Mycelial Growth, Sporulation, and Virulence to Apple Fruit of *Alternaria alternata* Isolates Resistant to Iprodione

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ABSTRACT

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Three isolates of *Alternaria alternata* from apple fruit were examined in the laboratory for sensitivity to iprodione; their EC_{50} values were 2.9, 2.4, and 2.7 μg/ml of iprodione. After 12 days on medium amended with iprodione at 25 or 250 μg/ml, resistant sectors occurred and six resistant isolates were selected and used for further study. Only resistant isolates grew on medium amended with 25 or 250 μg/ml of iprodione. Sporulation in culture varied with isolate and source; isolate WV/1 and its resistant selections produced the most conidia. The amount of fruit rot caused by resistant isolates was similar to that caused by the sensitive isolates when inoculated into nontreated fruit. Dips of apples in iprodione prior to inoculations reduced lesion development of sensitive isolates but had no effect on lesion development of resistant isolates. In this study, the ranges of fitness and virulence of resistant isolates overlapped those of sensitive isolates, and resistant isolates exhibited no discernible differences in cultural characteristics or virulence from the sensitive isolates.

Alternaria rot occurs in apple and pear fruit in most areas of the world. Although the disease seldom causes major losses, the apple cultivar Nittany is very susceptible to Alternaria rot. Nittany, a cross of York and probably Golden Delicious, has very good dessert and processing qualities (20). Rot of Nittany apple caused by *Alternaria alternata* (Fr.:Fr.) Keissl. results in significant losses in some years and is one of the major factors preventing more widespread planting of this cultivar. On most apple cultivars, Alternaria rot is characterized by round, brown to black, dry, firm, shallow lesions, often located around skin breaks, and symptoms often appear within 2 mo after the fruit are placed in refrigerated storage (18). On Nittany, initial infections can take place in the orchard, with lesions appearing either before harvest or during storage (3). Alternaria rot is not controlled with fungicides currently registered for use on apple in the United States, and no fungicide is registered for control of diseases caused by *Alternaria* spp. Iprodione, a dicarboximide fungicide, is moderately effective for control of Alternaria leaf blotch, caused by *A. mali* Roberts, on apple in North Carolina (4).

Resistance to dicarboximide fungicides has been reported for the fruit pathogens *Monilinia fructicola* (G. Wint.) Honey (12–14,21), *Penicillium expansum* Link (15), and *Botrytis cinerea* Pers.:Fr. (7,10). Resistant isolates have been shown to be less virulent and produce fewer conidia than sensitive isolates (5,10) or to be equally virulent (8). There is only one report of *A. alternata* resistance to iprodione, and this deals with laboratory selection of isolates from sweet cherries in British Columbia (9). The virulence of resistant isolates appeared similar to that of the sensitive isolates (9). There are no reports in the United States of *Alternaria* spp. from pome fruits showing resistance to dicarboximide fungicides. The purpose of this research was to contrast the parasitic fitness of iprodione-sensitive and iprodione-resistant isolates of *A. alternata* from apple.

MATERIALS AND METHODS

Sensitivity of initial isolates of *A. alternata* to iprodione and induction of resistant isolates. The three iprodione-sensitive *A. alternata* isolates used in the study were designated WV/1 (from a

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field trial of iprodione [Rovral] formulations where the fungicide failed to control Alternaria rot on Nittany apple), WV/2 (from a latent infection on Nittany apple fruit that emerged after treatment with 2,900 μg/ml of paraquat), and PA/1 (from Nittany apple fruit sampled after 2 mo in refrigerated storage). Mycelial plugs from these isolates were placed in 9-cm-diameter petri dishes containing 2% potato-dextrose agar (PDA) amended with 0, 0.25, 2.5, 25, or 250 μg/ml of iprodione (Rovral 50WP). Fungicide suspensions were prepared in sterile distilled water, and appropriate quantities were added to autoclaved, warm (45–50 C) PDA immediately prior to its being dispensed into petri dishes. Dishes with mycelial plugs were incubated in the dark at room temperature (21–23 C). Two measurements of colony diameter were made at right angles to each other after 4 days. The experiment was conducted three times with three replicates per treatment, and the data were analyzed by regression of the probit of the percent inhibition against the $\log_{10}$ concentration of iprodione (17). Resistant sectors that had developed from mycelial plugs after 12 days on medium containing 25 or 250 μg/ml of iprodione were transferred as mycelial tips to nonamended agar. To further refine the EC_{50} values, two additional experiments were conducted with nonamended medium and medium amended with 0.25, 2.5, 5, or 10 μg/ml of iprodione. The experiments were performed as above.

Mycelial growth and sporulation on amended agar. The three sensitive isolates and six derived iprodione-resistant isolates were assessed for mycelial growth and sporulation on nonamended medium and medium amended with 0.25, 2.5, 25, or 250 μg/ml of iprodione. A single 5-mm-diameter mycelial plug from each isolate growing on nonamended agar was transferred to each dish in the concentration series. Colony diameter was measured after 4 days. The experiment was performed three times with three replicates per isolate. Data were analyzed by linear regression of the mean colony diameter against the $\log_{10}$ concentration of iprodione in amended medium.

At the end of the growth study, each of the dishes was assessed for production of conidia. A 1-cm-diameter plug was removed from each colony with a cork borer, placed in a dish with five drops of 0.05% Tween 20, and scraped with a scalpel; spores were counted with a hemacytometer. To test the isolates for percent germination of conidia, a drop of conidial suspension was placed onto the surfaces of nonamended PDA and covered with a coverslip. After approximately 5.5 hr at room temperature (21–23 C), 50 conidia per isolate were examined for germination. A conidium was considered germinated if the length of the germ tube was greater than the width of the conidium. The data on numbers of conidia per isolate produced in relation to the concentration of iprodione in the medium were analyzed by linear regression (17). Data from the three runs on growth and sporulation were tested for homogeneity of error variance (Bartlett's test [19]), and it was determined that the data could not be combined. Thus, results from individual runs are reported separately or data from only one run are presented.

Virulence of iprodione-resistant isolates. The three initial iprodione-sensitive isolates and six resistant selections were grown on nonamended PDA for 7 days. Fruit of the apple cultivar Nittany were removed from refrigerated storage and surface-disinfested by dipping them into 0.625% NaOCl for 2 min, rinsing them in sterile distilled water, and drying them overnight before inoculation. Each fruit was inoculated on the side with a 5-mm-diameter mycelial plug placed into a 5-mm-diameter, 3-mm-deep wound made with a cork borer. The inoculation site was sealed with cellophane tape. Inoculated and control fruit were placed on paper trays in plastic boxes that were enclosed within opaque plastic bags. Controls consisted of sets of fruit wounded and either not inoculated or inoculated with plain agar. Temperature during incubation ranged from 21 to 23 C. Lesion diameter was measured in two directions at right angles to each other after 7 and 14 days of incubation. Five replicate fruit were used for each isolate, and the experiment was performed twice. After 14 days, isolations from inoculated fruit were made onto nonamended agar and agar amended with 250 μg/ml of iprodione to confirm the presence of resistant isolates in rotted tissues. Data were analyzed by analysis of variance (17). Where individual observations could not be used in the analyses because of contamination (mostly by *Penicillium* spp. after 14 days of incubation), unbalanced data were analyzed with the general linear models procedure (17).

Virulence of iprodione-resistant isolates on iprodione-treated fruit. Sets of three (runs 1 and 2) or 10 (runs 3 and 4) replicate fruit for each of the nine isolates were washed, surface-disinfested, and wounded as described above, then dipped for 90 sec in a suspension of iprodione at 1.18 g/L (2 lb/100 gal) and allowed to dry. Fruit were inoculated, incubated, and assessed as described above. A second set of fruit that was washed, surface-disinfested, and inoculated served as the control for the experiment. The experiment was performed four times, twice in 1993 (runs 1 and 2) and twice in 1994 (runs 3 and 4). Data were analyzed as described above.

RESULTS

Sensitivity of initial isolates of *A. alternata* to iprodione and induction of resistant isolates. Regression of the probit percent inhibition against the $\log_{10}$ concentration of iprodione provided EC_{50} values of 2.9, 2.4, and 2.7 μg/ml of iprodione for WV/1, WV/2, and PA/1, respectively (mean values from all five runs combined). There were no significant differences in EC_{50} values for the three isolates. After 12 days on amended medium, all three iprodione-sensitive isolates developed sectors on the medium amended with 25 or 250 μg/ml of iprodione. Six isolates were selected for further study.

Mycelial growth and sporulation on amended agar. In all runs, only the resistant isolates grew on PDA amended with 25 or 250 μg/ml of iprodione. Over the course of the three experiments, mycelial growth in 14 of the 18 tests of resistant strains was reduced relative to the control at these higher concentrations of iprodione. In four instances, growth of the resistant isolates was not reduced by the higher concentrations of iprodione (the R25 strain from WV/2 in the first and second runs and the R250 strain from WV/1 in the second and third runs). In all runs, comparison of EC_{50} values among sensitive and resistant isolates revealed significant differences. For example, in the first run, mean EC_{50} values for the sensitive isolates was 3.42 μg/ml, which was significantly different ($P \leq 0.05$) from the EC_{50} values of the R25 and R250 isolates (86.37 and 76.83 μg/ml, respectively). There were no significant differences in mycelial growth between the R25 and R250 isolates in all runs. Comparisons of the three source/isolate groups showed significant differences in mycelial growth among WV/1, WV/2, and PA/1, with the resistant isolates from WV/1 and PA/1 showing the fastest growth in the first and second runs, respectively, and WV/2 showing the slowest growth in the third run.

Sporulation in culture was highly variable among source/isolate groups across treatments (Table 1). In all runs, the sensitive isolate WV/1 and its resistant segregants produced more conidia (mean 52.3×10^4 conidia per milliliter) than the sensitive isolate and resistant isolates from PA/1 (mean 43.3×10^4 conidia per milliliter), which produced more conidia than those from WV/2 (mean 21.5×10^4 conidia per milliliter) ($P \leq 0.05$, data from experiment 2). In all experiments, sporulation of the three iprodione-sensitive isolates decreased as the concentration of iprodione increased (regression line slope significantly different from 0, $P \leq 0.05$, Table 1). Sporulation of the six resistant isolates in all runs was similar across the range of iprodione concentrations (regression line slope not significantly different from 0, $P \leq 0.05$, Table 1), and the two groups of resistant isolates (R25 and R250) produced similar numbers of conidia. Sensitive isolates and resistant isolates produced similar numbers of conidia at concentrations of 0, 0.25, and 2.5 μg/ml of iprodione. In

Table 1. Parameter estimates, coefficients of determination (R^2), and level of significance for the relationship between sporulation and concentration of iprodione in amended medium for three iprodione-sensitive isolates and six resistant isolates of *Alternaria alternata* from run two

Isolate	Source	b_0[z]	b_1[z]	R^2	Level of P
Sensitive	WV/1	35.15	−0.153	0.26	0.050
Sensitive	WV/2	16.54	−0.072	0.33	0.0002
Sensitive	PA/1	23.16	−0.101	0.23	0.0014
R250	WV/1	63.32	−0.062	0.09	0.29
R250	WV/2	24.05	0.023	0.04	0.49
R250	PA/1	54.73	0.061	0.08	0.31
R25	WV/1	71.93	−0.088	0.09	0.27
R25	WV/2	23.59	0.053	0.17	0.13
R25	PA/1	42.44	0.199	0.24	0.065

[z] b_0 = Intercept and b_1 = parameter estimates for the relationship $Y = b_0 + b_1X$, where Y = conidia per milliliter ($\times 10^4$) and X = concentration of iprodione (μg/ml). Sporulation was determined after 8 days of growth at 20–22 C.

Table 2. Virulence (rot diameter in millimeters) of iprodione-sensitive *Alternaria alternata* isolates and isolates resistant to iprodione inoculated as mycelial plugs into Nittany apple fruit[y]

Resistance level	Isolate/strain source		
	WV/1	WV/2	PA/1
Sensitive	16.4 a[z]	15.1 ab	20.2 a
R250	14.1 ab	17.7 a	22.2 a
R25	13.3 b	12.1 b	18.8 a

[y] Each observation is the mean of 10 replicates from data from two runs combined at 14 days postinoculation. Resistant populations were recovered from sectors of iprodione-sensitive isolates grown for 12 days on PDA amended with either 250 (R250) or 25 (R25) μg/ml of iprodione.

[z] Letters in columns denote significant differences according to Duncan's multiple range test ($P \leq 0.05$).

Table 3. Virulence (rot diameter in millimeters) of iprodione-sensitive *Alternaria alternata* isolates and isolates resistant to iprodione inoculated as mycelial plugs into Nittany apple fruit dipped in 1.18 g/ml of iprodione[y]

Isolate	Isolate/segregant source		
	WV/1	WV/2	PA/1
Sensitive			
Iprodione	6.1 b[z]	7.2 b	7.7 b
Control	12.8 a	13.5 a	12.7 a
R250			
Iprodione	10.1 a	13.0 a	13.0 a
Control	9.2 a	13.2 a	12.6 a
R25			
Iprodione	10.7 a	10.2 a	13.2 a
Control	10.6 a	10.8 a	12.9 a

[y] Each observation is the mean of 14–20 fruit from runs 3 and 4 combined at 7 days postinoculation. Resistant populations were recovered from sectors of iprodione-sensitive isolates grown for 12 days on PDA amended with either 250 (R250) or 25 (R25) μg/ml of iprodione. Wound diameter = 5.0 mm. Number of replicates varies because fruit that became contaminated were removed from the data set.

[z] Letters in columns within isolate and for each isolate/segregant source denote significant differences between treatment pairs according to Fisher's LSD ($P \leq 0.05$).

all experiments, percent germination of conidia on nonamended medium of iprodione-resistant isolates was significantly greater ($P \leq 0.01$) than that of sensitive isolates, ranging from 64% (sensitive) to 79 and 82% (R25 and R250 isolates, respectively) after 5.5 hr. Spore germination of WV/1 and its resistant selections (82%) and of PA/1 and its resistant selections (89%) was greater ($P \leq 0.01$) than that of WV/2 and its resistant selections (54%).

Virulence of iprodione-resistant isolates. With data from both runs combined, the main effects of isolate source and the concentration of iprodione upon which the resistant isolates were selected were significant ($P \leq 0.05$). When the data were analyzed by source, the PA isolates were more virulent than the two WV isolates, causing fruit lesions from 19 to 22 mm in diameter after 14 days, whereas the WV isolates caused fruit lesions ranging from 12 to 18 mm in diameter. The effect of selection pressure on virulence varied, with the R25 isolates causing less rot than the R250 isolates. Only one of the segregant groups (WV/1-R25) produced less rot relative to the iprodione-sensitive isolates (Table 2). All reisolations from rotted fruit confirmed the presence of resistant isolates in fruit inoculated with resistant isolates and of sensitive isolates in fruit inoculated with the iprodione-sensitive isolates.

Virulence of iprodione-resistant isolates on iprodione-treated fruit. For the first, third, and fourth runs, results from analysis of variance (GLM procedure) showed significant two-way interactions for fungicide × isolate and source × isolate after 7 days and only fungicide × isolate after 14 days (*data not shown*). To further explain these interactions, the data are presented comparing the fungicide-treated fruit with the nontreated fruit for each of the three sensitive and six resistant isolates (Table 3). Fungicide-treated fruit had significantly smaller lesions 7 and 14 days after inoculation than the nontreated fruit for only the iprodione-sensitive isolates. Fungicide-treated fruit inoculated with resistant isolates showed lesions similar in size to those in nontreated fruit (Table 3). Some variation among isolate source occurred, with the R25 strain from PA/1 causing more rot than the other R25 isolates and with the R250 strain from WV/1 causing less rot than the other R250 isolates. Results were similar in the second run (*data not shown*), except that strain R250 from WV/1 caused more rot in the iprodione-treated fruit relative to the control (10.2 vs. 7.7 mm, respectively, $P \leq 0.05$) after 7 days (but not after 14 days).

DISCUSSION

In the present study, EC_{50} values for three iprodione-sensitive isolates of *A. alternata* ranged from 2.4 to 2.9 μg/ml of iprodione, and these are similar to results found in other studies. The EC_{50} for growth of *A. mali* isolates from North Carolina on amended agar ranged from 0.78 to 2.68 μg/ml (4), and in a separate study, iprodione at 0.5 μg/ml was the most effective fungicide for inhibiting sporulation of *A. mali* on leaf disks (6). In British Columbia, linear growth and spore germination of *A. alternata* were limited by 5 and 1 μg/ml of iprodione, respectively (9). In Japan, minimum inhibitory concentration (MIC) of iprodione against *A. mali* was 6.25 μg/ml, with a small proportion of isolates having MIC values of 12.5 μg/ml (16). In the one case where field resistance of *A. mali* to iprodione has been reported (1), MIC values were 3.9–15.6 μg/ml for sensitive isolates and approximately 1,000 μg/ml for resistant isolates. The authors (1) indicated that isolates that grew at 125 μg/ml of iprodione could be transferred to 250 μg/ml and would resume growth. In the present study, isolates obtained from medium amended with 25 μg/ml resumed growth immediately when transferred to medium containing 250 μg/ml. The concentration of iprodione in our medium amended with 25 or 250 μg/ml probably does not reflect the true level of resistance of our isolates to iprodione because we did not use a solvent to solubilize the fungicide. Consequently, the EC_{50} values provided for our resistant isolates are probably lower than those reported above.

Fungi resistant to iprodione have been reported by Leroux et al (7), who demonstrated growth of *B. cinerea* at 30 μg/ml, and by Sztejnberg and Jones (21), who reported that isolates of *M. fructicola* grew and sporulated on PDA containing up to 3,000 μg/ml. McPhee (9) described the characteristics of resistant isolates of *A. alternata* isolated from sweet cherries. He found that most of the resistant isolates grew normally and that spore germination of these isolates was >90% on PDA containing up to 100 μg/ml. He noted differences in colony morphology between the resistant isolates and a sensitive isolate, the former possessing darker pigmentation and less aerial mycelium with fewer scattered

tufts. Resistant isolates retained their characteristics after five transfers on nonamended media, indicating the stability of the resistance. *Alternaria* spp. are heterokaryotic, and continued exposure to the fungicide could allow for the selection of resistant nuclei. A similar selection process for isolates of *Rhizopus stolonifer* (Ehrenb.:Fr.) Vuill. (23) and *B. cinerea* has been reported (22). The darker pigmentation, also noted for resistant isolates of *M. fructicola* (13), was not observed in the isolates of *A. alternata* used in the current study.

The generation of iprodione-resistant isolates is especially significant if the new isolates can cause disease at economic levels. Previous studies have shown that resistant isolates of *A. alternata* varied considerably in their virulence on cherries, ranging from 12 to 51% incidence for the resistant isolates, compared to 53% incidence for the sensitive isolate. In the present study, considerable variation also was noted, with resistant isolates having less, more, or similar virulence relative to the sensitive isolates. Resistant isolates generally sporulated at levels similar to the sensitive isolates, although spore germination percentages were higher for the resistant isolates.

The frequency of iprodione-resistant isolates within a population of *Alternaria* may be up to 20 times higher than the frequency of iprodione-resistant *M. fructicola* (9,21). Laboratory isolates of some fungi have shown induced resistance to fungicides, and this has been explained as adaptive enzyme formation (11), but this type of resistance seldom reaches a high level and usually is rapidly lost after transfer to fungicide-free medium. Studies of *A. alternata, M. fructicola*, and *B. cinerea* resistant to iprodione have shown that resistance is moderately stable and is maintained in consecutive transfers on nonamended media (2,9,14).

No field experiments testing the fitness of iprodione-resistant isolates of *Alternaria* have been reported. On the basis of the current laboratory data, it is not possible to predict what might happen if resistant isolates of *A. alternata* appeared. Data on iprodione-resistant *B. cinerea*, which revealed slower growth and reduced aggressiveness of the resistant isolates and reduced parasitic fitness relative to iprodione-sensitive isolates (10), could be used to infer that resistant isolates would not be likely to rapidly increase to a dominant level in the population. However, because resistant isolates can be isolated so easily in the laboratory, if they occur in the field and are able to persist, then selection pressure may be toward improved parasitic fitness. In many cases the detection of resistant isolates has not been associated with a lack of field control (2). Although resistant isolates generally are less fit and sporulate less than sensitive isolates (2), the range of virulence and fitness can overlap (12), as was observed in the present study. The present findings agree with those of Penrose et al (12), who observed that some of the resistant isolates of *M. fructicola* showed no discernible differences in cultural characteristics or virulence when compared with the sensitive isolates.

The potential for increased postharvest use of iprodione on apple may be limited because of the risk associated with the development of resistant pathogens, including the major postharvest apple pathogens *P. expansum, A. alternata,* and *B. cinerea.* Because of the relatively rapid selection of resistant isolates in the laboratory, caution should be exercised in the use of dicarboximide fungicides. In addition, the common occurrence of *P. expansum* isolates resistant to the benzimidazole fungicides (15) and the occurrence of *B. cinerea* isolates with resistance to both dicarboximides and benzimidazoles (10) may limit the ability to manage resistance by alternating or combining these fungicides. A problem of perhaps greater importance could develop if iprodione was registered for preharvest use on apples for controlling *A. alternata.* A resistant form selected in the orchard could spread throughout the orchard and infect a large percentage of fruit going into storage, rendering postharvest treatment with iprodione less effective.

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