Laboratory-Induced Resistance to Fosetyl-Al in a Metalaxyl-Resistant Field Isolate of *Pythium aphanidermatum*

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ABSTRACT

Sanders, P. L., Coffey, M. D., Greer, G. D., and Soika, M. D. 1990. Laboratory-induced resistance to fosetyl-Al in a metalaxyl-resistant field isolate of *Pythium aphanidermatum*. Plant Dis. 74:690-692.

Mutants of *Pythium aphanidermatum* resistant to both metalaxyl and fosetyl-Al were obtained following exposure of a metalaxyl-resistant field isolate to the chemical mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Selected mutants were resistant to metalaxyl and phosphorous acid in vitro and exhibited in vitro growth rates and zoospore production that differed little from the metalaxyl-resistant parent. Mutants were resistant to metalaxyl and fosetyl-Al on greenhouse-grown Penncross creeping bentgrass and showed virulence equal to that of the parental isolate.

Pythium blight (cottony blight, grease spot), caused by *Pythium aphanidermatum* (Edson) Fitzp., is a devastating foliar disease of turfgrasses. In hot, wet weather, this disease can decimate an established turf in 24 hr. The causal fungus is most aggressive under conditions of high humidity at air temperatures of 29–35 C (14).

The systemic fungicides metalaxyl (10), propamocarb (13), and fosetyl-Al (11) provide excellent control of Pythium blight. Metalaxyl was the first systemic fungicide registered for control of this disease. Because of its excellent control properties and residual action, it was used repeatedly and exclusively for Pythium blight management on many golf courses. After 3 yr of such use, the first verified metalaxyl control failure in the United States occurred on a Pennsylvania golf course (8). Other failures followed (9). Concern that resistance to other systemic fungicides that are widely used for control of Pythium blight might also develop provided the incentive for this study.

Both direct and indirect modes of action have been ascribed to fosetyl-Al and to its in vivo breakdown product, phosphorous acid (H_3PO_3; 2). Guest (7) reports identical defense responses in *Phytophthora nicotianae* Breda de Haan-inoculated tobacco seedlings treated with fosetyl-Al and H_3PO_3. Fenn and Coffey (4–6) report similarities between the two chemicals in studies of direct antifungal modes of action of fosetyl-Al and H_3PO_3.

Propamocarb and fosetyl-Al were registered for Pythium blight control in 1982 and 1986, respectively. To date, there have been no reported field control failures from fungal resistance to either chemical in the United States. There is, however, one report of isolation of a strain of *Phytophthora cinnamomi* Rands resistant to fosetyl-Al from *Chamaecyparis lawsoniana* (Andr. Murray) Parl. in a French nursery (15). In addition, resistance to fosetyl-Al has been induced in the laboratory with the chemical mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) in isolates of *Phytophthora capsici* Leonian, *P. palmivora* (E. J. Butler) E. J. Butler, and *P. parasitica* Dastur (1,3,6). Because no parallel studies have been conducted with *Pythium*, it was considered highly relevant to study and evaluate the potential for development of resistance to H_3PO_3 and fosetyl-Al in *P. aphanidermatum*. Additionally, production of mutants of *P. aphanidermatum*, which are resistant to H_3PO_3 in vitro and not controllable with fosetyl-Al on their host, would provide further evidence of a direct mode of action for phosphonate fungicides (2). The current experiments were undertaken to determine the response of a naturally occurring metalaxyl-resistant isolate of *P. aphanidermatum* to chemical mutagenesis and to examine the growth and pathogenicity characteristics of resultant double-resistant mutants (metalaxyl/fosetyl-Al).

MATERIALS AND METHODS

Mutagenesis procedures. The studies were conducted with a metalaxyl-resistant field isolate of *P. aphanidermatum* (P3207) recovered in 1986 from a site in Indiana where metalaxyl had failed to control Pythium blight. Wild-type isolates P32, P3203, and P3204, plus additional metalaxyl-resistant isolates P3206, P3208, and P3209, were used for comparison purposes in growth and sporulation studies with recovered double-resistant mutants.

P3207 was transferred to five plates of clarified V-8 juice-$CaCO_3$ (V8C) agar and incubated in the dark at 36 C for 48 hr, after which the colonized medium in each plate was cut into small squares. The squares were placed into 10 petri plates (100 × 15 mm), filled with sterile, distilled water to the surface of the mycelium, and incubated at 36 C for 24 hr. The water was then replaced with fresh sterile, distilled water, and the plates were returned to 36 C. After another 24 hr, the water was again replaced, and the plates were moved to a 20 C incubator for 24 hr for zoospore induction. Zoospores were harvested by decanting the water from all 10 plates into a sterile beaker and counted with the use of a hemacytometer.

A suspension of 4.4×10^4 zoospores/ml was used for mutagenesis experiments. Ten ml of the suspension was added to each of 25 petri plates. Two ml of V-8 broth was added to each plate, and the zoospores were left for 15 min to encyst. One ml of MNNG (300 μg/ml) was added to each plate and allowed to stand for 12 min. The MNNG and V-8 broth could be vacuum-aspirated because the encysted zoospores adhered to the bottom of the petri plates. A 1-ml wash of half-strength V-8 broth was placed in each plate to remove traces of the chemical mutagen. After 5 min, the V-8 broth wash was aspirated, and an overlay of cooled Difco cornmeal agar (CMA), amended with H_3PO_3 (250 μg/ml) buffered with KOH to pH 6.5, was poured into each plate. Plates were incubated at 24 C for 10 days. Following incubation, an additional overlay of H_3PO_3-amended CMA (500 μg/ml at pH 6.5) was added to each plate. Plates were then incubated at 24 C for an additional 7 days.

Contribution 1753, Department of Plant Pathology, The Pennsylvania Agricultural Experiment Station. Authorized for publication as Journal Series Paper 8274.

Accepted for publication 1 March 1990 (submitted for electronic processing).

After incubation, 27 colonies growing through the final overlay were transferred separately onto V8C agar. These isolates were transferred every 3 days for 8 wk and were evaluated for the stability of their H_3PO_3 resistance and the similarity of their growth rates on V8C agar compared with P3207.

Resistance to H_3PO_3 in vitro. After 8 wk, 14 selected mutants and the metalaxyl-resistant parent (P3207) were tested for resistance to H_3PO_3. Half-strength CMA (9 g of Difco CMA and 9 g of Difco agar per liter), free of fungicide or amended with 250 and 500 μg H_3PO_3/ml (pH 6.5 with KOH), were inoculated by placing 5-mm-diameter plugs in the center of each 100 × 15 mm petri plate. Three replicates of each concentration per isolate were used. Inoculated plates were incubated in the dark at 24 C for 24 hr, after which radial growth measurements were recorded. Mutants where growth was significantly different from the metalaxyl-resistant parent (P3207) on H_3PO_3-amended CMA were identified and compared to P3207 for similarity in zoospore production and growth rate on nonamended CMA. Three mutants (PY-M1, PY-M5, and PY-M7) were chosen for further study based on growth parameters and level of resistance to H_3PO_3.

Dose-response determinations. These were carried out by transferring 5-mm-diameter plugs of the selected mutants to half-strength CMA amended with metalaxyl (technical grade) at 0, 1, 2.5, 5, 125, 200, 250, and 300 μg/ml and H_3PO_3 (pH 6.5) at 0, 75, 125, 250, 500, 600, 1,000, 1,200, 2,400, 4,800, 7,200, 9,600, and 12,000 μg/ml. Six replications of each fungicide and concentration were used. Plates were incubated in the dark for 24 hr, after which radial growth measurements were recorded and EC_{50} values were determined with an MSTAT computer probit program (Michigan State University, Version 4.0, 1985).

Pathogenicity experiments. Greenhouse studies were conducted on Penncross creeping bentgrass (*Agrostis palustris* Huds.) grown in 10-cm-diameter plastic pots. Grass was seeded at 0.3 g per pot (5 × normal field seeding rate) into pasteurized sand and top-dressed with Terragreen calcined clay soil conditioner (Oil Dri Corporation of America, Chicago, IL). Pots were kept moist with mist until seedling emergence. Grass was irrigated daily thereafter and fertilized weekly with Peters 20:20:20 soluble fertilizer (W. R. Grace & Co., Fogelsville, PA). At 3 wk postplanting, chemical treatments were applied as foliar sprays in 11.1 liters of water per 93 m^2 equivalent. The following commercial fungicides and rates per 93 m^2 were tested: fosetyl-Al (Aliette 80W) at 227 g, metalaxyl (Subdue 2E) at 59 ml, and Aliette 80W at 227 g + Subdue 2E at 59 ml. Each treatment was replicated three times, and untreated checks were included for comparison. The three mutants (PY-M1, PY-M5, and PY-M7), the metalaxyl-resistant parent (P3207), and a wild-type reference isolate (P32) were tested for pathogenicity and fungicide sensitivity. Fungal inoculum was prepared by growing individual isolates on autoclaved rye grain. When the rye grain was colonized (2–3 days), inoculum of individual isolates was homogenized with sufficient sterile, distilled water to make thick homogenates. Inoculations were made by placing equal volumes of homogenate in the center of the grass area in the pots. Inoculation was carried out 24 hr after fungicide application. Following inoculation, pots were covered with transparent polyethylene bags to maintain high humidity and placed on a shaded greenhouse bench at 32–36 C for incubation. When grass in the nontreated pots was completely blighted (3 days postinoculation), plastic bags were removed and disease severity was evaluated. Disease was rated by measuring the diameter of the blighted grass area in each pot. Data obtained were subjected to analysis of variance and a Waller-Duncan *k*-ratio *t* test. The foregoing experiment was repeated, using the same experimental procedure, with the exception that an additional rate of Aliette 80W, 341 g (1.5 × field rate), was included. A third greenhouse experiment, also using the same procedures, was carried out to determine the sensitivity of PY-M1, PY-M5, and PY-M7 to propamocarb (Banol 6S) at 38.5 and 118.4 ml per 93 m^2.

RESULTS AND DISCUSSION

The mutants, PY-M1, PY-M5, and PY-M7, showed variable growth in culture. PY-M1 grew most vigorously and differed little from the metalaxyl-resistant parent (P3207). The remaining isolates were comparable to P3207 in colony morphology but grew more slowly in culture. All three mutants produced abundant zoospores.

Evaluation of the metalaxyl dose-response experiment shows that P32, P3203, and P3204 (the wild-type reference isolates) were more sensitive to metalaxyl (EC_{50} = 0.5–2.6 μg/ml) than the four metalaxyl-resistant field isolates, P3206, P3207, P3208, and P3209 (EC_{50} = 215–232 μg/ml), and the mutants PY-M1, PY-M5, and PY-M7 (EC_{50} = 205–215 μg/ml).

Results of the H_3PO_3 dose-response experiments (Fig. 1) demonstrated that the mutants were very resistant to the chemical in comparison with the parent, P3207 (EC_{50} = 275 μg/ml). This latter value is quite high when compared to typical EC_{50} values (5–90 μg/ml) for most wild-type *Phytophthora* spp. (2). Among the H_3PO_3-resistant mutants of *P. aphanidermatum*, the least sensitivity was shown by PY-M1 (EC_{50} = 4,700 μg/ml), followed by PY-M5 and PY-M7 (EC_{50} = 3,900 and 3,000 μg/ml, respectively). In comparison, H_3PO_3-resistant strains of several *Phytophthora* spp. possessed much lower EC_{50} values (118–815 μg/ml; 2).

Results of greenhouse experiments (Table 1) supported the in vitro findings of lack of sensitivity of the mutants to metalaxyl and H_3PO_3. PY-M5 and PY-M7 were not controlled in vivo by either fosetyl-Al or metalaxyl, while PY-M1 exhibited intermediate sensitivity to both fungicides. PY-M1, PY-M5, and PY-M7 showed virulence equal to the wild-type

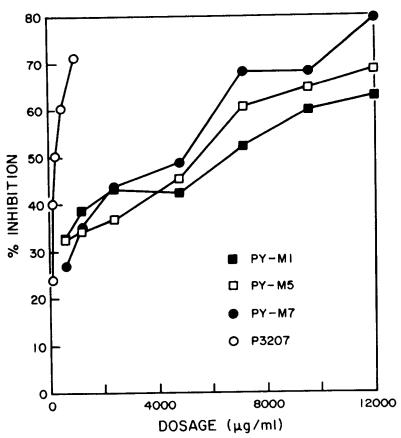

Fig. 1. In vitro dose response of field isolate and mutants of *Pythium aphanidermatum* to H_3PO_3.

Table 1. Control of wild-type (P32), metalaxyl-resistant parent (P3207), and MNNG mutants (PY-M1, PY-M5, and PY-M7) of *Pythium aphanidermatum* on pot-grown creeping bentgrass with metalaxyl and fosetyl-Al

Fungicide and rate/93 m^2	Disease severity[y]				
	P32	P3207	PY-M1	PY-M5	PY-M7
Nontreated check	10.0[z] a	10.0 a	9.3 a	10.0 a	10.0 a
Aliette 80W, 227 g	3.0 b	2.0 d	5.0 c	10.0 a	10.0 a
Aliette 80W, 341 g	3.3 b	3.0 c	4.8 c	10.0 a	10.0 a
Subdue 2E, 59 ml	0.0 c	10.0 a	6.7 b	10.0 a	10.0 a
Aliette, 227 g + Subdue, 59 ml	0.0 c	3.7 b	4.6 c	10.0 a	10.0 a
Aliette, 341 g + Subdue, 59 ml	0.0 c	3.0 c	4.3 c	10.0 a	10.0 a

[y] Diameter in centimeters of the blighted grass area in pots.
[z] Mean of three replications. Within columns, means followed by the same letter are not significantly different, using Waller-Duncan's *k*-ratio *t* test.

(P32) and the metalaxyl-resistant parent (P3207). Mutants exhibited sensitivity to propamocarb that was equivalent to that shown by the wild-type isolate (P32) and the metalaxyl-resistant parent (P3207).

Our research indicates that mutation of *P. aphanidermatum* to metalaxyl/fosetyl-Al double resistance is not necessarily linked to growth rate, zoospore production, and virulence or to propamocarb sensitivity. Furthermore, it implies that emergence of field resistance in *P. aphanidermatum* to fosetyl-Al and the eventual appearance of double resistance with metalaxyl are within the realm of possibility.

Findings of the current research and previous work (12) suggest that it would be prudent to eliminate metalaxyl use in sites where metalaxyl control of Pythium blight has failed and to employ fosetyl-Al in combination with other fungicides for Pythium blight control. Where no history of metalaxyl resistance exists, all three systemic fungicides, plus the contact fungicides registered for control of this disease, can provide the chemical diversity to reduce the risk of fungicide resistance development. Research evidence continues to accumulate that the continuous and exclusive use of any systemic fungicide risks selection of resistant pathogen populations and threatens the long-term usefulness of such chemicals.

LITERATURE CITED

1. Bower, L. A., and Coffey, M. D. 1985. Development of laboratory tolerance to phosphorous acid, fosetyl-Al, and metalaxyl in *Phytophthora capsici*. Can. J. Plant Pathol. 7:1-6.
2. Coffey, M. D., and Ouimette, D. G. 1989. Phosphonates: Antifungal compounds against Oomycetes. Pages 107-129 in: Nitrogen, Phosphorus and Sulphur Utilization by Fungi. L. Boddy, R. Marchant, and D. J. Read, eds. Cambridge University Press, Cambridge, England.
3. Dolan, T. E., and Coffey, M. D. 1988. Correlative in vitro and in vivo behavior of mutant strains of *Phytophthora palmivora* expressing different resistances to phosphorous acid and fosetyl-Na. Phytopathology 78:974-978.
4. Fenn, M. E., and Coffey, M. D. 1984. Studies on the in vitro and in vivo antifungal activity of fosetyl-Al and phosphorous acid. Phytopathology 74:606-611.
5. Fenn, M. E., and Coffey, M. D. 1985. Further evidence for the direct mode of action of fosetyl-Al and phosphorous acid. Phytopathology 75:1064-1068.
6. Fenn, M. E., and Coffey, M. D. 1989. Quantification of phosphonate and ethyl phosphonate in tobacco and tomato tissues and significance for the mode of action of two phosphonate fungicides. Phytopathology 79:76-82.
7. Guest, D. I. 1986. Evidence from light microscopy of living tissues that fosetyl-Al modifies the defense response in tobacco seedlings following inoculation by *Phytophthora nicotianae* var. *nicotianae*. Physiol. Mol. Plant Pathol. 29:251-261.
8. Sanders, P. L. 1984. Failure of metalaxyl to control Pythium blight on turfgrass in Pennsylvania. Plant Dis. 68:776-777.
9. Sanders, P. L. 1987. Failure of metalaxyl to control Pythium blight on Kentucky golf courses. (Abstr.) Phytopathology 77:121.
10. Sanders, P. L., Burpee, L. L., Cole, H., Jr., and Duich, J. M. 1978. Control of Pythium blight on Penncross bentgrass with CGA 48988. Plant Dis. Rep. 68:663-667.
11. Sanders, P. L., Houser, W. J., and Cole, H., Jr. 1983. Control of *Pythium* spp. and Pythium blight of turfgrass with fosetyl aluminum. Plant Dis. 67:1382-1383.
12. Sanders, P. L., Houser, W. J., Parish, P. J., and Cole, H., Jr. 1985. Reduced-rate fungicide mixtures to delay fungicide resistance and to control selected turfgrass diseases. Plant Dis. 69:939-943.
13. Sanders, P. L., Nutter, F. W., Jr., and Cole, H., Jr. 1980. Control of Pythium blight of turfgrass with SN66752. (Abstr.) Phytopathology 70:468.
14. Vargas, J. M., Jr. 1981. Management of Turfgrass Diseases. Burgess Publishing Co., Minneapolis, MN. 204 pp.
15. Vegh, I., Leroux, P., LeBerre, A., and Lanen, C. 1985. Détection sur *Chamaecyparis lawsoniana* 'Ellwoodii' dune souche de *Phytophthora cinnamomi* Rands résistante au phoséthyl-Al. P.H.M. Rev. Hortic. 262:19-21.