In Vitro Growth Response of *Phytophthora parasitica* var. *nicotianae* Isolates to Metalaxyl

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ABSTRACT

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Three hundred seventeen isolates of *Phytophthora parasitica* var. *nicotianae* were screened in vitro for sensitivity to metalaxyl. Sensitivity to metalaxyl was decreased by increasing the concentration of nutrients in the basal medium. Growth decreased linearly with metalaxyl concentrations from 0.1 to 10 μg/ml for the isolates screened resulting in a mean ED_{50} of 0.4 μg/ml. Isolates from six flue-cured tobacco fields varied greatly in their response to metalaxyl within as well as among fields. The range of mean inhibition in the six fields was 8–57, 46–85, 89–95, and 85–96% at 0.1, 1, 10, and 100 μg/ml of metalaxyl, respectively.

Since its introduction, metalaxyl (Ridomil) has been investigated widely for control of diseases caused by soilborne *Phytophthora* spp. (1,8,11,13). The systemic fungicide is currently used for control of Phytophthora root rots of many plant species including the black shank disease of tobacco, caused by *P. parasitica* Dast. var. *nicotianae* (Breda de Haan) Tucker. Metalaxyl controls black shank by preventing formation of sporangia of *P. parasitica* var. *nicotianae* from chlamydospores or infected tobacco roots and also by inhibiting mycelial growth and colonization of tobacco root tissue (13). Inhibition of mycelial growth in vitro has been used as the primary method of determining metalaxyl sensitivity of *Phytophthora* spp.

Development of resistance to metalaxyl by some fungal pathogens (2,11) has caused concern for the long-term effectiveness of this fungicide in controlling *Phytophthora* diseases. To detect shifts in metalaxyl sensitivity within a given *Phytophthora* sp., the natural range of sensitivity needs to be determined. In most studies, the in vitro response of a specific *Phytophthora* sp. to metalaxyl has been determined with only one or a few isolates of the pathogen (1,4,9,13). The sensitivities of these isolates were then used to represent the response of the whole species to the fungicide. In several studies, a larger number of isolates was screened to establish the range in sensitivity (3,6). Hunger et al (6) screened 35 isolates of *P. megasperma* from different hosts collected before exposure to metalaxyl to determine the natural range of metalaxyl sensitivity. Coffey et al (3) screened 100 isolates of *P. cinnamomi* and 20 isolates of *P. citricola* also collected before metalaxyl exposure.

Previous studies have not compared the observed range in metalaxyl sensitivity of a large number of isolates from a single field to pathogen populations in other fields and to the species as a whole. Several basal media and incubation times have been used in determining metalaxyl sensitivity in previous studies (1,3,9,13). The effects of these substrates on fungicide sensitivity have not been determined. The objectives of this research were to 1) compare in vitro the effects of different basal media on the sensitivity to metalaxyl, 2) determine the natural range in metalaxyl sensitivity of *P. parasitica* var. *nicotianae* before exposure to the fungicide, and 3) determine within- and between-field variation in metalaxyl sensitivity in isolates from six flue-cured tobacco fields.

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MATERIALS AND METHODS

Three hundred seventeen isolates of *P. parasitica* var. *nicotianae* were collected in 1980 and 1981 from flue-cured and burley tobacco before exposure to metalaxyl. Most isolates were obtained from single chlamydospore colonies on soil assay plates (12) and the remainder came from stock cultures and mass mycelial transfer of pathogen growth from infected tobacco root and stem tissue. Seventy-one percent of the isolates were collected from six flue-cured tobacco fields for within- and between-field comparisons. All isolates not originally obtained from stock culture collections were isolated from soil or tobacco roots on a selective agar medium (7,12). All isolates were maintained under oil on 5% clarified V-8 juice agar (5) slants until used.

Screening procedure. Metalaxyl 2EC (25.11% a.i.) was used in all studies to establish desired fungicide concentrations. All concentrations presented are expressed as active ingredients.

Initial tests determined the influence of basal medium on growth of *P. parasitica* var. *nicotianae* at metalaxyl concentrations of 0, 0.1, 1, and 5 μg a.i./ml of basal medium. Metalaxyl was added to cooled (45–50 C) agar media after autoclaving. Basal media used were 1) 5% clarified V-8 juice agar, 2) 10% clarified V-8 juice agar, 3) 20% clarified V-8 juice agar, 4) Difco cornmeal agar (CMA) with an additional 5 g of Bacto agar added per liter of medium, 5) nutrient agar (N) (10 g of peptone, 3 g of beef extract, and 17 g of Bacto agar per liter of deionized water), and 6) potato-dextrose agar (PDA) (200 g of sliced potatoes, 20 g of dextrose, and 17 g of Bacto agar per liter of deionized water). After addition of the fungicide, about 17 ml of medium was poured per 9-cm-diameter petri plate. Agar disks (6 mm in diameter) were transferred to the centers of plates from the growing margin of isolates on 5% V-8 agar. Mycelial growth along the largest radius was measured to the closest millimeter after 7 days of incubation in the dark at 22–25 C.

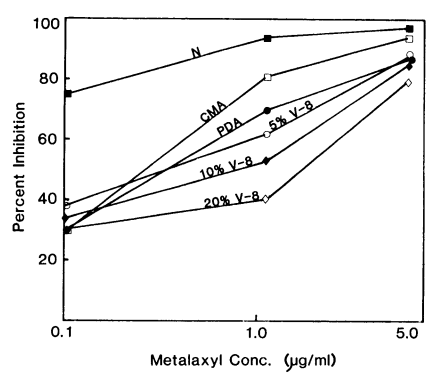

Fig. 1. Influence of six basal media and metalaxyl on mean inhibition of 42 isolates of *Phytophthora parasitica* var. *nicotianae* after 7 days of incubation in the dark at 22–25 C. N = nutrient agar; CMA = Difco cornmeal agar; PDA = potato-dextrose agar; and 5, 10, and 20% V-8 = different concentrations of V-8 juice agar.

Forty-two isolates selected at random were screened on each of the six basal media. There were two observations (plates) per treatment and the experiment was conducted twice. Data were analyzed by analysis of variance.

Results indicated that basal medium influenced the growth response of *P. parasitica* var. *nicotianae* isolates at various concentrations of metalaxyl. Therefore, all subsequent tests were conducted on 5% V-8 agar with metalaxyl concentrations of 0, 0.1, 1, 10, and 100 μg/ml. Mycelial growth at each metalaxyl concentration was expressed as percentage of inhibition compared with unamended 5% V-8 agar. There were three replicates for each isolate at each metalaxyl concentration. Analysis of variance was used to test for treatment effects and significant interactions.

RESULTS

Basal medium influenced the sensitivity of *P. parasitica* var. *nicotianae* isolates to metalaxyl (Fig. 1). Mean mycelial inhibition was greatest on N at all metalaxyl concentrations tested. A significant interaction ($P = 0.01$) occurred between basal medium and log concentration of metalaxyl. For example, mean inhibition at 0.1 μg/ml of metalaxyl was least on CMA but inhibition on CMA was next to the greatest at 1 and 5 μg/ml of metalaxyl. Mean inhibition was least on 20% V-8 at 1 and 5 μg/ml of metalaxyl. Sensitivity to metalaxyl decreased with increasing concentration of V-8 juice in the basal medium (Fig. 1).

Mean mycelial inhibition increased with increasing metalaxyl concentration (Table 1). Mean inhibition for the 317 isolates increased linearly (log scale) from 0.1 to 10 μg/ml of metalaxyl, but mycelial growth was similar at 10 and 100 μg/ml. The mean ED_{50} for the 317 isolates screened was about 0.4 μg/ml of metalaxyl.

Although mean inhibition increased with increasing metalaxyl concentrations, the response of individual isolates to metalaxyl varied greatly (Fig. 2). For example, inhibition of isolate 85 decreased with increasing metalaxyl concentration and isolate 359 was almost completely inhibited at 0.1 μg/ml (Fig. 2). Furthermore, mycelial growth of some isolates was the same or greater on metalaxyl-incorporated agar at 0.1 and 1 μg/ml than on fungicide-free 5% V-8 agar (Table 2).

Mean sensitivity of *P. parasitica* var. *nicotianae* isolates to metalaxyl varied ($P = 0.01$) among fields. The range of inhibition was 8–57, 46–85, 89–95, and 85–96% at 0.1, 1, 10, and 100 μg/ml, respectively, for the six fields (Table 2). Isolates from fields 3 and 4 were the most sensitive, with mean inhibition greater than 50% at 0.1 μg/ml in both fields. Although mean sensitivity varied with field of origin, there was a significant overlap in the range of sensitivities among isolates from the six fields (Table 2). For example, at 0.1 μg/ml of metalaxyl, inhibition of growth was +18–94% of the control in field 3 isolates, which were most sensitive to metalaxyl, and +21–34% of the control in field 2 isolates, which were least sensitive (Table 2).

Isolate sensitivity varied with field and metalaxyl concentration. At 1 μg/ml of metalaxyl, the percentage of all isolates inhibited less than 50% compared with fungicide-free 5% V-8 agar ranged from 6% in field 3 to 67% in field 2 (Fig. 3).

DISCUSSION

Previous studies on the sensitivity of *Phytophthora* spp. to metalaxyl (1,3,6,9,13) have not addressed the role of substrate in growth response. In this

Table 1. Influence of metalaxyl concentration on growth of *Phytophthora parasitica* var. *nicotianae* in vitro

Metalaxyl conc. (μg/ml)	Growth[a] (mm)	Growth inhibition (%)
0.0	20.5 ± 0.5	...
0.1	14.9 ± 0.4	31
1.0	7.6 ± 0.4	63
10.0	1.8 ± 0.1	91
100.0	1.8 ± 0.1	91

[a] Mean growth of 317 isolates on 5% V-8 juice agar after 7 days in the dark at 22–25 C plus-minus standard error of mean.

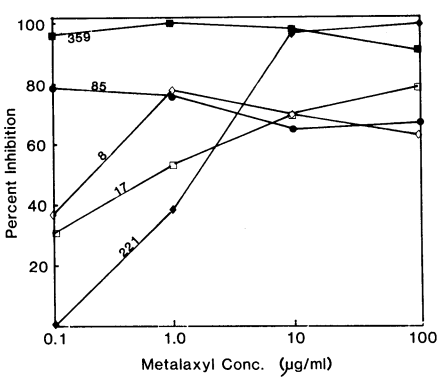

Fig. 2. Growth responses of five isolates of *Phytophthora parasitica* var. *nicotianae* to metalaxyl on 5% V-8 juice agar after 7 days of incubation in the dark at 22–25 C.

Table 2. Influence of metalaxyl concentration on inhibition of growth of *Phytophthora parasitica* var. *nicotianae* isolates collected from six flue-cured tobacco fields

Field	No. of isolates	Growth inhibition (%)[a] at indicated metalaxyl conc. (μg/ml) 0.1	1	10	100
1	82	25 (+2–43)	59 (9–89)	91 (76–100)	94 (82–100)
2	37	8 (+21–34)	46 (0–97)	95 (86–100)	96 (73–100)
3	33	57 (+18–94)	85 (23–100)	89 (35–100)	85 (35–97)
4	44	50 (+3–97)	84 (34–100)	94 (82–100)	89 (74–100)
5	23	18 (+34–65)	48 (13–97)	94 (87–100)	96 (85–100)
6	5	23 (16–27)	70 (62–76)	89 (83–91)	95 (93–97)

[a] Mean inhibition compared with growth on fungicide-free 5% V-8 juice agar after 7 days in the dark at 22–25 C. Values in parentheses indicate the range in percentage of growth inhibition. Plus values indicate growth was greater on metalaxyl-incorporated agar than on fungicide-free agar for some isolates.

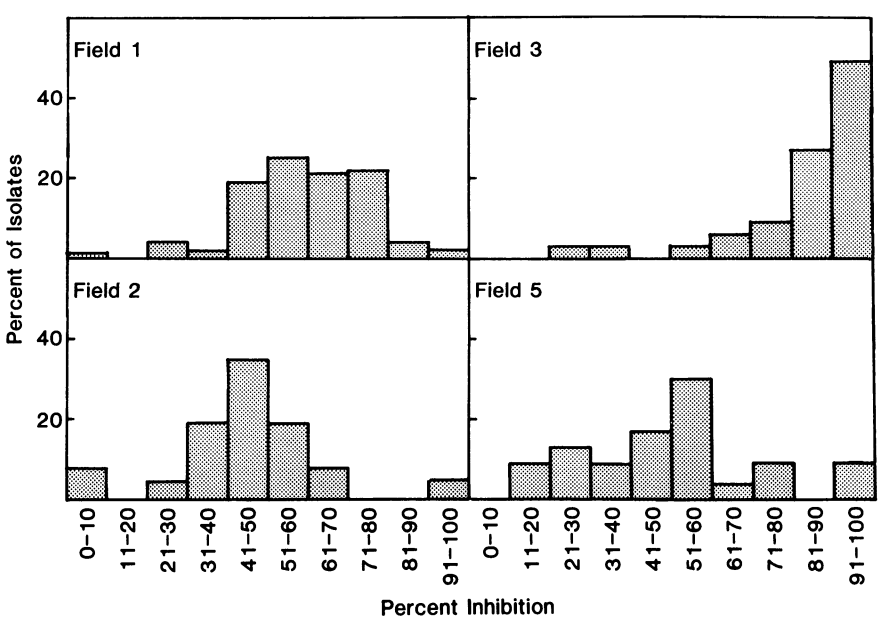

Fig. 3. Frequency distribution of growth responses among isolates from four flue-cured tobacco fields in response to metalaxyl at 1 μg a.i./ml in 5% V-8 juice agar. Field 1 = 82 isolates, field 2 = 37 isolates, field 3 = 33 isolates, and field 5 = 23 isolates.

study, growth of *P. parasitica* var. *nicotianae* depended on basal medium and fungicide concentration, resulting in a different mean ED_{50} for each medium for the 42 isolates screened. In addition, the rankings of isolates in response to a given metalaxyl concentration changed with basal medium used. These data indicate that comparisons of metalaxyl sensitivity among isolates of the same species should be made by screening on the same basal medium. Increasing the nutrient status of the basal medium decreased the sensitivity of *P. parasitica* var. *nicotianae* to metalaxyl. An analogous situation may also occur under field conditions. Metalaxyl is not recommended for black shank control if highly susceptible tobacco cultivars are planted. One reason metalaxyl does not control black shank at standard field rates on susceptible cultivars may be related to the susceptible nature of the substrate (root tissue) available to the pathogen. If this hypothesis is true, factors that increase root tissue susceptibility to *P. parasitica* var. *nicotianae*, such as the root galling caused by root-knot nematodes (10), should decrease the effectiveness of metalaxyl in black shank control. Such a decrease in effectiveness of metalaxyl in black shank control in the presence of root-knot nematodes has been observed (H. D. Shew, *unpublished*).

Staub and Young (13) reported an ED_{50} for mycelial growth of 0.2 μg/ml for an isolate of *P. parasitica* var. *nicotianae*. The mean ED_{50} of the 317 isolates screened in this study was about 0.4 μg/ml of metalaxyl. Because Staub and Young used 10% V-8 agar in their tests, which decreases sensitivity to metalaxyl compared with 5% V-8 agar, the difference in ED_{50} values is greater than 0.2 μg/ml. Although these two values are relatively close, a wide range in metalaxyl sensitivity in the species was apparent. The significance of this variation in relation to development of tolerance to metalaxyl is not known, but it does indicate that the species is genetically diverse with regard to metalaxyl sensitivity before exposure to the fungicide. Other researchers have observed similar diversity in other *Phytophthora* spp. Coffey et al (3) concluded that it was unlikely that loss of fungicidal efficacy would be an immediate problem with *P. cinnamomi* and *P. citricola*. Hunger et al (6), on the other hand, concluded that the amount of diversity and the level of tolerance in *P. megasperma* collected from different hosts present before exposure to metalaxyl may indicate that selection of tolerant strains of the fungus to metalaxyl will occur rapidly in nature. After 3 yr of use for black shank control (1980–1983), metalaxyl has not failed to control the black shank disease in tobacco in North Carolina. However, a shift to a lower in vitro metalaxyl sensitivity in *P. parasitica* var. *nicotianae* isolates collected from some flue-cured tobacco fields after repeated use of metalaxyl has been observed (H. D. Shew, *unpublished*).

More important than overall mean inhibition of isolates to metalaxyl is the percentage of total isolates in a field that show a low level of sensitivity at a given fungicide concentration. Assuming equal virulence, these less-sensitive isolates would be the most likely to infect roots and cause disease in the presence of metalaxyl, resulting in an even greater percentage of total isolates that can grow and infect at a given metalaxyl concentration. Frequency distribution plots of data on inhibition at 1 μg/ml of metalaxyl illustrate the difference in isolate sensitivity to metalaxyl in four of the six fields sampled. In this study, most isolates from field 3 were very sensitive to metalaxyl, whereas isolates from fields 1, 2, and 5 showed a greater range of initial metalaxyl sensitivity.

Morton et al (8) reported that in the field, rates of 1–2 μg/ml of metalaxyl provided control of black shank under low to moderate disease pressure when used in conjunction with a moderately resistant tobacco cultivar. Some *P. parasitica* var. *nicotianae* isolates in this study showed little or no growth inhibition at 1 μg/ml of metalaxyl. Because only inhibition of mycelial growth was determined, and metalaxyl acts by inhibiting spore production as well as mycelial growth, direct comparisons cannot be made between the in vitro growth response and disease control in soil. In addition, host resistance plays a role in the effectiveness of black shank control by metalaxyl in vivo. Development of an in vivo screening procedure that takes into account host and pathogen factors needs to be developed to compare in vitro growth response and root infection in vivo. Results indicate that *P. parasitica* var. *nicotianae* is extremely variable in vitro in response to metalaxyl, and continued monitoring of this species for shifts in sensitivity to this fungicide is warranted.

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