In Vitro Variability Among Isolates of Six *Phytophthora* Species in Response to Metalaxyl

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ABSTRACT

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A comparison of isolates of *Phytophthora parasitica* and *P. citrophthora*, mainly from citrus, revealed a range of inhibitions of mycelial growth from 69.1 to 83.6% for the former, and only from 0 to 14.6% for the latter, at a metalaxyl concentration of 0.1 μg/ml. Similarly, *P. palmivora* was much more sensitive than either *P. citrophthora* or *P. capsici* (all from cacao) to the fungicide at 0.1 μg/ml. Among isolates of *P. megasperma*, those of *P. megasperma* f. sp. *medicaginis* from alfalfa were extremely sensitive to metalaxyl, followed by *P. megasperma* f. sp. *glycinea* from soybean. Isolates of *P. megasperma* from Douglas-fir were much less sensitive to metalaxyl. In contrast, isolates of *P. infestans* from potato did not display characteristic in vitro responses to metalaxyl, but variation in inhibition of mycelial growth ranged from a low of 11.8 to a high of 92.8% with metalaxyl at 1.0 μg/ml. For various *Phytophthora* spp., ED_{50} values for inhibition of mycelial growth in vitro ranged from 0.013 μg/ml for an isolate of *P. megasperma* f. sp. *medicaginis* to 0.95 μg/ml for an isolate of *P. megasperma* from Douglas-fir.

Additional key words: *Phytophthora boehmeriae*, *Phytophthora cinnamomi*, *Phytophthora citricola*, and *Phytophthora heveae*.

A number of recent studies have established the high in vitro sensitivity of several *Phytophthora* spp. to the acylalanine fungicide metalaxyl. The typical ED_{50} value for in vitro inhibition of mycelial growth by metalaxyl was ~0.1 μg (a.i.)/ml (1,6,7,10,22,24). These investigations generally have involved only a few isolates of each *Phytophthora* sp., and consequently, the potential for variability within populations has not been assessed.

One study of 35 isolates of *P. megasperma* revealed wide variation in sensitivity to metalaxyl (13). Highly sensitive isolates were inhibited 25% at only 0.01 μg/ml, whereas tolerant isolates never exceeded 50% inhibition even at the highest concentration tested of 100 μg/ml. Recently, a similar investigation of over 100 isolates of *P. cinnamomi*, including both A1 and A2 mating types, revealed a much more restricted range of in vitro ED_{50} values for metalaxyl, from 0.07 to 0.29 μg/ml (6). In contrast, the ED_{50} values for isolates of another avocado species *P. citricola* were much higher and ranged from 1.18 to 4.61 μg/ml.

In view of the wide variation in response to metalaxyl encountered in species such as *P. megasperma* (13), and the slight variation found in species such as *P. cinnamomi* and *P. citricola* (6), we decided to investigate the in vitro responses of other *Phytophthora* species to metalaxyl.

MATERIALS AND METHODS

Isolates of *Phytophthora* were from the collection at the University of California, Riverside. In all cases, the dates of isolation either preceded the use of metalaxyl, or isolates were from locations where the fungicide had not been used. A wettable powder formulation of the fungicide metalaxyl (*N*-[2,6-dimethylphenyl]-*N*-[methoxyacetyl]-alanine methyl ester) containing 25% a.i. was used in all tests. This fungicide was prepared as a concentrated stock solution in sterile distilled water and added to media after autoclaving. Final concentrations of metalaxyl in the media are given as micrograms (actual ingredient) per milliliter.

To determine linear growth response, metalaxyl was added to Difco cornmeal agar (CMA), or to rye seed medium (RSM) (5) in the case of *P. infestans* (Mont.) deBary and *P. megasperma* Drechs. The test fungus was added to each medium in petri plates as a single 5-mm-diameter disk, using three replicate plates per treatment. Plates were incubated in the dark at 25 C and linear growth was measured after 7 days. The percentage inhibition of radial growth compared to growth on unamended media was determined at 7 days.

RESULTS

A comparison of isolates of *P. parasitica* Dastur and *P. citrophthora* (Sm. and Sm.) Leonian, mainly from citrus, revealed significant differences in response to metalaxyl at the low concentration of 0.1 μg/ml (Table 1). Individual isolates of *P. parasitica* were inhibited by 69.1–83.6%, contrasting with a range of 0–14.6% for isolates of *P. citrophthora*. Differences in response between these species were much less apparent with metalaxyl at 1.0 μg/ml.

Significant variation existed among isolates of the *P. palmivora* Butler complex from cacao (*Theobroma cacao* L.). With 0.1 μg/ml of metalaxyl, isolates of *P. palmivora* were generally more sensitive than those of either *P. citrophthora* or *P. capsici* Leonian (Table 2). Again, these differences were not apparent at 1.0 μg/ml. Examination of the response of additional isolates of *P. capsici* from *Capsicum* spp. showed a similar range of responses to metalaxyl, the majority being inhibited by between 15 and 30% at 0.1 μg/ml (Table 3).

In a comparison of isolates of *P. megasperma*, those from arrowleaf clover (*Trifolium vesiculosum* Savi.), alfalfa (*Medicago sativa* L.), and chick-pea (*Cicer arietinum* L.) responded similarly, being extremely sensitive to metalaxyl at 0.1 μg/ml (Table 4). Soybean (*Glycine max* L.) isolates were a little less sensitive, and isolates from other hosts such as *Ficus* and Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), exhibited much more tolerance to metalaxyl. Two large-oospore isolates from alfalfa (groups AL2-508 and AL2-509 of *P. megasperma*) also were much less sensitive to metalaxyl (Table 4). A closer examination of a larger number of isolates of *P. megasperma* f. sp. *glycinea* (17) from soybean and *P. megasperma* f. sp. *medicaginis* from alfalfa revealed that their responses to metalaxyl were characteristic of those particular formae speciales (Table 5).

Among individual isolates of *P. infestans*, all from potato, there was a marked variation in response to metalaxyl with 7.6–94.4% inhibition at 0.1 μg/ml and 11.8–92.8% inhibition at 1.0 μg/ml (Table 6). This variation appeared unrelated to the geographical

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TABLE 1. In vitro responses of isolates of *Phytophthora parasitica* and *P. citrophthora* to metalaxyl

Phytophthora species	Isolate	Host	Origin	Inhibition of radial growth (%)[z] by metalaxyl at: 0.1 μg/ml	1.0 μg/ml
parasitica	T131	citrus	California	83.6 a	91.6 ab
	M134	citrus	Brazil	75.5 a	93.3 a
	M141	citrus	California	72.1 a	88.3 abc
	M152	citrus	California	69.7 a	96.7 abcd
	M114	citrus	California	69.1 a	86.7 abcd
citrophthora	M131	citrus	Argentina	20.8 b	81.3 bcde
	P776	cacao	Brazil	14.6 bc	93.3 a
	M143	citrus	California	6.3 bc	78.8 cde
	P1163	citrus	California	5.0 bc	75.0 e
	P1212	cacao	Brazil	4.6 bc	87.5 abcd
	M117	citrus	California	4.2 bc	77.1 de
	M142	citrus	California	2.1 c	71.7 e
	M132	citrus	Brazil	0.0 c	41.7 f

[z] Means followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's new multiple range test.

TABLE 2. In vitro responses to metalaxyl among cacao isolates of the *Phytophthora palmivora* complex

Phytophthora species	Isolate	Origin	Inhibition of radial growth (%)[w] by metalaxyl at: 0.1 μg/ml	1.0 μg/ml
palmivora[x]	P1055	Thailand	98.0 a	92.2 d
palmivora	P922	Malaysia	94.7 a	97.7 abc
palmivora	P832	Trinidad	93.6 ab	96.8 abcd
palmivora	P1020	Nigeria	87.2 abc	92.0 d
palmivora[y]	P1182	Florida	87.1 abc	96.7 abcd
palmivora	P987	Nigeria	81.1 c	100.0 a
citrophthora	P1212	Brazil	58.7 d	98.6 ab
citrophthora	P776	Brazil	51.2 de	96.2 abcd
citrophthora	P1213	Brazil	47.9 e	98.6 ab
citrophthora	P1201	Brazil	47.6 e	98.6 ab
capsici[z]	P558	Puerto Rico	33.3 f	93.3 cd
palmivora	P736	Ghana	32.9 f	94.5 bcd
capsici	P1195	Mexico	17.5 g	60.9 e
capsici	P782	Cameroun	13.7 g	43.9 f
capsici	P632	Brazil	7.7 g	94.2 bcd

[w] Means followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's new multiple range test.
[x] *P. palmivora* from rubber.
[y] *P. palmivora* from milkweed.
[z] *P. capsici* from black pepper.

TABLE 3. In vitro responses to metalaxyl among isolates of *Phytophthora capsici* from *Capsicum*

Isolate	Origin	Inhibition of radial growth (%)[z] by metalaxyl at: 0.1 μg/ml	1.0 μg/ml
P504	Mexico	54.5 a	85.6 bc
P525	Mexico	33.9 b	81.5 c
P725	Mexico	29.9 bc	81.4 c
P528	Mexico	28.3 bcd	89.4 ab
P524	Mexico	24.4 cde	94.9 a
P531	Mexico	23.7 def	80.6 c
P526	Mexico	21.3 efg	68.7 d
P529	Mexico	21.1 efg	79.5 c
P527	Mexico	20.9 efg	84.6 bc
P890	Mexico	18.1 fgh	67.6 d
P1109	Costa Rica	16.2 ghi	46.9 e
P588	Costa Rica	15.5 ghi	80.9 c
P765	Mexico	15.3 ghi	39.9 f
P411	Guatemala	12.8 hi	84.0 bc
P891	Mexico	11.6 i	39.1 f

[z] Means followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's new multiple range test.

origin of the isolates.

Dosage-response curves were constructed for single isolates of seven species, forms, or groups of isolates with a relatively narrow spectrum of responses to metalaxyl (Fig. 1). For the more tolerant isolates, a range of metalaxyl concentrations from 0.1 to 5.0 μg/ml was used, and with more sensitive isolates the concentrations used ranged from 0.025 to 0.25 μg/ml. The percentage of inhibition of radial growth was plotted against the logarithm of the metalaxyl concentration (Fig. 1). The regression lines obtained for these isolates clearly demonstrate the diversity of in vitro response to low concentrations of metalaxyl. The isolates of *P. heveae* Thomson (P1085), *P. megasperma* f. sp. *glycinea* (P1316), and *P. boehmeriae* Saw. (P1257) were included (Fig. 1) as they were the most sensitive isolates found during screening of a broader range of *Phytophthora* species. The ED_{50} and ED_{90} values (Table 7) that were calculated from the regression lines (Fig. 1) also demonstrated the broad range of responses to metalaxyl. ED_{50} values ranged from 0.015 μg/ml for *P. boehmeriae* (P1257) to 1.17 μg/ml for *P. citricola* (P1273). Corresponding ED_{90} values ranged from 0.048 μg/ml for an isolate of *P. boehmeriae* to 7.1 μg/ml for an isolate of *P. citricola* Saw.

DISCUSSION

A limited comparison of the ED_{50} values of a single isolate of *P. megasperma* f. sp. *glycinea* (17) with those of four isolates of *P. capsici* revealed a surprising range of in vitro responses to metalaxyl (19). A comparison of the response of 35 isolates of *P. megasperma* isolated from 14 different hosts revealed a remarkable diversity in response to metalaxyl, ranging from >95% inhibition to only 27% inhibition by metalaxyl at 1 μg/ml (13). In contrast, for a large number of A2 isolates of *P. cinnamomi*, ED_{50} values had a restricted range from 0.07 to 0.14 μg/ml; values for A1 isolates were much wider, while values for *P. citricola* were much higher and ranged from 1.18 to 4.61 μg/ml (6). Recently, a study of 180 isolates of *P. cinnamomi* revealed a high degree of uniformity among the majority of A2 isolates, but a high variability among the A1 isolates, in terms of 20 isozyme loci representing 13 different enzymes (18).

In the current study, the aim was to observe a large number of isolates so that the response of different *Phytophthora* spp. could be compared more broadly. Isolates of *P. parasitica* from citrus and of *P. citrophthora* from citrus and cacao had characteristic responses to metalaxyl. Although *P. parasitica* was highly sensitive and *P. citrophthora* was highly insensitive to metalaxyl at 0.1 μg/ml such differences were not apparent at 1.0 μg/ml. Thus, the large difference in metalaxyl sensitivity originally observed with single isolates of *P. parasitica* and *P. citrophthora* (10) can be considered characteristic for the species.

The *P. palmivora* "complex" may involve four different species: *P. palmivora, P. megakarya, P. capsici,* and *P. citrophthora* (2,3,15,27). This study shows that responses to metalaxyl clearly differentiate most isolates of *P. palmivora, P. citrophthora,* and *P. capsici* from each other. Again, these different responses occurred with 0.1 μg/ml of metalaxyl, but not with 1.0 μg/ml.

A comparison of isolates of *P. megasperma* revealed inhibition ranging from 9–100% with metalaxyl at 0.1 μg/ml. The responses of isolates from clover, alfalfa, and chick-pea were very similar, all being highly sensitive to metalaxyl. The soybean isolates were less sensitive to metalaxyl. The types isolated from Douglas-fir and alfalfa in Oregon (13) were quite distinct from the more typical alfalfa and soybean types (17).

Controversy exists over the taxonomy of *P. megasperma* (2,9,12,17). The classical approach based on morphological characters (27) divides the species into small-oospore types, *P. megasperma* var. *sojae*, and large-oospore types, *P. megasperma* var. *megasperma* (2,9). Cytogenetic evidence has revealed that isolates of *P. megasperma* var. *sojae* have 10–15 chromosomes, and *P. megasperma* var. *megasperma* 22–27 chromosomes (2,21). Kuan and Erwin (17) observed that "there are no distinct differences in oospore morphology" between 30 isolates of *P. megasperma* from 14 different hosts, though "isolates from alfalfa and soybean were pathogenic only to the host from which they were isolated." They proposed the use of pathogenicity to erect two subgroups or formae speciales: *P. megasperma* f. sp. *glycinea* on soybean and *P. megasperma* f. sp. *medicaginis* on alfalfa (17).

The data obtained in this study indicates different metalaxyl responses for these formae speciales. Recently, Irwin and Dale (14) demonstrated that their chick-pea isolates had identical temperature optima and protein profiles to *P. megasperma* f. sp. *medicaginis*, but differed significantly in this respect from *P. megasperma* f. sp. *glycinea*. These results parallel the responses to metalaxyl obtained in this study.

In contrast to most other *Phytophthora* species investigated in this study, 12 isolates of *P. infestans* exhibited marked differences in responses to metalaxyl. The range of responses of individual isolates of *P. infestans* to metalaxyl was similar to that obtained with the different *Phytophthora* spp. tested (Fig. 1). There were no relationships with geographical origin, since different isolates from Mexico and Wales varied greatly in their response.

A wide variation in response to metalaxyl in vitro would not necessarily imply a similar variation in vivo. This has been clearly demonstrated for *P. infestans* isolates. Several passages in vitro through media amended with acylalanines decreased their in vitro sensitivity quite dramatically (ED_{50} values up to 65 μg/ml), but did not affect their in vivo behavior (25). Similarly, one isolate of *P. infestans* was unaffected in vitro by metalaxyl up to 350 μg/ml, and yet in vivo only 1.0 μg/ml applied on the day of inoculation completely prevented lesion expansion (4).

Metalaxyl would appear useful, especially at low concentrations of around 0.1 μg/ml, as an aid in identification of *Phytophthora* species. While current morphological criteria (26) provide the basic framework for classification and identification of *Phytophthora* species, there is justification for development of a revised taxonomy partly based on nonmorphological criteria (11). Such criteria could include cytogenetic data, particularly chromosome information (2,23), as well as serological data (11) and isoenzyme profiles (2,11).

Metalaxyl acts on the fungus by inhibiting nucleic acid synthesis (7,16) and probably specifically by interference with RNA synthesis

TABLE 4. In vitro responses to metalaxyl at 0.1 μg/ml among isolates of *Phytophthora megasperma* from different hosts

Isolate	Host	Origin	Inhibition of radial growth (%)[v]
102[w]	arrowleaf clover	Mississippi	100.0 a
P1057	alfalfa	California	100.0 a
P1316	alfalfa	California	100.0 a
P844	alfalfa	California	98.8 a
P1253	chick-pea	Australia	98.6 a
P405	soybean	Mississippi	73.3 b
P1139	soybean	Wisconsin	65.8 b
P1258	*Ficus*	New Guinea	51.6 c
AL2-508[x,y]	alfalfa (AL2)	Oregon	41.5 d
D1-306[x,z]	Douglas-fir (D1)	Oregon	41.0 de
D2-C17[x,y]	Douglas-fir (D2)	Oregon	37.1 de
D1-304[x,y]	Douglas-fir (D1)	Oregon	36.3 de
AL2-509[x,y]	alfalfa (AL2)	Oregon	30.4 e
P147	sugarcane	Louisiana	9.2 f

[v] Means followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's new multiple range test.
[w] Isolate 102 is from R. G. Pratt (20).
[x] AL2, D1, and D2 are the isolate designations used by Hansen and Hamm (12).
[y] Large-oospore isolates (12).
[z] Small-oospore isolates (12).

TABLE 5. In vitro responses to metalaxyl at 0.1 μg/ml among isolates of *Phytophthora megasperma* from alfalfa, soybean, and chick-pea

Isolate	Host	Origin	Inhibition of radial growth (%)[z]
P1316	alfalfa	California	96.2 a
P844	alfalfa	California	95.4 ab
1133-2	alfalfa	California	94.6 ab
P1057	alfalfa	California	94.2 ab
P1253	chick-pea	Australia	92.8 ab
1129-4	alfalfa	California	91.9 b
P348	alfalfa	Mississippi	84.6 c
P510	soybean	Mississippi	69.7 d
P508	soybean	Mississippi	68.9 de
P405	soybean	Mississippi	68.5 de
P509	soybean	Mississippi	66.8 de

[z] Means followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's new multiple range test.

TABLE 6. In vitro responses among A1 isolates of *Phytophthora infestans* to metalaxyl

Isolate	Host	Origin	Inhibition of radial growth (%)[z] by metalaxyl at: 0.1 μg/ml	1.0 μg/ml
M4	potato	Mexico	94.4 a	92.8 a
P1298	potato	Wales	86.6 ab	84.2 ab
P1294	potato	Scotland	86.6 ab	89.9 a
65	potato	Mexico	80.5 b	78.1 abc
P1302	tomato	California	77.8 b	83.0 ab
P1292	potato	Wales	65.6 c	81.1 ab
57	potato	Mexico	62.1 cd	69.6 bcd
P4	potato	Mexico	58.3 cd	64.6 cde
P1295	potato	Scotland	52.4 de	61.9 de
P1293	potato	Scotland	45.8 ef	52.1 ef
P1300	potato	Wales	34.2 fg	41.7 fg
M3	potato	Mexico	7.6 h	11.8 h

[z] Means followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's new multiple range test.

TABLE 7. ED_{50} and ED_{90} values[a] for inhibition of radial growth expressed in micrograms per milliliter for individual isolates of *Phytophthora* species[b] varying in response to metalaxyl

Isolate	*Phytophthora* species	ED_{50}	ED_{90}
P1316	*megasperma* f. sp. *medicaginis*	0.013 ± 0.003	0.19 ± 0.02
P1257	*boehmeriae*	0.015 ± 0.006	0.05 ± 0.005
P1085	*hevea*	0.025 ± 0.004	0.05 ± 0.003
P405	*megasperma* f. sp. *glycinea*	0.033 ± 0.001	0.16 ± 0.005
Pc402	*cinnamomi*	0.057 ± 0.002	1.19 ± 0.12
M134	*parasitica*	0.097 ± 0.040	0.52 ± 0.09
P1163	*citrophthora*	0.44 ± 0.036	2.98 ± 0.37
P1273	*citricola*	0.44 ± 0.158	7.08 ± 1.37
D1-306[c]	*megasperma* (D1)	0.95 ± 0.059	6.70 ± 0.78

[a] Isolates of *Phytophthora* were grown on cornmeal agar at 25 C.
[b] Mean ± standard deviation of the mean based on a linear regression of the response (percent mycelial inhibition) plotted against the dosage (log concentration of metalaxyl).
[c] Isolate 306 is from Douglas-fir; D1 refers to the terminology used by Hansen and Hamm (12).

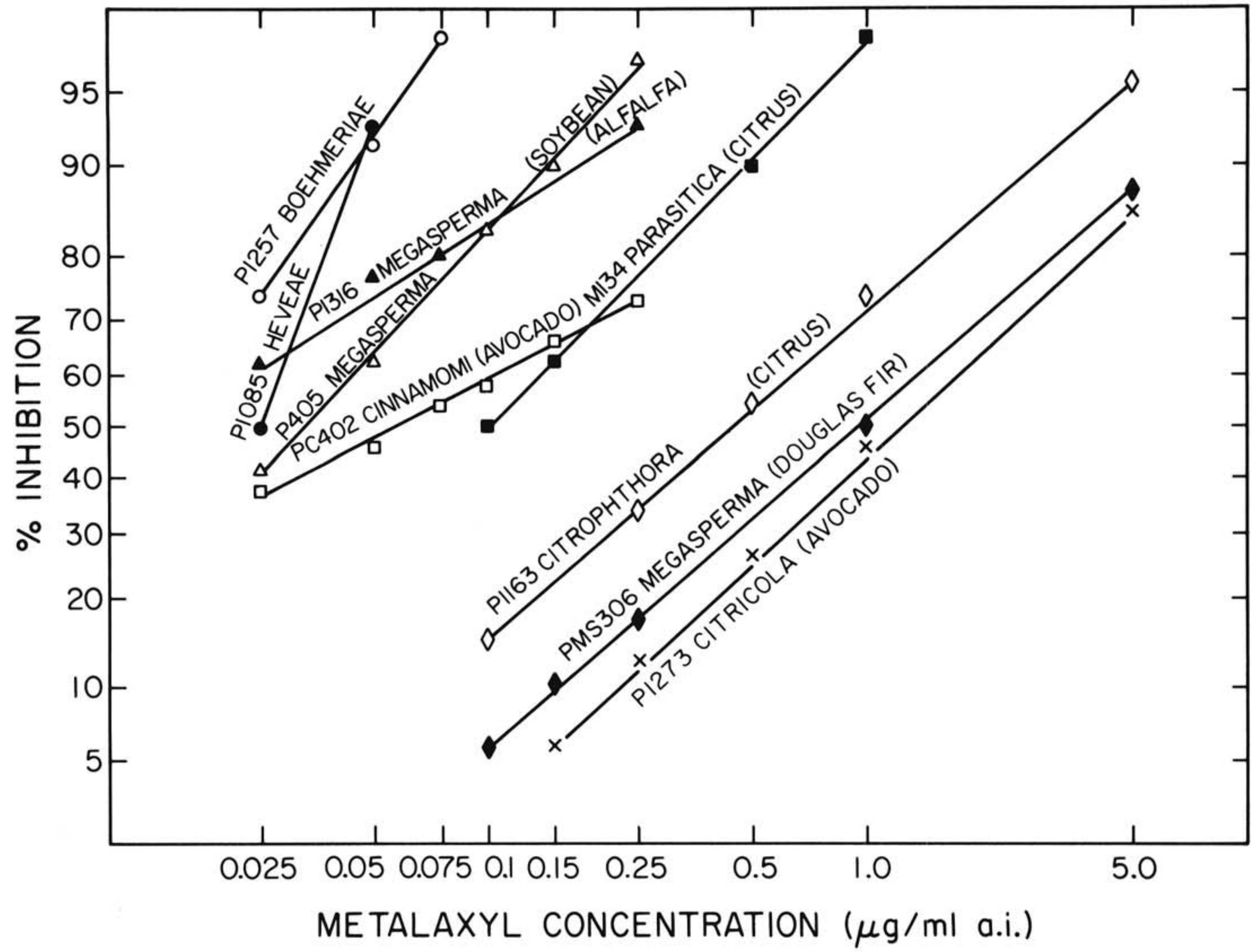

Fig. 1. Dosage-responses of nine isolates of *Phytophthora* species representing the range of sensitivities of the species in vitro. The correlation coefficients for the nine regression lines were all highly significant ($P < 0.01$).

(7). In fact, recent evidence points to inhibition of an α-amanitin-insensitive RNA polymerase-template complex (8). Thus, metalaxyl sensitivity, and perhaps related aspects of RNA transcription, should be added to the list of nonmorphological criteria which could prove useful as diagnostic tools in future studies of the taxonomy and evolution of *Phytophthora*.

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