Competitiveness of Mutant and Wild-type Isolates of Colletotrichum gloeosporioides f. sp. aeschynomene on Northern Jointvetch

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ABSTRACT


The competitiveness of mutant and wild-type isolates of Colletotrichum gloeosporioides f. sp. aeschynomene was determined. Experiments were conducted in growth chambers constructed within a greenhouse in which available free moisture and splash dispersal were used to control successive infection cycles. Isolates of nitrate non-utilizing (nit) mutants or benomyl-resistant mutants were introduced with a wild-type isolate onto susceptible northern jointvetch plants in the growth chambers at initial population ratios of 1:1 or 9:1. The proportion of each isolate infecting northern jointvetch was determined at each of seven to 10 subsequent infection cycles. From initial lesion populations, benomyl-resistant lesions decreased to an apparent equilibrium level of 10 to 20% of the total lesion population after three to five infection cycles. In experiments with nit mutants and a wild-type isolate, the population of lesions caused by the nit mutant decreased to an apparent equilibrium of 10 to 30% of the total population in two experiments when initial ratios were 1:1. Comparison of infection-cycle components of mutants with their wild-type parent suggest that the decrease in competitiveness of mutants was associated with a decrease in infectivity, longer latent periods, reduced lesion size, and reduced sporulation.

Additional keyword: mycoherbicides.

Biological control of weeds with fungal plant pathogens has been investigated for nearly three decades, and three fungal pathogens currently are registered as products for biological control of weeds in the U.S. and Canada (3,7,15,20). There is increasing interest in development of specialized and more effective isolates of these and other fungi for biological control of weeds (1,4,10,25,28). The use of mutants selected for resistance to pesticides and the potential use and release of transformed isolates of pathogenic fungi raise questions regarding survival, fitness, and competitiveness of resistant isolates relative to wild-type populations. Fitness and competitiveness are used as defined by Zadoks and Schein (31).

Previous field and greenhouse studies indicated that fungicide-resistant isolates may successfully establish significant populations in the presence of sensitive wild-type isolates (2,8,18,22,24). Schuepp and Kung (22) reported that populations of Botrytis resistant to methyl benzimidazole carbamate (MBC) from grape were stable at nearly 60% of the total population even in the absence of any selection for 4 years. Although Ruppel (21) reported that benomyl-tolerant populations of Cercospora beticola Sacc. declined in mixed populations, Dovas et al. (8) reported that resistant isolates of C. beticola in Greece were more competitive, becoming a greater proportion of the total population, than sensitive isolates in controlled field experiments. McGee and Zuck (19) compared resistant and sensitive isolates of Venturia inaequalis (Cooke) G. Wint. through several sporulation cycles on apple seedlings under greenhouse conditions and found that there were no changes in the original ratios of a combined grouping of resistant/sensitive isolates taken from the same orchard. Lalancette et al. (17) have suggested that due to differences in the fitness of the resistant isolates among orchards, removal of benomyl may allow reversion of the population of V. inaequalis to sensitivity in one orchard but not in another.

Colletotrichum gloeosporioides (Penz.) Penz. & Sacc. in Penz. f. sp. aeschynomene has been used to control the leguminous weed northern jointvetch, Aeschynomene virginica (L.) B. S. P., since 1982 (3,29). Infection by the fungus produces well-defined oval lesions on stems. Use of the fungus has several advantages for the study of multigeneration and inter-isolate competitiveness for both crop pathogens and mycoherbicides. First, the pathogen is easily disseminated by splashing rain (30), infects northern jointvetch under broad temperature conditions, sporulates profusely on lesions, and has a short latent period (3 to 4 days) (29). Second, benomyl-resistant and nitrate non-utilizing (nit) mutant isolates are available. Third, there are no known reports addressing competitiveness with respect to multigeneration studies that utilize splash dispersal for reinoculations between generations. Fourth, there are no known studies on multigeneration competitiveness for any Colletotrichum species. The comparison of mutant, genetically engineered, and wild-type isolates of mycoherbicides may provide data to demonstrate the epidemiological parameters for the assessment of the overall competitiveness of introduced mutants in a wild population.

Previous methods for study of fungal competition under controlled conditions generally require collecting inoculum from previously inoculated plants or detached leaves followed by reinoculation of healthy plants in greenhouse experiments (2,11,12,14). The role of epidemiological parameters such as dispersal and survival are excluded in the process of evaluation. The results

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of these competition studies generally show that isolates sensitive to fungicide are undetectable after a number of generations.

The objective of this study was to determine the relative fitness of wild-type and selected mutants of *C. g. f. sp. aescynomene* in controlled microcosms over multiple generations and infection cycles.

**MATERIALS AND METHODS**

**Derivation of mutant isolates.** A single isolate of *C. g. f. sp. aescynomene*, 3-1-3, was used to generate all mutant isolates and was further used for comparisons of competitiveness. Cultures of all isolates were maintained in cryogenic storage at −80°C.

Benomyl-resistant mutants were generated previously by ethylmethanesulfonate mutagenesis of parental isolate 3-1-3 (25). Isolates of benomyl-resistant mutants grew on Torula agar amended with MBC at 100 μg per ml (TAB) but the wild-type did not grow at 1 μg per ml. On Torula agar without MBC (TA), benomyl-resistant mutants grew more slowly than the wild-type isolate. Benomyl-resistant mutants were maintained on TA and grown on TAB to verify resistance. Two mutants, B18 and B21, were selected for this study.

*Nit* mutants were generated as described previously (5,16). Mycelial plugs taken from colonies of wild-type isolate 3-1-3 were placed on chlorate medium and incubated at 28°C. After incubation, sectors or colonies emerging from the plugs were isolated onto TA medium. Mutant isolates were further characterized by plating to differential media (5,16). All benomyl-resistant and *nit* mutants appeared to be stable during these experiments following routine subculture or after reinoculation from plants onto TA or on selective nitrate media.

**Competition.** Competition experiments were conducted in 1991, 1992, and 1993. Polyethylene enclosed chambers (4 × 1.2 × 1.2 m) permitting maintenance of semi-aquatic conditions used in the experiments were built within a greenhouse. Each chamber was separated into two smaller, equal-size test plots with a polyethylene sheet placed vertically at the center of the chamber.

Supplemental lighting was supplied daily with four 40W fluorescent lamps placed 0.6 m above the chamber between 1600 and 2100 h. The temperature within the greenhouse was maintained between 20 and 30°C.

Surface-disinfested seeds of northern jointvetch were planted in plastic pots (7 × 7 × 6 cm) filled with vermiculite (four seeds per pot). A total of 72 pots, 4 plants per pot, were placed within each experimental plot (288 plants per plot) in a 6 × 12 matrix. The experimental plots were flooded to keep plants in water 5 cm in depth. Plants were fertilized once weekly with a solution of Peter's fertilizer (Grace-Sierra Horticultural Products, Milpitas, Calif.) (20-20-20 for N-P-K).

When plants were about 50 cm in height, a wild-type isolate and a mutant were introduced into the experimental plots. The isolates used were 3-1-3 (wild-type), B21 (benomyl-resistant), and 3-1-3/3A (*nit* mutant). Inoculum was obtained from the parental, benomyl-resistant, and *nit* mutant isolates grown on TA at 28°C. Spores were collected from the surface of 7-day-old cultures, and suspensions of each isolate were adjusted to 5 × 10⁵ spores per ml. Plants, 30 cm in height, were sprayed with spore suspensions and incubated in a dew chamber at 28°C for 2 days. Those that developed eight to 10 lesions were selected as sources of initial inoculum for competition experiments.

Twenty diseased plants (2 plants per pot, 10 pots) were introduced into each experimental plot. For each isolate, the number of pots introduced into an experimental plot depended on the initial ratio desired (Table 1). For the competition experiments between the *nit* mutant and the wild-type isolate, only an initial 1:1 ratio of lesions was tested. In the four experiments with benomyl-resistant isolate, three experiments were conducted in which initial lesion ratios were 1:1 (mutant/wild-type) while the fourth experiment had an initial lesion ratio of 9:1 (mutant/wild-type). Infected plants were evenly distributed at five points within a plot for the 1:1 ratio or centered as a point source for the 9:1 ratio.

There were two replicates (experimental plots), except for the 1992 experiment in which there were four replicates, and each replicate had a total of 308 plants (288 healthy plus 20 plants introduced as the sources of initial inoculum).

There were seven to 10 disease cycles (periods from dispersal to sporulation) in each experiment. Because of the sticky nature of spore matrix, rain splashing is necessary for spore dissemination (30). A 10-min overhead sprinkler irrigation was provided to each plot for each disease cycle at approximately 1800 h to facilitate dispersal of the inoculum from lesions to healthy tissues. The top of the chamber was then covered and the entire chamber misted for 14 h with a humidifier. Thus, inoculation and infection of plants between each cycle were controlled by rain splashing and misting. Symptoms were observed 2 to 3 days after inoculation, and after 7 days all lesions had sporulated. Plants were usually killed after two to three disease cycles, and dead plants were replaced with healthy plants.

Six days after the inoculation of each cycle, 2 × 2 mm of diseased tissue was excised from the edge of each of about 100 new lesions. Excised tissue was placed in 0.5% sodium hypochlorite solution for 40 sec, transferred to TA medium, and incubated at 28°C for 2 days. Hyphal tips from the leading margins of colonies were then transferred to TAB medium in the experiments for identification of benomyl-resistant mutant colonies. In the experiments with the *nit* mutant, the mycelia from the leading edges of colonies were transferred to minimal NO₃ medium (6) for further characterization of *nit* mutant colonies. On minimal nitrate medium, the wild-type isolate grew as expansive aerial colonies while the *nit* isolate grew as very thin, submerged colonies.

In all experiments, the frequency at which the mutant was recovered from the lesions collected after each cycle was determined as the proportion of isolates differentiated from the wild-type isolate on selective media to the total number of colonies isolated on TA media. Data from replicates were combined for each experiment to calculate means and standard deviation using SAS (SAS Institute, Cary, N.C.).

**Disease development.** The development of disease, measured as the number of lesions per plant during the first three disease cycles, was monitored in two experiments initiated in spring and fall utilizing mutant B21 and wild-type isolate 3-1-3 at an initial

<table>
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<th>Replicate number</th>
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<td>6</td>
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lesion ratio of 1:1. In these experiments, the number of lesions was counted on each plant for the first three disease cycles, and the sum of the lesions was calculated for each plot to determine the size of the pathogen population.

**Disease components.** Two experiments were conducted to compare the infection components (latent period, infectivity, lesion size, and sporulation) of the wild-type isolate and mutants. Seeds of northern jointvetch were germinated on moistened filter paper in petri dishes at 28°C for 24 h. Uniformly germinated seeds were then planted in vermiculite in 7 × 7 × 6 cm plastic pots, 4 seeds per pot, and placed in growth chambers (Model E7 Conviron, Control Environments, Pembine, N. Dak.) at 28°C with a 15-h photoperiod (approximately 185 μE). Plants were thinned to three plants per pot, and only plants with a diameter of 3.5 mm and a height of approximately 30 cm were used. For each isolate, a 5-ml spore suspension, prepared as described above, was sprayed evenly onto each of three pots (replicates). Following inoculation, all plants were placed into a dew chamber at 28°C for 24 h. All plants were then moved to the growth chambers for further incubation and observation.

Plants were observed every 24 h after inoculation to quantify latent period, the number of lesions per plant, and lesion size. On each day, new lesions on each plant were marked and numbered. The width and length of each lesion were measured every day until day five. When lesions merged with adjacent lesions, no further measurements were taken. Lesion size was calculated with the formula area = (length × width)/2. The total number of lesions per plant were counted. The latent period for each isolate was calculated as a weighted mean using the number of lesions per day as the weight. The amount of sporulation for each isolate was determined by submerging each of ten lesions of uniform size (approximately 3 × 10 mm) in 4 ml of water, agitating for 2 min and counting the number of spores in suspension with a hemacytometer. Infection efficiency was measured as the number of lesions per plant.

**RESULTS**

**Mutants.** All mutants appeared to remain stable during the course of experiments as evidenced by recurrent isolation on TA or TAB and nitrate media. The mutants also remained pathogenic on northern jointvetch, producing lesions in all experiments.

**Disease progress over time.** The number of lesions in the spring experiment and fall experiment increased with time (Fig.

![Fig. 1](image)

**Fig. 1.** The total number of lesions caused by benomyl-resistant mutant B21 and the wild-type parental isolate 3-1-3 on 288 northern jointvetch plants in greenhouse microcosms during the first three infection cycles in two experiments conducted in May or September of 1991.

![Fig. 2](image)

**Fig. 2.** Proportions of benomyl-resistant mutant B21 and wild-type isolate 3-1-3 of *Colletotrichum gloeosporioides* f. sp. * aeschynomene* in four experiments over several induced infection cycles following introduction of isolates to healthy northern jointvetch plants. In experiments 1 through 3, the introduced populations of mutant B21 and isolate 3-1-3 were equal. In experiment 4, the benomyl-resistant mutant was introduced at 9 times the level of the wild-type isolate.
In the spring experiment, the number of lesions increased from 361 in the first infection cycle to 1,011 lesions per plot by the second infection cycle, while in the fall experiment the number of lesions increased from 544 lesions per plot in the first infection cycle to 2,541 lesions per plot by the third cycle. After the second infection cycle, a few plants near the initially introduced diseased plants were killed by infection with C. g. f. sp. aescynomenae. The average number of lesions per plant for the May and September experiments were 1.25 and 1.88 for the first, and 3.51 and 8.8 for the third generation, respectively. The disease population reached a maximum after three infection cycles.

Competitiveness of the benomyl-resistant isolate. Lesions caused by the wild-type isolate often could be distinguished from lesions caused by mutant B21. Lesions caused by B21 were generally smaller than those of the wild-type isolate and had fewer acervuli and visibly reduced sporulation. The number of infected plants in the plots generally progressed to 100% after three infection cycles, and some plants were killed after three infection cycles.

An apparent equilibrium, at which a mutant isolate had consistent proportion over generations, was established in each competition experiment. In experiment 1 (Fig. 2), the mutant lesion population fell to approximately 15% of the total population (proportion = 0.15) within one infection cycle, rebounded slightly to about 25% in the second infection cycle, then stabilized at approximately 10 to 15% of the population for all remaining cycles. In experiment 3 (Fig. 2), the population of lesions caused by benomyl-resistant mutant decreased to about 15% of the total population after the first two infection cycles, then increased slightly for two infection cycles before declining to less than 10% of the population. The proportion of the benomyl-resistant lesions appeared to stabilize at approximately 10% of the total population at ten generations in both experiments.

In experiment 2 (Fig. 2), the benomyl-resistant mutant decreased to about 20% of the total lesion population by the third infection cycle and then increased again. In the last three infection cycles (late spring), there was no significant difference (P = 0.05) between wild-type and mutant lesion populations (Fig. 2).

In experiment 4 (Fig. 2), in which the number of mutant lesions was introduced into the plots nine times the level of the wild-type lesions, the proportion of mutant lesions decreased at a constant rate from the initial proportion of 90% to only 24% of the population sampled by seven infection cycles.

The rate of decrease of benomyl-resistant lesion populations with respect to the wild-type populations was about 9.4% of the total population per infection cycle when the mutant lesion population was nine times greater than the wild-type (Fig. 2). In comparison, the rate of decrease was 13% for the first three generations in the first three experiments in which populations were initiated at equal levels.

Competitiveness of nit mutants. In experiment 5 (Fig. 3), the population of lesions caused by the nit mutant decreased to about 30% after two infection cycles and then varied between 30 and 40%. In experiment 6 (Fig. 3), the proportion of mutant isolate decreased to about 15% after one infection cycle and appeared to stabilize at a level of 10% for the remainder of the experiment.

Disease components. There were significant (P = 0.05) differences in the number of lesions per plant, lesion size, and sporulation among the isolates. The latent periods of benomyl-resistant mutants B18 and B21 were significantly greater than that of the wild-type isolate, 3-1-3 (P = 0.05). The latent period for mutant B18 was 5.5 days, 1.4 days greater than for isolate 3-1-3 and 0.6 days longer than for mutant B21. The latent periods for the nit isolates were not significantly different from the latent period for isolate 3-1-3.

The infection efficiency, measured as lesions per plant, of benomyl-resistant mutants was significantly less than that of isolate 3-1-3 (P = 0.05) (Table 2). Mutants B18 and B21 caused an average of only 3.7 and 5.2 lesions per plant, respectively, while isolate 3-1-3 caused an average of 13.7 lesions per plant. The nit mutants varied in infectivity to northern jointvetch. Although mutant 3-1-3/A produced as many lesions per plant as did the wild-type isolate, mutant 3-1-3/E produced only an average of 9.8 lesions per plant. Both nit mutants produced more lesions per plant than did the benomyl-resistant mutants.

Lesion size on plants varied considerably among isolates, as shown in Table 2. In general, lesions produced by the nit mutants and the parental isolate were not significantly different, ranging from 17.3 to 22.7 mm² after 5 days incubation in the growth chamber at 28°C. Although the sizes of the lesions produced by

![Fig. 3. Proportions of nitrate non-utilizing (nit) mutant 3-1-3/A and wild-type isolate 3-1-3 of Colletotrichum gloeosporioides f. sp. aescynomenae in two experiments over several induced infection cycles following introduction of isolates to healthy northern jointvetch. In both experiments, the populations of the nit mutant and wild-type isolate were initially equal.](image)
mutant B21 were generally smaller (averaging only 11.1 mm²) than those of isolate 3-1-3, they were not statistically significantly different. However, mutant B18 produced lesions significantly smaller than any of the other isolates tested. Lesions produced by mutant B18 were, on average, only 5.3 mm², approximately one-half to one-fourth the size of lesions produced by other mutants tested.

Sporulation by nit and benomyl-resistant mutants was reduced from levels produced by the parental wild-type isolate 3-1-3 (Table 2). Lesions caused by isolate 3-1-3 produced, on average, approximately 1 million spores per lesion, while the benomyl-resistant mutants B18 and B21 caused an average of less than 2 x 10⁵ spores per lesion, only one-fifth the number produced by isolate 3-1-3. Lesions caused by nit mutant 3-1-3/A produced 6.4 x 10⁵ spores per lesion, approximately two-thirds the number produced by isolate 3-1-3.

**DISCUSSION**

The results of these experiments show broadly that nit and benomyl-resistant mutants of C. g. f. sp. aescynomone are less competitive than a wild-type isolate in greenhouse experiments. The reduced competitiveness of the benomyl-resistant mutants may have resulted from a combination of longer latent periods, decreased sporulation on lesions, decreased infectivity, and reductions in lesion size. The microcosms in this study include important epidemiological parameters, such as survival and dispersal, to reflect population dynamics and were effective in testing multitemperature competition. Our design gives a more accurate prediction of how mutants would compete under field conditions.

The rates of decrease for mutant lesion populations (9 to 13% per generation) suggest a strong competition of wild-type isolates with mutant isolates in our study. Such competition may be explained by the nature of the pathosystem in which rain splashing is the major dispersal mechanism for C. gloeosporioides f. sp. aescynomone. In a dispersal study in which simulated rain was used (30), Yang and TeBeest demonstrated that about 95% of the infections occurred within the portions of plant stems 10 cm above ground surface. The height of vertical dispersal is limited by the height of rebounding (30). Such a vertical dispersal mechanism limits the number of available infection sites, which may result in competition for resources, as indicated by the results of experiments 1, 2, 3, and 6. The proportion of mutants reached an apparent equilibrium after the third infection cycle (Figs. 2 and 3) when the number of lesions was at its maximum (Fig. 1). Our component experiments (Table 3) showed that the mutants produced fewer infections and smaller lesions. As a result, mutants may produce fewer infective spores (reduction in fitness) than does their parent in the competition for infection sites. Previous experiments (12) with metalaxyl-resistant isolates of *Phytophthora infestans* have related reduction in fitness to the reduction in competitiveness of mutants.

Although the benomyl-resistant mutants were less competitive and less fit than the parental wild-type isolate, an equilibrium was nevertheless established (Fig. 2, experiments 1, 2, and 3) and the isolate did not become extinct during the course of the competition experiments. In previous studies (12) with metalaxyl-resistant isolates of *Phytophthora infestans*, in which only competition related to infection was examined, the more competitive isolates reached 100% in frequency, and no equilibrium was demonstrated. The fact that apparent equilibria were reached in our experiments suggests that less-fit benomyl-resistant mutants may have a higher than expected potential to survive under natural conditions without continuous selection pressure to favor their survival.

Studies (23) with *Venturia pyrina* Aderhold indicate that benomyl-resistant isolates persisted in pear orchards for 7 years in the absence of fungicide selection. Shabb et al. (23) suggested this may be due to a lack of marked deficiencies in the mutants. Similarly, Spotts and Cervantes (24) showed that the frequency of isolates of *Penicillium* spp. and *Botrytis* spp. resistant to benomyl did not change in a pear orchard that had not been sprayed with benomyl for 5 years. The fact that benomyl-resistant mutants reached an equilibrium as in our studies may provide an explanation of Spotts and Cervantes’s observations. Furthermore, the persistence of mutants at apparent equilibrium level may help to explain why genetic diversity is widespread and maintained in *C. gloeosporioides*.

Many plant pathogens have been investigated as potential mycoherbicides, and recent reviews have discussed increasing the effectiveness of potential mycoherbicides by employing recombinant DNA techniques (26,27). Data describing the survival and competitiveness of genetically transformed isolates under controlled conditions would be required before future field tests of genetically engineered mycoherbicides could be done. This information also would be important to the assessment of any risk associated with the release of genetically engineered mycoherbicides since the fate of a modified microbe is a major component in risk assessment (9).

The test chambers used in our study have one particular advantage over other methods in which successive infection cycles are used to estimate competitiveness. Other methods generate disease in successive generations by first collecting spores from old leaves and then spraying them onto new plants. Collection of spores prevents evaluation of important epidemiological factors, such as dispersal and survival of spores, while our experiments permitted a more comprehensive test and evaluation of these critical factors. However, one limitation is that chambers such as the ones we used require a large amount of space in which to conduct the tests and in which to grow replacement plants for the experiments. Space limitations frequently reduced the number of infection cycles and replicates that could be completed at one time.

Our results also demonstrated a potential limitation for competition experiments conducted in greenhouses. The results of experiment 2, conducted during the summer, contrasted sharply with other experiments. During the last three infection cycles, there was little difference in the frequency between the benomyl-resistant mutant and the parental isolate. These contrasting results may be due to the higher temperatures in the greenhouse during the early summer. It was observed that lesion sizes appeared to be smaller and less sporulic in a greenhouse during the summer months. Katsuya and Green (13) previously showed that temperature affected the competitiveness of two races of *Puccinia graminis* on wheat.

**LITERATURE CITED**

8. Dovas, C., Skylakakis, G., and Georgopoulos, S. G. 1976. The adaptability of benomyl-resistant population of *Cercospora beticola* in north-