In Vitro Insensitivity to Metalaxyl of Isolates of *Phytophthora citricola* and *P. parasitica* from Ornamental Hosts in Southern California

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ABSTRACT

Ferrin, D. M., and Kabashima, J. N. 1991. In vitro insensitivity to metalaxyl of isolates of *Phytophthora citricola* and *P. parasitica* from ornamental hosts in southern California. Plant Dis. 75:1041-1044.

In vitro sensitivity to metalaxyl was evaluated for 13 isolates of *Phytophthora citricola* and 26 isolates of *P. parasitica* recovered from ornamental hosts or soil from 12 locations in southern California. One isolate of *P. citricola* from azalea and two isolates of *P. parasitica* from periwinkle were insensitive to metalaxyl. Mean EC_{50} values for inhibition of linear growth were 219.9 μg a.i./ml for the metalaxyl-insensitive isolate of *P. citricola* and 717.4 and 742.4 μg a.i./ml for the two metalaxyl-insensitive isolates of *P. parasitica*. In contrast, mean EC_{50} values ranged from 0.087 to 4.970 μg a.i./ml and from 0.255 to 3.080 μg a.i./ml for metalaxyl-sensitive isolates of *P. citricola* and *P. parasitica*, respectively. Based on mean EC_{50} and slope values of the linearized dose-response curves, the metalaxyl-sensitive isolates of *P. citricola* could be divided into three distinct groups, whereas the metalaxyl-sensitive isolates of *P. parasitica* formed a single heterogeneous group.

Additional keywords: fungicide resistance, Subdue

Metalaxyl, the active ingredient of the oomycete-specific fungicides Subdue and Ridomil, is very effective in the control of diseases caused by *Phytophthora*, *Pythium*, and the downy mildew fungi. Root and crown rots caused by species of *Phytophthora* are major problems on many ornamental plants both in nursery and landscape settings. Since its registration, metalaxyl has been the primary fungicide used by nurserymen to control these diseases. Resistance to this fungicide has developed in many pathogen populations against which it was initially highly efficacious, including *Phytophthora infestans* (Mont.) de Bary and various species of downy mildew fungi (4) as well as *Pythium aphanidermatum* (Edson) Fitzp. (14,15). Additionally, considerable variation in sensitivity to metalaxyl exists naturally both within and across species of *Phytophthora* (2,3,7) and *Pythium* (20)

Naturally occurring resistance to metalaxyl in species of *Phytophthora* causing root and crown rots has not been documented, although such resistance has been demonstrated and induced in the laboratory (5,8,18). The continuous use of metalaxyl as the primary means of controlling diseases caused by *Phytophthora* increases the likelihood for the development of resistance to it, particularly among populations on woody ornamentals where fungicide applications are repeated over several years.

The objective of this study was to assess the in vitro sensitivity to metalaxyl of populations of two species of *Phytophthora*, *P. parasitica* Dastur and *P. citricola* Sawada, commonly isolated

This research was supported by a grant from the University of California Cooperative Extension.

Accepted for publication 3 April 1991 (submitted for electronic processing).

from ornamental hosts in southern California.

MATERIALS AND METHODS

The isolates of *P. citricola* and *P. parasitica* used in this study are identified in Table 1. Isolates collected before the registration and widespread use of metalaxyl were obtained from the culture collection at the University of California, Riverside. Single-zoospore isolates of species collected from ornamental hosts or soil from nurseries in southern California since the registration of metalaxyl were established after isolation from diseased tissue on a medium selective for species of *Phytophthora* (12).

The fungicide metalaxyl (Subdue 2E, 25.1% a.i.) was diluted in sterile deionized water and added to media that had been autoclaved and cooled to 45 C. The concentrations used for determination of EC_{50} values were 0.0, 0.025, 0.05, 0.075, 0.1, 0.25, and 0.5; 0, 50, 100, 250, 500, 750, and 1,000; 0.0, 0.05, 0.1, 0.5, 1.0, 2.5, and 5.0; and 0, 250, 500, 750, 1,000, 1,250, and 1,500 μg a.i./ml for the metalaxyl-sensitive and metalaxyl-insensitive isolates of *P. citricola* and *P. parasitica*, respectively.

Linear growth responses were determined by incorporation of metalaxyl into Difco cornmeal agar (CMA) for *P. citricola* and into clarified V8 juice-$CaCO_3$ (V8C) agar for *P. parasitica*. The center of each plate was inoculated with a 4-mm-diameter plug taken from the leading edge of 4-day-old colonies of *P. citricola* growing on CMA or 5- to 6-day-old colonies of *P. parasitica* growing on V8C agar. Plates were incubated in the dark at 25 C for 4 days for *P. citricola* and 5 or 6 days for *P. parasitica*. Three colony diameter measurements were made on each of three replicate plates per isolate per concentration. The mean colony diameter per replicate plate was calculated and used to calculate the mean colony diameter per treatment. All experiments were conducted five times.

Data were analyzed by linear regression with the general linear models procedure (GLM) of the Statistical Analysis System (SAS Institute Inc., Cary, NC) after calculation of percent inhibition of radial growth with respect to growth on nonamended media. Percent inhibition data were transformed by probits and metalaxyl concentrations were transformed by $\log_{10}$ before analysis. The metalaxyl concentrations that resulted in 50% inhibition of linear growth (EC_{50}) were calculated from the linear equations relating fungicide concentration to percent inhibition. Analysis of covariance was used to obtain common regression lines to describe the dose-response relationship for each isolate across individual experiments.

Table 1. Isolates of *Phytophthora citricola* and *P. parasitica* tested for sensitivity to metalaxyl in vitro

Isolate[x]	Mating type	Year isolated	Host	Location[y]
Phytophthora citricola				
P-767	NA[z]	?	*Syringa vulgaris*	Canada
P-1039	NA	?	*Eucalyptus* sp.	California
P-1189	NA	?	*Pieris japonica*	North Carolina
T-569	NA	1982	*Rhododendron* sp.	California
T-598	NA	1986	*Rhododendron* sp.	California
P-040F	NA	1989	Soil	B
P-045F	NA	1989	*Olea europaea*	C
P-051F	NA	1989	*Rhamnus californica*	D
P-053F	NA	1989	*Ceanothus griseus* var. *horizontalis*	D
P-054F	NA	1989	*Rhamnus californica*	D
P-059F	NA	1989	Soil	D
P-073F	NA	1989	*Gardenia jasminoides*	E
P-074F	NA	1989	*Rhododendron* sp.	E
Phytophthora parasitica				
P-1083	A1	?	*Gypsophila paniculata*	California
P-1098	A2	?	*Simmondsia chinensis*	California
P-001F	A2	1986	*Myrtus communis*	F
P-008F	A2	1986	*Myrtus communis*	F
P-012F	A1	1987	*Catharanthus roseus*	G
P-013F	A1	1987	*Catharanthus roseus*	G
P-014F	A1	1987	*Catharanthus roseus*	H
P-015F	A1	1987	*Catharanthus roseus*	H
P-024F	A2	1987	*Myrtus communis*	F
P-025F	A2	1987	*Myrtus communis*	F
P-031F	A2	1988	*Boronia heterophylla*	I
P-032F	A2	1988	*Boronia heterophylla*	I
P-033F	A2	1988	*Boronia heterophylla*	I
P-034F	A1	1988	*Ceanothus* sp.	B
P-046F	A2	1989	*Ceanothus griseus* var. *horizontalis*	D
P-048F	A1	1989	*Rhamnus californica*	D
P-065F	A2	1988	*Pinus pinea*	J
P-068F	A2	1989	*Catharanthus roseus*	K
P-070F	A2	1989	*Catharanthus roseus*	K
P-071F	A2	1989	*Catharanthus roseus*	K
P-072F	A2	1989	*Catharanthus roseus*	K
P-075F	A2	1989	*Gardenia jasminoides*	E
P-076F	A1	1989	*Gardenia jasminoides*	L
P-077F	A2	1989	*Gardenia jasminoides*	E
P-078F	A1	1989	*Hibiscus rosa-sinensis*	L
P-079F	A1	1989	*Hibiscus rosa-sinensis*	L

[x] Isolates designated by P are from the culture collection at the University of California, Riverside; those designated by T are from the collection of P. H. Tsao; those followed by F are from the collection of D. M. Ferrin.

[y] Isolates with the same letter designation were collected from the same location.

[z] Mating type is not applicable for *P. citricola*.

RESULTS

One of the 13 isolates of *P. citricola* tested was less sensitive to metalaxyl than the others (Table 2). The mean EC_{50} value for this isolate was 219.9 μg a.i./ml, with a mean slope value of 2.03. The 12 sensitive isolates could be subdivided into three groups based on their EC_{50} values and the slopes of their linearized dose-response relationships (Table 2 and Fig. 1). Mean EC_{50} values for the seven isolates in group I ranged from 0.087 to 0.198 μg a.i./ml; the mean slope values ranged from 2.26 to 2.74. Mean EC_{50} values for the three isolates in group II ranged from 0.306 to 0.606 μg a.i./ml; the mean slope values ranged from 1.42 to 1.59. Mean EC_{50} values for the two isolates in group III were 4.524 and 4.970 μg a.i./ml; their mean slope values were 0.74 and 0.75, respectively.

Two of the 26 isolates of *P. parasitica* tested were insensitive to metalaxyl (Table 3 and Fig. 2). The mean EC_{50} values for these isolates were 717.4 and 742.4 μg a.i./ml; their mean slope values were 2.93 and 2.41, respectively. The mean EC_{50} values for the sensitive isolates ranged from 0.255 to 3.080 μg a.i./ml; the mean slope values ranged from 0.63 to 1.53. No distinctive groups were observed among the metalaxyl-sensitive isolates of *P. parasitica*.

DISCUSSION

Mean EC_{50} and slope values for the metalaxyl-sensitive isolates of *P. citricola* examined in the present study were within the ranges reported previously for *P. citricola* (2,3,8). However, a wider variation in response to metalaxyl was observed for isolates collected from a variety of hosts worldwide (3). EC_{50}

values for the metalaxyl-sensitive isolates of *P. parasitica* examined in the present study were somewhat greater than those reported previously for *P. parasitica* from citrus from California (6) or Brazil (2) or for *P. parasitica* var. *nicotianae* (Breda de Haan) Tucker (11,16,19).

Within the metalaxyl-sensitive isolates of *P. citricola* examined, there were three distinct subpopulations that could be distinguished by their EC_{50} and slope values. Within the metalaxyl-sensitive isolates of *P. parasitica* examined, there were no identifiable subpopulations, but there was considerable heterogeneity in their response to metalaxyl. The distinct groups occurring within *P. citricola* may be a reflection of the intraspecific diversity and occurrence of distinct subpopulations demonstrated by isozyme analysis, whereas the lack of distinct subpopulations within *P. parasitica* may be a reflection of the lack of intraspecific diversity as demonstrated by isozyme analysis (13).

Two types of resistance to metalaxyl were described for *P. megasperma* Drechs. f. sp. *medicaginis* T. Kuan & D. C. Erwin (5). Low levels of resistance were associated with mycelial adaptation and mass selection of untreated zoospores on a metalaxyl-amended medium. Isolates exhibiting this type of resistance had ED_{50} values of less than 5 μg/ml, generally were less fit and less virulent than the parent isolates, and did not show a high degree of resistance in vivo. High levels of resistance were associated with the mass selection of zoospores after treatment with mutagenic agents. Isolates exhibiting this type of resistance had ED_{50} values greater than 100 μg/ml, generally were as fit and as virulent as the parent isolate, and expressed resistance in vivo. These two types of resistance to metalaxyl have been observed for other species of *Phytophthora* as well.

Annual applications of metalaxyl to field soils infested with *P. p. nicotianae* resulted in an increase in the ED_{50} from 0.4 to 1.2 μg/ml within 3 yr (17). Resistant isolates of *P. citricola* were not obtained by mass selection on metalaxyl-amended media, but isolates exhibiting both types of resistance were recovered after treatment with mutagenic agents (8). Isolates of *P. m. medicaginis* with both types of resistance were recovered after mass selection of untreated zoospores on a metalaxyl-amended medium (18).

This is the first report of naturally occurring insensitivity to metalaxyl in *P. citricola* and *P. parasitica*. The metalaxyl-insensitive isolate of *P. citricola* had an EC_{50} of 219.9 μg/ml, compared with 0.198 μg/ml for a metalaxyl-sensitive isolate from the same nursery. This level of resistance is approximately five times greater than the highest recommended rate for use of metalaxyl and was just slightly less than that observed for laboratory-produced metalaxyl-resistant isolates of *P. citricola*, which had EC_{50} values ranging from 230 to 505 μg/ml, compared with 0.26 μg/ml for the metalaxyl-sensitive parent isolate (8).

The metalaxyl-insensitive isolates of *P. parasitica* had EC_{50} values of 717.4 and 742.4 μg/ml. This level of resistance is approximately 15 to 16 times greater than the highest recommended rate for use of metalaxyl. One of the isolates of *P. parasitica* (P-015F) is insensitive to metalaxyl in vivo and appears to be as virulent as sensitive wild-type isolates (D. M. Ferrin, *unpublished*), as has been reported for *P. infestans* (1,9,10) and *P. citricola* (8). Thus, the insensitivity to metalaxyl of the isolates identified in this study appears to be of the second type identified by Davidse (5).

Widespread failures of metalaxyl to control diseases caused by *Phytophthora* were not observed in the nurseries from which the metalaxyl-insensitive isolates were recovered. For nurseries where plants are grown in containers, widespread failures in control would not necessarily be expected immediately after the appearance of resistance because of the low frequency with which such

Table 2. Influence of metalaxyl on linear growth of isolates of *Phytophthora citricola* on amended agar

		Means[v]		Parameter estimates[w]		
Isolate	Group[u]	EC_{50}[x]	Slope[y]	EC_{50}	Slope	R^{2}[z]
P-053F	I	0.087 (0.024)	2.74 (0.21)	0.098	2.72	0.980
P-054F	I	0.102 (0.025)	2.46 (0.16)	0.106	2.46	0.987
P-1039	I	0.104 (0.023)	2.41 (0.12)	0.104	2.41	0.984
P-059F	I	0.107 (0.027)	2.39 (0.10)	0.105	2.39	0.989
P-040F	I	0.111 (0.027)	2.26 (0.16)	0.115	2.26	0.983
P-051F	I	0.112 (0.029)	2.58 (0.12)	0.115	2.57	0.983
P-073F	I	0.198 (0.045)	2.40 (0.17)	0.170	2.40	0.987
P-045F	II	0.306 (0.060)	1.59 (0.23)	0.306	1.57	0.957
P-1189	II	0.465 (0.111)	1.42 (0.20)	0.405	1.42	0.963
P-767	II	0.606 (0.181)	1.51 (0.26)	0.497	1.51	0.937
T-598	III	4.524 (2.043)	0.74 (0.11)	2.168	0.74	0.914
T-569	III	4.970 (1.948)	0.75 (0.13)	2.275	0.75	0.916
P-074F	IV	219.900 (10.400)	2.03 (0.18)	201.500	2.03	0.978

[u] Group designations reflect natural groupings based on the ranges in EC_{50} and slope values of the isolates.
[v] Means of parameter estimates from five experiments. Numbers within parentheses are standard deviations of the estimates.
[w] Parameter estimates of common regression lines obtained by analysis of covariance of all experiments combined.
[x] Concentration (μg a.i./ml) required for 50% inhibition of linear growth calculated from the regression equations describing the dose-response relationship.
[y] Slope of the regression equation describing the dose-response relationship.
[z] Coefficient of determination; all regressions were significant at $P < 0.05$.

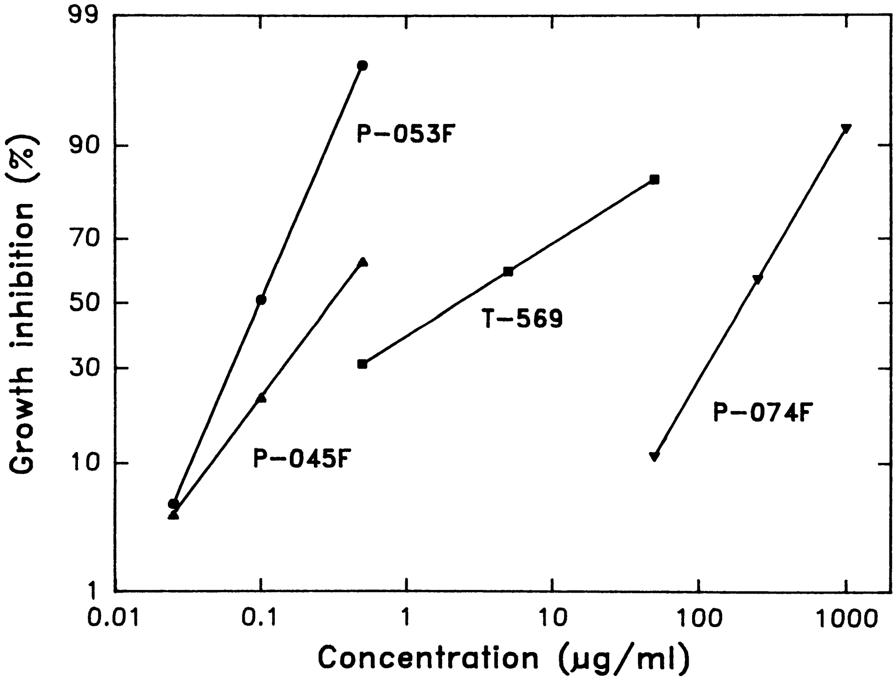

Fig. 1. Dose-response relationships for representative isolates of *Phytophthora citricola* demonstrating the range of sensitivities to metalaxyl of the subpopulations within this species.

resistance appears (5,8,18). However, this may pose a threat to the nursery industry, particularly should such resistance appear in wholesale nurseries that supply local retail nurseries and landscapers. Furthermore, current regulations requiring nurseries to recirculate all run-off water increases the risk of recycling fungicide insensitive pathogen populations. Thus, the appearance of resistance could eventually result in severe control failures if the treatment of recirculated water is not sufficient to eliminate propagules of *Phytophthora* spp.

Table 3. Influence of metalaxyl on linear growth of isolates of *Phytophthora parasitica* on amended agar

	Means[v]		Parameter estimates[w]		
Isolate	EC_{50}[x]	Slope[y]	EC_{50}	Slope	R^2[z]
P-1083	0.255 (0.066)	1.09 (0.16)	0.226	1.09	0.944
P-076F	0.275 (0.053)	0.83 (0.06)	0.302	0.83	0.951
P-077F	0.279 (0.079)	0.81 (0.09)	0.367	0.81	0.946
P-065F	0.289 (0.064)	0.85 (0.10)	0.330	0.85	0.959
P-048F	0.315 (0.103)	0.79 (0.04)	0.404	0.79	0.920
P-033F	0.366 (0.081)	1.35 (0.16)	0.358	1.35	0.934
P-1098	0.482 (0.115)	1.04 (0.14)	0.490	1.04	0.961
P-034F	0.502 (0.107)	0.90 (0.10)	0.289	0.90	0.817
P-079F	0.527 (0.089)	0.87 (0.04)	0.615	0.87	0.813
P-068F	0.546 (0.134)	0.94 (0.14)	0.520	0.94	0.960
P-001F	0.582 (0.053)	1.32 (0.20)	0.608	1.32	0.895
P-078F	0.679 (0.206)	1.16 (0.17)	0.577	1.14	0.900
P-012F	0.715 (0.176)	0.78 (0.03)	0.864	0.78	0.975
P-070F	0.720 (0.346)	0.95 (0.16)	0.609	0.88	0.983
P-075F	0.740 (0.094)	0.95 (0.12)	0.639	0.95	0.934
P-071F	0.780 (0.393)	0.88 (0.07)	0.535	0.88	0.964
P-008F	0.894 (0.439)	1.01 (0.20)	1.297	0.99	0.898
P-046F	0.951 (0.105)	1.14 (0.09)	1.054	1.14	0.947
P-072F	1.005 (0.372)	1.08 (0.16)	0.928	1.08	0.938
P-031F	1.157 (0.510)	1.53 (0.53)	0.770	1.64	0.925
P-032F	1.255 (0.818)	0.94 (0.11)	1.729	0.94	0.926
P-013F	1.278 (0.745)	0.63 (0.19)	0.780	0.57	0.924
P-025F	1.651 (1.229)	0.84 (0.07)	1.068	0.84	0.931
P-024F	3.080 (0.991)	0.97 (0.29)	3.213	1.09	0.884
P-015F	717.400 (77.100)	2.93 (0.95)	755.700	3.02	0.839
P-014F	742.400 (67.700)	2.41 (0.71)	887.100	2.58	0.860

[v] Means of parameter estimates from five experiments. Numbers within parentheses are standard deviations of the estimates.
[w] Parameter estimates of common regression lines obtained by analysis of covariance of all experiments combined.
[x] Concentration (μg a.i./ml) required for 50% inhibition of linear growth calculated from the regression equations describing the dose-response relationship.
[y] Slope of the regression equation describing the dose-response relationship.
[z] Coefficient of determination; all regressions were significant at $P < 0.05$.

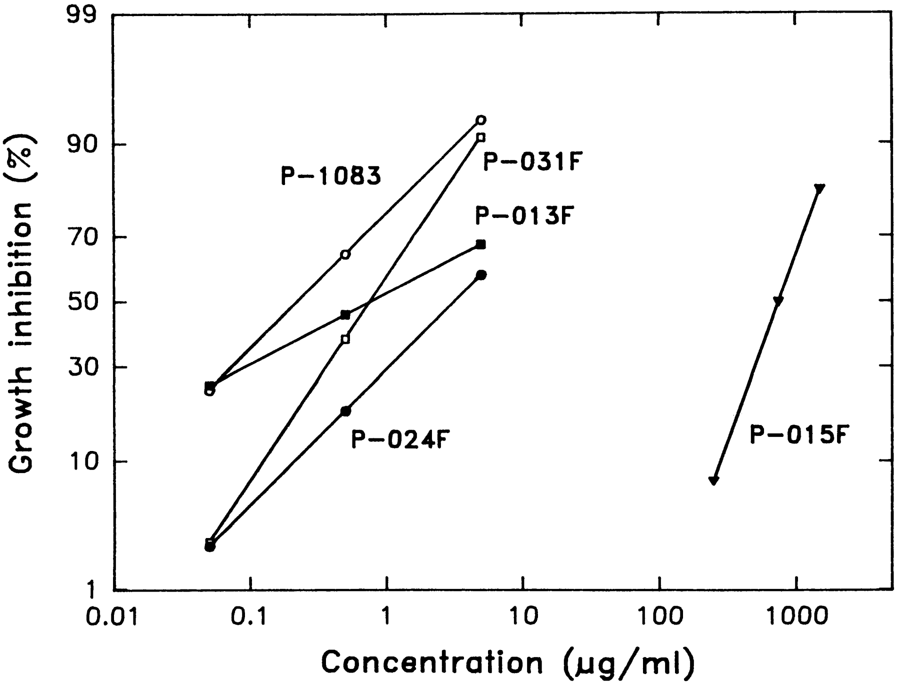

Fig. 2. Dose-response relationships for representative isolates of *Phytophthora parasitica* demonstrating the range of sensitivities to metalaxyl within this species.

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