruption of the first uredium (*LP1*); latent period (days) to eruption of 50% of uredia (*LP50*); mean number of uredia per leaf disk recorded daily; cumulative number of uredia per leaf disk (*ULD*), assessed at the end of disease monocycle (when at least 80% of the pustules commence disintegrating), indicative of infection efficiency; number of uredia produced per leaf disk per day (*UPD*), indicative of the rate of disease progression, was the ratio of *ULD* to the length of the disease monocycle in days; and number of urediospores produced per square millimeter of leaf surface (*USM*), indicative of reproductive potential. Short *IPF*, *LP1*, and *LP50* and high *ULD*, *UPD*, and *USM* are indicative of high aggressiveness of an isolate on a cultivar.

For the calculation of *USM* after *ULD* had been recorded, 10 randomly selected leaf disks, in two groups of five, were transferred to 10 ml of a solution of 0.1% agar, containing 10 drops per liter of polyxyethylene monolaurate (Tween-20), in McCartney bottles. The urediospores were dislodged by vigorous shaking of the bottles containing the solution for 45 min on a mechanical shaker and spores were counted with a haemocytometer.

Although occasional uredia occur on cultivar T-173 in the field, as a precautionary measure in the present study the leaf disks were steam sterilized before being discarded.

Statistical analysis. The experimental design was of a factorial treatment consisting of six isolates × two cultivars with 15 replications (leaf disks) per treatment (Table 1). For all the disease parameters, data were tested for the requirements of homoscedasticity and normality of error variance (8). The data for *ULD* was square root transformed and that of *USM* was $\log_e + 1$ transformed for ANOVA and to compute significance of differences between means, to satisfy the above requirements. However, the untransformed values for these two traits have been presented in Table 2. The data were subjected to analysis of variance by using a fixed-effect ANOVA model employing the statistical package GENSTAT (1) with the inclusion and exclusion of race 5A in separate analyses. Signficance of differences between transformed means of the isolates were tested with Student's *t*-test based on least significant differences.

For all isolates on both cultivars, individual curves of disease progress were constructed based on daily observations of numbers of uredia per leaf disk (recorded from 2 days after the date of eruption of first uredium; mean of 15 replications) over the length

TABLE 1. Summary of variance[a,b] for six aggressiveness traits[c] of five mutant lines of *Melampsora medusae* on two cultivars of poplar (*Populus* × *euramericana* cultivar I-488 and *Populus deltoides* cultivar T-173

Source	Degrees of freedom	*IPF*	*LP1*	*LP50*	*ULD*	*UPD*	*USM*
Mutants	4	0.61	2.61***	5.21***	14.41***	5.17***	2.83***
Cultivars	1	72.50***	423.36***	879.84***	67.92***	71.81***	13.93***
Mutants × cultivars	4	0.61	3.21***	3.88*	4.32***	1.56***	1.35***
Residual[d]	140	0.59	0.48	0.66	0.92	0.38	0.16
Total[d]	148	1.08	3.44		1.84	1.03	0.40

[a] Mean sum of squares.
[b] Asterisks *** and * indicate values that are very highly significant ($P \leq 0.001$) and significant ($P \leq 0.05$).
[c] The six aggressiveness traits are: incubation period to flecking (*IPF*), latent period to eruption of first uredium (*LP1*), latent period to eruption of 50% uredia (*LP50*), cumulative uredial number per leaf disk (*ULD*; square root transformed), uredia produced per day (*UPD*), and urediospores produced per square millimeter (*USM*; $\log_e + 1$ transformed).
[d] Residual and total degrees of freedom for *USM* are 109 and 119, respectively.

TABLE 2. Means[v,w] of six aggressiveness traits[x] of the wild-type race 5A and five virulent mutants of *Melampsora medusae* on the susceptible cultivar I-488 and the resistant cultivar T-173

	Cultivar I-488 (S)						Cultivar T-173 (R)					
	5A	M1	M2	M3	M4	M5	5A[y]	M1	M2	M3	M4	M5
IPF	5.0 a	5.2 a	5.1 a	5.1 a	5.0 a	5.1 a	···	6.3 bc	6.2 b	6.8 c	6.2 b	6.7 c
LP1	9.0 b	8.5 a	8.4 a	8.3 a	8.0 a	8.3 a	···	11.3 cd	10.9 c	12.3 e	11.6 d	12.3 e
LP50	12.1 b	10.6 a	10.8 a	10.8 a	10.7 a	10.8 a	···	14.4 c	15.3 d	16.2 e	16.2 e	15.7 de
ULD[z]	75.8 a	32.7 de	37.4 cd	33.5 de	51.2 b	42.6 bc	0.0 h	11.7 g	30.6 de	23.7 f	27.2 e	34.7 de
UPD	5.0 a	2.1 d	2.5 cd	2.2 d	3.4 b	2.8 c	0.0 h	0.6 g	1.5 e	1.1 f	1.3 e	1.7 e
USM[z]	1299.0 a	439.9 cd	566.2 c	393.4 d	1051.5 ab	817.4 b	0.0 e	172.8 d	373.8 d	401.9 d	348.1 d	388.5 d

[v] Each value is a mean of 15 observations.
[w] Within a trait, isolates followed by the same letter do not differ significantly ($P < 0.05$) based on Student's *t*-test.
[x] The six aggressiveness traits are incubation period to flecking (*IPF*), latent period to eruption of first uredium (*LP1*), latent period to eruption of 50% of the uredia (*LP50*), cumulative uredial number per leaf disk (*ULD*), uredial number per day (*UPD*), and urediospores produced per square millimeter (*USM*).
[y] Race 5A was avirulent on cultivar T-173.
[z] The significance of difference computed by using transformed data (square root for *ULD*, and $\log_e + 1$ for *USM*).

of the monocycle. The curves were fitted by regression analysis (16) employing the following model:

$$y_i = b_0 + b_1 x_i + b_2 x_i^2 + e_i$$

Here, y_i is the fitted disease level on day x_i, and the best fit was decided on maximal coefficient of determination obtained. The three groups of curves (5A/I-488, mutants/I-488, and mutants/T-173) were compared pairwise as follows (16): The residual sum of squares of the fitted curves for a certain pair in a specific combination were added to obtain a total residual sum of squares (r_1) with f_1 degrees of freedom. Then, a single curve was fitted to the combined data for the particular pair to yield a residual sum of squares (r_2) with f_2 degrees of freedom. The difference, $r_0 = r_2 - r_1$ (with $f_0 = f_2 - f_1$ degrees of freedom), is the sum of square of residuals due to the deviation from the hypothesis that curves are the same (the null hypothesis) and was tested for significance by employing an F-test. Mean *UPD* was also computed for these three groups.

RESULTS

Race 5A was avirulent on cultivar T-173 and produced no macroscopic symptoms of the disease. For the traits of aggressiveness, irrespective of the exclusion (Table 1) or inclusion of race 5A (details not presented) in the analysis of variance, cultivar was the most important ($P < 0.001$), and isolate a lesser but significant ($P < 0.001$) (except for *IPF* [NS, $P > 0.05$]), determinant of variability, while the isolate × cultivar interaction was usually a significant contributor to such variation. When the variance of the interaction was included in that of the residual, the differences in traits among isolates and between cultivars still were highly significant (except that of isolates for *IPF*) and can, therefore, be discussed independently despite the significance of the isolate × cultivar interaction (2).

The five mutant lines were more aggressive on susceptible cultivar I-488 than on resistant cultivar T-173 (Fig. 1, Table 2). However, on I-488, the wild type was more aggressive in terms of *IPF, ULD, UPD*, and *USM*, while the mutants were more aggressive for *LP1* and *LP50* (Table 2). When the isolates were ranked in the order of decreasing aggressiveness for all the traits, although on the basis of *ULD* and *UPD* the ranking was relatively consistent, it was inconsistent among other traits, or within traits between cultivars. Such nonuniformity in ranking of mutants within a trait between the cultivars explains the significant isolate ×cultivar interaction in the ANOVA (Table 1). This indicates the quantitative specificity of the isolates on the cultivars for some traits; in some instances this involved reversal of ranking for aggressiveness. For example, on the basis of *USM*, M3 is the least aggressive isolate on I-488 but is the most aggressive mutant on T-173. Similarly, M1 and M2 had the longest latent period (*LP1*) on I-488 but the shortest on T-173 (Table 2).

When the isolates were ranked in the order of decreasing aggressiveness on cultivar I-488, despite the significant differences among them, the mutants formed a cluster distinctly removed from the value for race 5A, in all traits. The apparent differences between the mutants and the wild type were greater for amount of disease produced (basis *ULD*, *UPD*, and *USM*), than in the timing of disease expression (basis *IPF*, *LP1*, and *LP50*) (Table 2).

All three groups of curves for disease progress of the three cultivar × isolate combinations (Fig. 1) differed significantly ($P < 0.01$), indicating that the differences in numbers of uredia per leaf disk of the three groups are not artifacts of the time of observation of cumulative *ULD*. Further, within a group, particularly on cultivar I-488, the ranking of the isolates for cumulative *ULD* (Table 2) parallels that of ranking for disease progress (Fig. 1). The ranking of the race × cultivar combinations for mean uredia produced per day (*UPD*) was 5A/I-488 (5.04) > mutants/I-488 (2.63) > mutants/T-173 (1.24).

The irradiation dose under which each of the five mutants virulent on T-173 was produced was not consistently related to their ranking for aggressiveness for any trait.

The major results were reproduced when the experiment was repeated using one mutant (M2) and race 5A on both the cultivars.

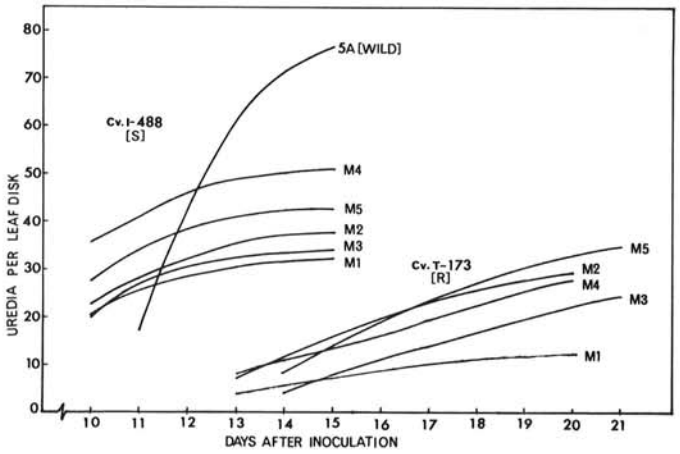

Fig. 1. Disease progress curves (basis, cumulative number of uredia produced daily over the period of the monocycle) of the wild type, race 5A, and the mutants of *Melampsora medusae* on *Populus* × *euramericana* I-488 (susceptible cultivar) and T-173 (resistant cultivar). Note: Race 5A is avirulent on cultivar T-173.

DISCUSSION

The mutant series was selectively isolated from race 5A by their virulence on cultivar T-173. In a separate study, when inoculated on a range of poplar cultivars, the spectrum of virulence was uniform among the mutants, although broader for these than for race 5A (14). Thus, the mutants can be considered to be near isolines of race 5A, differing in virulence genes, but sharing a similar background genotype with the wild type (5–7,15). On the susceptible cultivar, I-488, the mutants, compared to the wild type, were lower in infection efficiency and sporulation capacity but had a shorter latent period (Table 2). Such changes in aggressiveness in the mutants were associated with aquisition of virulence on cultivar T-173 and on certain other cultivars of *P. deltoides* (14), i.e., the virulence factor (or factors) unnecessary for compatibility with I-488. This suggests a negative relationship between the increased virulence spectrum of these isolates and their aggressiveness (for traits of disease severity) on a universal suscept. The minor, albeit significant, differences among the mutants for aggressiveness may be due to the changes in modifying loci. However, the overall virulence/aggressiveness relationship in these results suggests that, while biotypes with differing aggressiveness can occur within a race, the major variation in aggressiveness is associated with differences in the virulence/avirulence makeup of the genotype. This conclusion is supported by the consistency with which, in a rank order test for any trait of aggressiveness, the mutants form a cluster distinct from the values of race 5A.

The cause of reduction in aggressiveness associated with the increase in virulence range (termed 'cost of virulence' [7]) may be direct (i.e., avirulence alleles themselves may behave additively as aggressiveness factors in the absence of corresponding resistance factors [4,11] in the host, and the mutation of such avirulence alleles to virulence would result in reduced aggressiveness) or indirect (i.e., certain aggressiveness factors may be linked to avirulence genes and deletion of this chromosomal segment would result in reduced aggressiveness).

The reduced aggressiveness in the mutants is not due to the residual effect of the irradiation, since irradiation dosage and aggressiveness levels were not correlated. Further, similar reduced aggressiveness was also observed in natural virulent mutants of *M. medusae* (*unpublished*). Finally, the residual effects of irradiation are nonheritable (14), while the reduced aggressiveness of the mutants was reproduced in successive generations (*unpublished*)

and appears to result mainly from its association with increased virulence.

On cultivar I-488, the *ULD*, *UPD*, and *USM* of the mutants were lower than those of the wild type, suggesting that the latter would be a better competitor in an epidemic on a susceptible cultivar. Despite these apparent epidemiological disadvantages, such mutants may survive in low frequencies in polycyclic disease situations due to their shorter latent period (Table 2).

The cultivar constitution was the most important contributor to variation in the constituent traits of aggressiveness (Tables 1 and 2) and a significant factor in disease progress (Fig. 1). Possibly, the disparate geographic origins of these cultivars accounts for their considerable contribution to such observed variation. Although the mutants overcame the major-gene resistance of T-173 to race 5A, they were still confronted with an array of rate-reducing mechanisms in this cultivar. Within a monocycle, the increased latent period and reduced rate of disease progress for the mutants on T-173 (Fig. 1) would delay the initiation of the following cycle by 4–5 days and considerably reduce the inoculum available for that cycle, compared with that on I-488. This indicates an association between rate of disease progress and initial disease inoculum (9).

The rate-reducing mechanisms in T-173 (Fig. 1) were not observed in *P.* × *euramericana* 'I-154,' the reaction of which to race 5A changed from resistant (mesothetic) to highly susceptible to these mutants in an earlier study (14). Thus, the usefulness in a breeding program of a host cultivar which contains a 'defeated' resistance gene probably depends on the background genotype of the host (17). The newly virulent gene may not necessarily enjoy high aggressive advantage on the corresponding resistance gene (c.f., '*a*' values, as used by Leonard [6]).

Watson (19) and Leonard (6) have suggested that for a virulent mutant to be epidemiologically successful, it should arise in an aggressive genotype. While our results support this view, these emphasize that increased virulence was associated with reduced infection and sporulation on the susceptible cultivar. Thus, for a virulent mutant to be epidemiologically competitive on a susceptible cultivar distinct from the resistant cultivar, it should arise in a biotype with aggressiveness higher than that of the mean of the avirulent population, so that, despite the cost of virulence or genetic load, its fitness would still approximate that of the mean of the avirulent population. However, in the presence of the resistant cultivar, the epidemiological success of such a virulent mutant may essentially depend on the frequency of the resistant cultivar and the consequent selection pressure in the host population.

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