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Title: Chemical Control of Hop Downy Mildew (Pseudoperonospora humuli) and Tolerance by Phytophthora Isolates to the Systemic Fungicide Metalaxyl.

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Dr. Chester E. Horner

Four systemic fungicides (metalaxyl, M 9834, propamocarb, and efosite aluminum) were evaluated for control of the downy mildew disease (Pseudoperonospora humuli) of hops (Humulus lupulus). Metalaxyl applied at 0.3 gm/plant in mid-April resulted in nearly complete control of the disease and increased yields significantly. Treatment with M 9834 at 0.3 gm/plant resulted in control comparable to metalaxyl up to four weeks after application, but disease incidence increased sharply after six weeks. Propamocarb and efosite aluminum treatments were less effective than metalaxyl or M 9834, but did result in substantial disease control.

The possibility that metalaxyl tolerance occurs in Phytophthora, fungus related to Pseudoperonospora was examined in vitro. Thirty-five isolates of Phytophthora megasperma were tested for tolerance to metalaxyl. Tolerance was measured by comparing isolate growth, and oogonia and sporangia formation on amended and control media. Based on growth at 0, 1, and 10 µg metalaxyl/ml, 15 isolates were highly

sensitive, 8 moderately tolerant, and 12 highly tolerant to the fungicide. No isolates in the highly sensitive group formed oogonia at 1 µg metalaxyl/ml, whereas six isolates from the moderately tolerant group, and eight from the highly tolerant group did. The number of isolates forming sporangia and the mean number of sporangia formed per isolate decreased with increasing fungicide concentration. At least two of 280 single zoospore isolates were more tolerant to metalaxyl than their parents, suggesting that commercial applications of metalaxyl may select for naturally occurring tolerant strains which may lead to loss of disease control.

Response to metalaxyl was examined more extensively using ED_{50} values and slopes of dosage-response curves from 16 Phytophthora isolates. Percent inhibition of growth determined by comparing hyphal extension on metalaxyl amended and unamended media was used to generate the dosage-response curves. One isolate each of P. cinnamomi and P. drechsleri, and 14 isolates of P. megasperma were examined. P. cinnamomi and P. drechsleri isolates were highly sensitive to metalaxyl (ED_{50} values < 0.1 µg metalaxyl/ml, and slope values > 1.0). P. megasperma isolates ranged from highly sensitive (ED_{50} values > 30 µg metalaxyl/ml, and slope values < 0.65). Response to metalaxyl would not be helpful in identifying isolates to species, but may be useful in separating P. megasperma isolates into morphologically distinct groups if used in addition with morphological traits.

CHEMICAL CONTROL OF HOP DOWNY MILDEW (*PSEUDOPERONOSPORA HUMULI*) AND TOLERANCE BY *PHYTOPHTHORA* ISOLATES TO THE SYSTEMIC FUNGICIDE METALAXYL

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CHAPTER I

INTRODUCTION

The common hop plant (*Humulus lupulus* L.) produces the hops of commerce. Although there are reports alluding to the medicinal value of *H. lupulus* (35), the sole economic market for the hops of commerce is in the brewing of beer and similar malt beverages.

The United States has been the leading worldwide producer of hops during most of the twentieth century (48). The only exception was from 1968-1979 when West Germany was the leading hop producer. In 1981, the United States produced 79.0 million lb (35,833,797 kg) of hops compared to 74.1 million lb (33,611,196 kg) produced by West Germany. These figures represent 27.4 and 25.6% of the total world production (288.8 million lb: 130,997,480 kg) in 1981 (48).

Within the United States, hops are cultivated commercially only in Washington, Oregon, Idaho, and California. The acreages and production figures from each state in 1981 were (48):

1. Washington: 31,300 acres (12672.1 ha); 59.47 million lb (26,975,138 kg).
2. Oregon: 7200 acres (2915.0 ha); 12.384 million lb (5,617,287.1 kg).
3. Idaho: 3400 acres (1376.5 ha); 5.61 million lb (2,544,653.2 kg).
4. California: 1200 acres (485.8 ha); 1.68 million lb (762,035.2 kg).

Historically, the primary disease problem on hops has been hop downy mildew caused by the fungus Pseudoperonospora humuli (Miyabe et Takahashi) Wilson. This disease was first observed in the United States on native, wild hops in Wisconsin in 1909 (33), and in Washington in 1929 (39). Shortly after 1930, hop downy mildew was found widespread in hopyards throughout the Pacific Northwest.

Losses resulting from this disease are frequently substantial. For example, in 1957 an estimated 12 and 30% of the United States and California hop crops were destroyed by P. humuli, respectively (47). In addition, statements made by hop growers continuously indicate concern regarding economic losses from downy mildew, and reflect the need for more effective measures to control this disease.

Thus, the objectives of this research were to identify a fungicide that effectively controls hop downy mildew, and examine the possibility that tolerance to the systemic fungicide metalaxyl exists in Phytophthora isolates; a fungus related to P. humuli.

CHAPTER II

LITERATURE REVIEW

Description of H. lupulus.

The perennial rootstock of the common hop plant (H. lupulus) has fleshy storage roots, slender feeder roots, and underground rhizomes connected to a woody crown (47). Because H. lupulus is dioecious with staminate and pistilate flowers on separate plants, hop plants are genetically heterozygous. Consequently, hop varieties are propagated with rhizome sections so genetic uniformity is maintained (38).

Numerous buds are formed each fall on hop crowns. These buds emerge in April and give rise to annual vines. During late April, twine is strung from wires suspended 18-20 ft (5.5-6.1 m) above the soil surface on a system of poles to individual hop plants (hills) (47). Two or three strands of twine are attached to each hill. During May, 6-9 vines from each hill are trained onto the twine. Trained vines grow rapidly and produce many side branches. Female flowers (burrs) produced on side branches and main vines form strobiles (cones) consisting of a central stem (strig) that bears bracts and bracteoles (petals) (38, 47). Cones are oblong, yellowish-green, and 1-4 in (2.5-10.2 cm) long. Numerous glandular bodies (lupulins) found in mature cones contain the essential oils and resins that give beer its aroma and bitter taste (38, 47). Dried and baled mature cones are the hops of commerce used directly (or as pellets or extracts) to brew beer.

Description of Hop Downy Mildew.

Hop downy mildew caused by P. humuli is a fungus in the family Peronosporaceae, order Peronosporales, class Oomycete, subdivision Mastigomycotina, division Eumycota (1). P. humuli is an obligate parasite and can not survive or reproduce if separated from a suitable host. This fungus overwinters as systemic hyphae in hop crowns (9, 39, 44). Oospores are formed, but their role in the disease cycle is uncertain (9, 39, 44).

In hills with diseased crowns, infected buds emerge in spring and form distorted shoots called primary basal spikes. Spikes are infected systemically with P. humuli, and appear silvery or pale green, rigid, stunted, and brittle (36). Zoosporangia are produced primarily on the abaxial leaf surfaces of spikes, appearing as a purplish-black mat (39). Zoosporangia are dispersed to all plant parts by wind or splashing rain. With adequate free moisture and temperatures between 5-28 C (15-20 C optimum), zoosporangia germinate and release zoospores that usually encyst near stomata (9, 33, 39, 44). Germ tubes from encysted zoospores penetrate stomata and result in secondary infections (9, 39, 41, 44). When secondary infections occur on shoot meristems, they become systemic and result in the formation of secondary basal spikes, lateral stem spikes, or terminal spikes. Localized infections also occur, causing leaf spots, flower blight, and partial to complete fruit blight. Healthy hop crowns become infected either by movement of hyphae down systemically infected shoots, or by zoospores (10, 39, 44).

Severe crown infection, or infection of hop varieties highly susceptible to P. humuli (e.g. Cluster, Comet, and Talisman) may result

in rotting of the crown and hill death. For this reason, these varieties are cultivated in Washington, Idaho, and California where hot and dry climates usually suppress the disease. However, weather in these areas occasionally favors severe disease and subsequent economic losses.

Hop varieties with some resistance to the crown infection phase of this disease are cultivated in the Willamette Valley of Oregon (e.g. Fuggle, Cascade, Brewer's Gold, and Bullion). However, mildew control in Oregon is especially important because shoots, leaves, and fruits of these varieties are susceptible to the disease. In addition, mild temperatures and rainfall during April and May in the Willamette Valley favor disease development. Thus, if growers are able to control hop downy mildew during the wet and mild spring, hot and dry weather beginning in late June usually suppresses further disease.

Measures employed to control hop downy mildew in the Willamette Valley include (47):

1. Deep pruning of hop hills in early spring to remove buds and tissue infected with P. humuli.
2. Removal of trained vines with terminally infected shoots, and retraining healthy shoots.
3. Stripping infected lower leaves on otherwise healthy trained vines to remove secondary inoculum sources.
4. After vines are trained, periodic killing of new basal growth with chemicals and/or covering basal growth with soil (hilling-up). This removes secondary inoculum and juvenile, succulent growth highly susceptible to infection.

5. Spraying or dusting foliage with zineb (zinc ethylene-bisdithiocarbamate) or various copper formulations.

Employing these control measures does not ensure a successful crop of hops. For example, reduced yields are imminent if healthy vines are not trained by late May, or if fewer than six vines are trained per hill (30). Zineb and coppers are protectant fungicides, and are not redistributed after application. Thus, growth of hops following application and foliage covered incompletely are susceptible to infection. Consequently, weekly applications of zineb or copper have been recommended when weather conditions favor disease. The expense of weekly fungicide applications added to the salaries of labor crews hired to remove infected shoots and retrain healthy vines is considerable. Furthermore, residue problems have occurred in beer brewed with hops treated with zineb.

Several systemic fungicides were developed during the late 1970's that are highly effective against many oomycete fungi (Fig. 1) (8, 43, 50). Systemic fungicides penetrate plant tissue and are translocated in the phloem or the evapotranspiration stream to new growth and foliage (16). Because of the high cost and inefficiency of current controls, hop growers in Oregon as well as the rest of the United States anxiously awaited the testing of these compounds for control of hop downy mildew. However, the effectiveness of many systemic fungicides used to control diseases has been lost because the fungus became tolerant to the fungicide. This phenomenon is an important consideration when using systemic fungicides and has been the subject of several review articles (12, 13, 14, 16, 20).

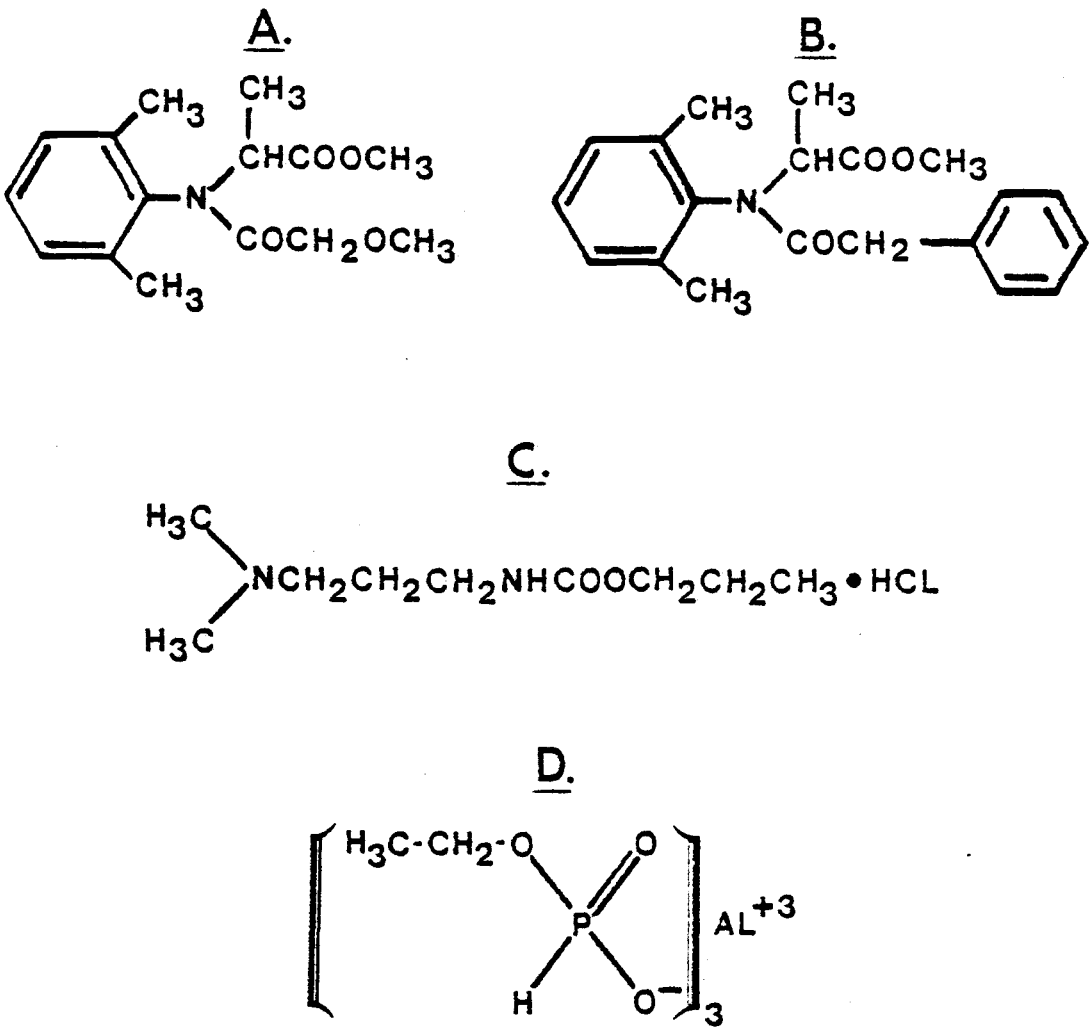

Fig. 1. Structures of systemic fungicides recently developed and reported effective against many oomycete fungi: A. N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-alanine methyl ester (metalaxyl; Ciba-Geigy Corp.); B. methyl-2-[N-phenylacetyl-N-(2,6-dimethyl phenyl) amino] propanoate (M 9834; Montedison Research Labs); C. [3-(dimethyl-amino) propyl] carbamate monohydrochloride (propamocarb; Rhone-Poulenc, Inc.); D. aluminum tris (-O-ethyl Phosphonate) (LS 74 783; Nor-Am, Inc.). All structures taken from technical bulletins supplied by the companies.

The following three chapters present data regarding control of hop downy mildew with systemic fungicides (Chapter III), and the occurrence of tolerance to the systemic fungicide metalaxyl in Phytophthora isolates (Chapters IV and V).

CHAPTER III

Control of Hop Downy Mildew with Four Systemic Fungicides

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ABSTRACT

Hunger, R. M., and C. E. Horner. 1982. Control of hop downy mildew with four systemic fungicides. Plant Disease

Four systemic fungicides (metalaxyl, M 9834, propamocarb, and efosite aluminum) were evaluated for control of the systemic infection phase of hop downy mildew during 1979-1981. Metalaxyl applied to the soil surface over the perennial hop crown area in April resulted in nearly complete control and increased yields significantly. Treatment with M 9834 resulted in control comparable to metalaxyl up to four weeks after application, but disease incidence increased sharply after six weeks. Propamocarb and efosite aluminum were less effective than metalaxyl or M 9834 but gave substantial disease control. In addition, hop crowns treated with metalaxyl as a post-harvest soil spray in September-October had a lower disease

incidence the following year than control plants. The possibility of using metalaxyl and a broad spectrum fungicide in alternate years to avoid selection of fungal strains tolerant to metalaxyl is discussed.

Additional key words: metalaxyl, Ridomil, M 9834, Galben, propamocarb, Previcur N, efosite aluminum, Aliette, fungicide tolerance, fungicide resistance, Pseudoperonospora humuli.

Hop downy mildew, caused by Pseudoperonospora humuli (Miy. et Tak.) Wils., was introduced into the Pacific Northwest in 1929 (39) and has become a major problem on hops (Humulus lupulus L.) in all producing areas of the United States (39, 47). P. humuli overwinters in the perennial rootstock (crown) of infected hop plants (9, 39, 44). Systemically infected buds emerge in spring and form distorted shoots called primary basal spikes. Zoosporangia produced on basal spikes function as primary inoculum. Abundant moisture and mild temperatures favor sporulation, spread of zoosporangia, and secondary infection by zoospores (9, 39, 44). When secondary infections occur on shoot meristems, the infections become systemic resulting in the formation of secondary basal spikes, lateral stem spikes, or terminal spikes. Localized infections also occur causing leaf spots, flower blight, and partial to complete fruit blight. Healthy hop crowns become infected either by movement of mycelium down systemically infected shoots or by zoospores (10, 39, 44). Oospores are produced,

especially in infected fruits, but their role in the disease cycle has not been clearly established (9, 39, 44).

Crown infection of highly susceptible varieties (e.g. Cluster, Comet, and Talisman) may result in severe crown rot and complete plant death. Thus, these varieties are grown in areas of Washington, Idaho, and California where hot and dry climates usually suppress disease development. However, weather in these areas occasionally favors severe disease and losses. In contrast, varieties with some tolerance to crown rot (e.g. Fuggle, Cascade, Bullion, and Brewer's Gold) are cultivated in the Willamette Valley of Oregon where rainfall and mild temperatures during April and May usually favor disease development. Mildew control in Oregon is especially important because shoots, leaves, and fruits of these varieties are susceptible to the disease. Further, if healthy shoots are not trained onto trellis systems by mid-May vines do not have sufficient time to develop fully and produce reasonable yields.

The protectant fungicide zineb (zinc ethylenebisdithiocarbamate) and various formulations of copper have been used to control hop downy mildew. However, relatively poor levels of control are achieved with these nonsystemic fungicides even with the multiple applications required to protect new growth. Thus, field trials were conducted in Oregon during 1979-1981 to identify a systemic fungicide effective in controlling hop downy mildew.

MATERIALS AND METHODS

Field trials with metalaxyl and M 9834. Metalaxyl (Ridomil 2 EC, Ciba-Geigy Corp.) was the only compound tested in 1979. Test

plots were located in five commercial hop fields in the Willamette Valley planted with the varieties Cascade, Bullion, or Brewer's Gold. A total of 2029 plants were treated with metalaxyl at 0.3 gm per plant in 50 ml water. Fungicide was applied in mid-April with a model 1730A Root-Lowell sprayer (8 liter capacity) by distributing a coarse spray directly over the crown area when the longest emerging shoots were 5-10 cm long. Spikes (basal, lateral, and terminal) on metalaxyl treated and control plants were counted at the time of metalaxyl application and at two week intervals for 12 weeks.

Yield trials were conducted at two fields in 1979. Three replications of five plants/rep were selected from each treatment (metalaxyl and control) by use of a random numbers table. Vines were removed and picked with a small mechanical hop picker. Fresh weights of hop fruits were recorded, and yields were extrapolated to kg dried hops/ha and lb/acre.

Metalaxyl (0.3 gm/plant/50 ml water) also was applied to the crown area of plants as a post-harvest spray in September 1979 and in October 1980 in fields where downy mildew had been severe for several consecutive years. Three-hundred plants and 50 plants were treated in 1979 and 1980, respectively. Treated plants received no additional fungicide. Spikes in treated and control plants were counted six times and five times during the spring and summer of 1980 and 1981, respectively.

Metalaxyl was tested at three commercial fields in 1980. Metalaxyl was applied to the crown area at 0.3 and 0.6 gm/plant in 100 ml water in mid-April. Eighty-four plants/rate were treated at each field plot. Disease control was determined by comparing total spikes

in metalaxyl treated and control plants. In addition, the occurrence of spikes during 1980 on plants treated with metalaxyl in 1979 was observed, although these plants were not treated with any fungicide in 1980.

Control of hop downy mildew by metalaxyl and M 9834 (Galben 20% LC, Montedison Research Laboratories) was compared in 1981 at two commercial fields. Fungicides were applied in mid-April at 0.3 and 0.6 gm/plant in 50 ml water using 50 plants/treatment at each location. Spikes in treated and control plants were counted one, two, four, six, and ten weeks after application. Yields were compared in one field plot by harvesting hop fruits from six plants/treatment selected with a random numbers table. Hops were harvested as in 1979, and fresh weights were extrapolated to kg dried hops/ha and lb/acre.

Field trials with propamocarb and efosite aluminum. Propamocarb (Previcur N 6 LC, Nor-Am, Inc.) and efosite aluminum (Aliette WP 80%, Rhone-Poulenc, Inc.) were tested at 3 commercial fields. Propamocarb was applied at 1.17, 2.34, and 4.68 gm/plant. Efosite aluminum (efosite Al) was applied at 0.22, 0.44, and 0.88 gm/plant. Fungicides were applied in 100 ml water in mid-April to 50 plants at each field plot. Disease control was determined by counting spikes in treated and control plants every two weeks after application for eight weeks.

RESULTS

Field trials with metalaxyl and M 9834. Results of field trials with metalaxyl in 1979 and 1980 are presented in Table 1. A total of

Table 1. Control of hop downy mildew in Oregon with metalaxyl and M 9834 during 1979-1981.

Date of application	Treatment[W] (gm/plant)	Number of plants treated[X]	Number of spikes at time of fungicide application	Number of spikes observed after fungicide application (weeks)							Total
				1	2	4	6	8	10	12	
April 17, 1979	Control 0.0	2029	1058	--	3620	4065	4709	6035	756	673	19,858
	Metalaxyl 0.3	2029	869	--	26	15	9	27	6	7	90
April 17, 1980	Control 0.0	252	66	--	147	205	210	117	--	135	814
	Metalaxyl 0.3	252	27	--	1	0	0	0	--	0	1
	Metalaxyl 0.6	252	16	--	1	0	0	0	--	0	1
April 25, 1981	Control 0.0	100	24	45	74	359	926	--	564	--	1968
	M 9834 0.3	100	11	6	0	0	92	--	199	--	297
	M 9834 0.6	100	11	4	0	0	34	--	101	--	139
	Metalaxyl 0.3	100	10	5	0	0	1	--	1	--	7
	Metalaxyl 0.6	100	7	2	0	0	5	--	0	--	7

[W] Fungicides were applied in 50 ml water/plant in 1979, and in 100 ml water/plant in 1980 and 1981.

[X] Tests were located at five commercial hop fields in 1979, three fields in 1980, and two fields in 1981.

19,858 spikes were present in 1979 on 2029 control plants compared to 90 spikes observed on an equal number of plants treated with metalaxyl. In 1980, 814 spikes were observed in 252 control plants compared to one spike each in 252 plants treated at 0.6 and 0.3 gm metalaxyl/plant. Totals do not include spikes counted at the time of fungicide application because these spikes resulted from the overwintering of the pathogen in the hop crown and were present before fungicide application. On plants treated with metalaxyl and M 9834, spikes became necrotic after 1-2 weeks and were no longer inoculum sources. Some chlorosis and necrosis of leaf margins occurred on plants treated with 0.3 gm metalaxyl and was more pronounced at 0.6 gm metalaxyl. However, phytotoxic effects were transient and affected shoots usually appeared normal after 3-4 weeks. This phytotoxicity caused no production problems and was largely avoided by applying metalaxyl prior to shoot emergence.

Yields were increased greatly by metalaxyl treatment in 1979 (Table 2). Cascade and Brewer's Gold plants treated with metalaxyl (0.3 gm ai/plant) yielded 76.6% and 24.6% more hop fruits than control plants, respectively.

Plants treated with metalaxyl as a post-harvest spray in 1979 had fewer spikes in 1980 than control hills (Table 3). On 300 control plants, 1048 spikes were present compared to 60 spikes observed in an equal number of plants treated the previous fall with metalaxyl. The difference in total spikes during 1981 was not as striking in plants treated in 1980; however, the incidence of spikes during April and May of 1981 was considerably lower on treated plants than on control plants. Further, more spikes occurred in 1980 on control

Table 2. Effect of metalaxyl and M 9834 treatments on hop yields.

Variety and year harvested	Treatment (gm/plant)	Fresh weight of hop fruits[x] (gm)		Extrapolation to hop yield[y] kg/ha	lb/acre
Brewer's Gold 1979	Control 0.0	16,666.7		1416.3	1264.0
	Metalaxyl 0.3	20,766.7	*	1764.7	1575.0
Cascade 1979	Control 0.0	13,483.3		1145.8	1022.6
	Metalaxyl 0.3	23,816.7	*	2023.9	1806.2
Cascade 1981	Control 0.0	3233.3	a	1373.8	1226.1
	M 9834 0.3	3566.7	a	1515.5	1352.5
	M 9834 0.6	4016.7	ab	1706.7	1523.1
	Metalaxyl 0.3	4516.7	b	1919.1	1712.7
	Metalaxyl 0.6	4650.0	b	1975.7	1763.3

[x] Fresh weights are the averages of three reps (five plants/rep) and six reps (one plant/rep) in 1979 and 1981, respectively. In 1979, * indicates significant difference (unpaired T-test, P = 0.05). In 1981, fresh weights not followed by the same letter are significantly different at P = 0.05 by Duncan's multiple range test.

[y] Extrapolation is based on 1912 hop plants/ha (774 plants/acre), 20% dry matter content of hop fruits, plus 10% moisture added to hops sold commercially.

Table 3. Occurrence of spikes on hop plants during the spring and summer following metalaxyl application as a post-harvest spray in fall.

Date of application	Treatment[y] (gm/plant)	Number of plants[z]	Number of spikes observed during the spring and summer following application						Total
			4-22	5-2	5-16	5-29	6-17	7-24	
Sept. 29, 1979	Control 0.0	300	78	175	244	250	140	161	1048
	Metalaxyl 0.3	300	3	13	8	24	6	6	60
Oct. 2, 1980	Control 0.0	50	3	20	23	320	628	--	994
	Metalaxyl 0.3	50	2	0	5	212	447	--	666

[y] Metalaxyl was applied in 50 ml water/plant. No additional metalaxyl was applied during the subsequent spring and summer.
[z] Tests were located at three commercial hop fields in 1979, and at one field in 1980.

plants than on plants treated with metalaxyl in 1979 (Table 4). In 1980, 831 spikes were counted on control plants compared to 19 spikes on an equal number of plants treated with metalaxyl a year earlier.

Excellent disease control was obtained with metalaxyl and M 9834 in 1981 for four weeks after application (Table 1). However, after six weeks, 92 and 34 spikes were observed on 100 plants treated with 0.3 and 0.6 gm M 9834/plant, respectively. Ten weeks after application counts increased to 199 and 101 spikes, respectively. In comparison, few spikes were observed in plants treated with metalaxyl during the entire ten week period. No phytotoxicity occurred on plants treated at 0.3 gm M 9834, and only a slight leaf chlorosis was observed on plants treated at 0.6 gm M 9834. Phytotoxicity following application of metalaxyl was similar as described previously.

Plants treated with metalaxyl in 1981 at 0.3 and 0.6 gm/plant yielded 39.7% and 43.8% more hop fruits than untreated plants (Table 2). Plants treated with M 9834 at 0.3 and 0.6 gm/plant yielded 10.3% and 24.2% more than control plants. Further, plants treated at 0.6 gm metalaxyl yielded 15.8% more than those treated at 0.6 gm M 9834; and plants treated at 0.3 gm metalaxyl yielded 26.6% more than those treated at 0.3 gm M 9834 (Table 2).

Treatment with propamocarb and efosite Al resulted in statistically significant control of downy mildew (Table 5). This was especially true at the higher rates of propamocarb. No phytotoxicity occurred following application of either fungicide. Some spikes present at the time of fungicide application became necrotic, but many were unaffected and continued to support sporulation by the fungus.

Table 4. Occurrence of spikes in 1980 on hop plants treated with metalaxyl in the spring of 1979

Treatment[y] (gm/plant)	Number of spikes observed in control and treated plants during 1980[z]						Total
	4-22	5-2	5-16	5-29	6-17	7-24	
Control (0.0)	22	192	135	263	127	92	831
Metalaxyl (0.3)	1	1	0	0	16	1	19

[y] Metalaxyl was applied on April 17, 1979 in 50 ml water/plant. No additional fungicide was applied during 1980 to plants treated with metalaxyl in 1979.

[z] Values are the sum of spikes counted in 300 plants at three commercial hop fields.

Table 5. Control of hop downy mildew in Oregon with propamocarb and efosite aluminum during 1980.

Treatment[w] (gm/plant)	Number of spikes at time of fungicide application[x]	Number of spikes observed after fungicide application (weeks)[x] 2	4	8	Total	Mean[y]
Control 0.00	39	88	121	195	404	134.7 a
Efosite Al 0.22	20	50	82	134	266	88.7 b
Efosite Al 0.44	34	57	45	76	178	59.3 c
Efosite Al 0.88	27	59	60	55	174	58.0 c
Propamocarb 1.17	30	67	31	60	158	52.7 cd
Propamocarb 2.34	20	23	41	47	111	37.0 de
Propamocarb 4.68	20	22	11	52	85	28.3 e

[w] All chemicals were applied in 100 ml water/plant on April 17, 1980.
[x] Each value is the sum of spikes counted on 150 plants at three commercial hop fields.
[y] Means not followed by the same letter are significantly different (P = 0.05) according to Duncan's multiple range test and split-plot-in-time analysis of variance.

DISCUSSION

Field trials in Oregon from 1979-1981 demonstrated that metalaxyl was the most effective fungicide tested for control of hop downy mildew. A single application at 0.3 gm/plant (574 gm/ha) in mid-April provided almost complete control until hot, dry weather in early July naturally inhibited the disease. In comparison, shoots trained onto the trellis system from control plants frequently became severely infected during May and June, requiring growers to replace terminal spikes with healthy shoots. In 1979, re-training healthy shoots was not completed until mid-June; and as a result, control plants yielded significantly less than metalaxyl treated plants.

Treatment with M 9834 provided disease control comparable to metalaxyl for four weeks after application. However, after four weeks many lateral spikes appeared on vines of plants treated with M 9834. Because hop cones are produced on side branches and terminal shoots, the high frequency of lateral spikes resulted in lower yields from plants treated with M 9834 compared to those treated with metalaxyl.

Zaki, et al. (53) studied the translocation of radioactive carbon (^{14}C) in Persea indica plants following application of labeled metalaxyl as a soil drench. They concluded that labeled fungicide was readily taken up by P. indica roots, and radioactivity was translocated uniformly to the above plant parts. Hops have an extensive root system and may penetrate soil more than 4.5 m (47). The abundant rainfall in the Willamette Valley of Oregon during April insures that metalaxyl is drenched into the root zone, and we suspect that the high level of disease control obtained with metalaxyl may result

from extremely efficient uptake of chemical by the roots and subsequent translocation to all young shoots. M 9834 is not as water soluble as metalaxyl (37 and 7100 ppm at 20 C: technical bulletins from Montedison Labs and Ciba-Geigy, Inc., respectively), and may not be as effectively taken up by hop roots. Less efficient uptake of M 9834 could explain the higher disease incidence observed after six weeks on plants treated with M 9834 as compared to those treated with metalaxyl.

Data from 1980 and 1981 demonstrated that metalaxyl controlled hop downy mildew when applied as a post-harvest spray. Most of the spikes observed in treated hills were terminal and lateral spikes. These spikes resulted from inoculum disseminated from basal spikes on control plants. Thus, the effectiveness of metalaxyl as a post-harvest spray probably resulted from preventing *P. humuli* from infecting and overwintering in hop crowns rather than from residual activity of metalxyl.

Propamocarb and efosite Al were not as effective as metalaxyl or M 9834 in controlling hop downy mildew. However, *P. humuli* strains tolerant to metalaxyl probably would be tolerant to M 9834 because cross tolerance has been demonstrated *in vitro* (7). Thus, propamocarb and efosite Al may be desirable alternates to control this disease if strains tolerant to metalaxyl occur because their chemical structures are unrelated to metalaxyl or M 9834.

Metalaxyl was applied in 1981 under emergency registration to all commercial hop fields in Oregon planted with the varieties Cascade, Bullion, and Brewer's Gold. Although weather conditions were extremely favorable for mildew development from mid-April

through late June, virtually complete control of downy mildew was obtained by growers. Thus, large financial losses were avoided as indicated by the low yields from control plants in our plots in 1981.

Alternating applications of multi-site fungicides with site-specific fungicides has been suggested to avoid development of fungal strains tolerant to site-specific fungicides (51). Thus, we feel that the occurrence of P. humuli strains tolerant to metalaxyl may be delayed or averted entirely if treatment with metalaxyl is alternated yearly with zineb or a copper fungicide. Hop plants treated in 1979 received no additional fungicide in 1980 or 1981. Occurrence of spikes in these plants in 1980 was nearly zero, although basal spikes were abundant in neighboring untreated plants. In 1981, metalaxyl was applied to entire hop fields; and based on observations of plants treated in 1979 and 1980, an extremely low amount of primary inoculum is expected in 1982. Thus, metalaxyl nearly eliminates the primary inoculum source for the following year; and application of zineb or a copper fungicide in alternate years should decrease the pressure to select for P. humuli strains tolerant to metalaxyl. This may be important because stable tolerance to metalaxyl has been reported in another species of Pseudoperonospora following intensive use of metalaxyl (21, 29).

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CHAPTER IV

Tolerance to Metalaxyl by *Phytophthora megasperma* Isolates

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ABSTRACT

HUNGER, R. M., P. B. HAMM, C. E. HORNER, and E. M. HANSEN. 1982. Tolerance to metalaxyl by *Phytophthora megasperma* isolates. Plant Disease

Thirty-five isolates of *Phytophthora megasperma* were tested *in vitro* for tolerance to the systemic fungicide *N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl) alanine methyl ester (CGA-48988, metalaxyl). Isolates had been collected before commercial use of metalaxyl. Tolerance was measured by comparing isolate growth and oogonia and sporangia formation on amended and control media. Based on growth at 0, 1, and 10 µg/ml metalaxyl, 15 isolates were highly sensitive, 8 moderately tolerant, and 12 highly tolerant to the fungicide. No isolates in the highly sensitive group formed oogonia at 1 µg/ml, whereas six

isolates from the moderately tolerant group, and eight from the highly tolerant group did. The number of isolates forming sporangia and the mean number of sporangia formed per isolate decreased with increasing fungicide concentration. At least two of 280 single zoospore isolates were more tolerant to metalaxyl than their parents suggesting that commercial applications may select for naturally occurring tolerant strains which may lead to loss of disease control.

Commercial applications of systemic fungicides can favor selection of tolerant strains. As a result, higher rates or numerous applications may be required for disease control. Eventually fungicidal effectiveness may be completely lost as tolerant strains multiply, disseminate, and replace the initially susceptible population. This phenomenon is well documented (5, 12, 42) and is a major concern to growers who sustain reduced crop yields, and agricultural chemical companies who lose commercial sales.

Many factors determine the speed at which tolerance to systemic fungicides develops. Some of these factors are: the number of genes regulating tolerance; gene mutability; the involvement of a polycyclic or monocyclic pathogen; and whether or not tolerant strains existed prior to fungicide applications (12, 13).

Few studies regarding the preexistence of tolerant strains have been reported. Wuest *et al.* (52) evaluated three *Verticillium malthousei* Ware isolates *in vitro* for tolerance to benomyl and concluded that one isolate was tolerant by linear extension of hyphae,

sporulation, and spore germinability. These isolates had been collected prior to release of benomyl as a commercial fungicide. Others (6, 11, 23, 37, 49) have reported tolerance to fungicides, but preexisting tolerance in the natural population could not be determined because the chemicals were used prior to collection of isolates.

To increase information concerning this phenomenon, the following work was initiated to determine: (i) if *in vitro* tolerance to the systemic fungicide metalaxyl existed in 35 *Phytophthora megasperma* Drech. isolates collected before commercial release of metalaxyl, (ii) to what degree isolates were tolerant, and (iii) if isolates obtained from asexual spores (zoospores) were differentially tolerant from parents.

MATERIALS AND METHODS

Thirty-five isolates of *P. megasperma* collected before commercial release of metalaxyl were obtained from four continents and 14 hosts (Table 6). Eight single zoospore isolates (SSI) were obtained from each parent isolate (PI) as described elsewhere (25). Effect *in vitro* of metalaxyl on growth, and oogonia and sporangia formation was determined. Results were expressed as percent inhibition as compared to controls. A 2 EC formulation of metalaxyl (Ridomil, Ciba-Geigy Corp.) was used in all studies.

Growth. Growth inhibition was determined on Difco corn meal agar (CMA) with additional Bacto agar added to make 2% CMA amended at 0, 1, and 10 µg/ml metalaxyl. The fungicide was sterilized with a

Table 6. Sources of *Phytophthora megasperma* isolates

Isolate		Host	Location	Source
1	(W1)	Alfalfa	Washington	Christen[1]
2	(S1)	Alfalfa	Salem, Oregon	OSU[2]
3	(P1)	Alfalfa	Corvallis, Oregon	OSU
4	(M1)	Alfalfa	Medford, Oregon	OSU
5	(PC3)	Alfalfa	Princeton, Oregon	OSU
6	(PC5)	Alfalfa	Klamath Falls, Oregon	OSU
7	(S2)	Alfalfa	Salem, Oregon	OSU
8	(P3)	Alfalfa	Corvallis, Oregon	OSU
9	(5b)	Alfalfa	Wisconsin	Maxwell[3]
10	(DA)	Alfalfa	Wisconsin	Maxwell
11	(K2)	Almond	Red Bluff, California	Mircetich[4]
12	(K3)	Almond	Red Bluff, California	Mircetich
13	(K10)	Grape Soil	Napa Co., California	Mircetich
14	(K11)	Grape Soil	Napa Co., California	Mircetich
15	(B3A)	Douglas fir	Brownsville, Oregon	OSU
16	(B217)	Douglas fir	Brownsville, Oregon	OSU
17	(345)	Douglas fir	Brownsville, Oregon	OSU
18	(336)	Douglas fir	Toledo, Washington	OSU
19	(C17)	Douglas fir	Elkton, Oregon	OSU
20	(520)	Douglas fir	Brownsville, Oregon	OSU
21	(NF1)	Noble fir	Corvallis, Oregon	OSU
22	(908)	Soybean (Race 1)	Wisconsin	Grau[5]
23	(909)	Soybean (Race 3)	Wisconsin	Grau
24	(105)	Clover	Mississippi	Pratt[6]
25	(117)	Clover	Mississippi	Pratt
26	(102)	Clover	Mississippi	Pratt
27	(T14)	Apple	New Zealand	CMI[7] 144023
28	(T47)	Apple	New Zealand	CMI 147131
29	(PA)	Rose	Japan	Nagai[8]
30	(PB)	Rose	Japan	Nagai
31	(K1)	Cherry	Stockton, California	Mircetich
32	(K8)	Pear	Walnut Grove, California	Mircetich
33	(K9)	Juniper	Davis, California	Mircetich
34	(T56)	*Brassica* sp.	United Kingdom	CMI 56348
35	(T28)	*Populus robusta*	United States	ATCC[9] 28765

[1] A. Christen, I.A.R.E.C., Prosser, Washington.
[2] OSU = P. B. Hamm, Oregon State University.
[3] D. P. Maxwell, University of Wisconsin, Madison.
[4] S. Mircetich, USDA, University of California, Davis.
[5] C. R. Grau, University of Wisconsin, Madison.
[6] R. G. Pratt, USDA, Mississippi State University, Mississippi.
[7] CMI = Commonwealth Mycological Institute, Kew, Surrey, England.
[8] Y. Nagai, Laboratory of Plant Pathology, Chiba, Japan.
[9] ATCC = American Type Culture Collection, Rockville, Maryland.

Seitz bacterial and fungal filter. CMA was prepared and cooled to 45 C before addition of the fungicide to obtain the specified metalaxyl concentrations. Twenty ml aliquots of amended CMA were pipetted aseptically into sterile petri plates and the medium was inoculated with small blocks (3 x 5 mm) cut from actively growing colony margins cultured on CMA + 20 µg/ml pimaracin. One replication per PI and SSI per concentration was used. Growth along the largest radius was measured after 5 days at room temperature (22-25 C). Average growth inhibition of each isolate set (PI and 8 SSI) at 1 and 10 µg/ml metalaxyl was used to compare tolerance between the 35 PI.

Results from this initial study were used to separate isolates for two additional growth tests. In the first test, PI were separated into three groups to facilitate evaluation of tolerance levels. These groups were categorized highly sensitive, moderately tolerant, or highly tolerant to metalaxyl. Representative PI from each of these sensitivity groups were inoculated onto CMA amended with 0, 0.01, 0.1, 1, 10, 50, and 100 µg/ml metalaxyl. Four replications per PI per concentration were used. The largest growth radius of each colony was measured after 5 days incubation at room temperature (22-25 C). The average growth inhibition at each concentration was used to compare tolerance groups.

The second test compared growth of PI with their SSI. Growth of two SSI, one with greater and one with lesser inhibition than the parent, were compared on CMA amended at 0, 1, and 10 µg/ml using four replications per isolate per concentration. Preparation and inoculation of CMA was done as in the previous studies. Average percent inhibition after 5 days was calculated as before.

Oogonia Formation. Inhibition of oogonia formation was determined on clarified V-8 juice agar (V-8A) amended with metalaxyl at 0, 1, 10, and 100 µg/ml. Parent isolates and those SSI showing differences in growth inhibition from their parents on amended CMA were tested. V-8A plates were inoculated as in the growth study and incubated in the dark at room temperature (22-25 C). After 4 weeks, the area of each colony containing oogonia was delineated. From this area three random samples (2 mm diameter) were selected, and the number of normal and aborted oogonia in each sample were counted. Average number of these structures formed by each PI and SSI on amended agar was compared to controls (0 µg/ml) to determine percent inhibition.

Sporangia Formation. Two methods were used to determine inhibition of sporangia formation by PI and selected SSI. Both methods used colonies incubated for 7 days in pea broth (46) at room temperature (22-25 C). In one method, colonies were rinsed with distilled water, and incubated overnight in soil extract water containing 0, 1, 10, or 100 µg/ml metalaxyl. The second method differed from the first by rinsing colonies with autoclaved distilled water and incubating rinsed colonies in autoclaved soil extract water. Empty and full sporangia were counted in three randomly selected fields of view at 100x. The presence of released zoospores was noted for each isolate. The average number of sporangia formed was used to calculate inhibition of sporangia formation by metalaxyl.

RESULTS

Growth. Fifteen parent isolates were highly sensitive (> 95% inhibition at both concentrations), eight PI were moderately tolerant (51-90 and 52-92% inhibition at 1 and 10 µg/ml, respectively), and 12 PI were highly tolerant (27-47 and 29-63% inhibition at 1 and 10 µg/ml, respectively) (Fig. 2A).

Tolerance levels observed for all PI in the initial study were confirmed following extensive testing of selected PI on amended CMA (Fig. 3). PI (#'s 2, 5, 22, 23) from the highly sensitive group were inhibited 24.8 and 98.9% at 0.01 and 10 µg/ml, respectively. Complete growth inhibition of this group occurred at ≥ 50 µg/ml. PI (#'s 15, 16, 17, 20) from the moderately tolerant group showed < 2% growth inhibition at 0.01 µg/ml, and 82% inhibition at 10 µg/ml. Growth by this group was inhibited 80-90% between 10-100 µg/ml metalaxyl. PI (#'s 3, 8, 11, 18, 19, 32) from the highly tolerant group were inhibited < 2% at 0.01 µg/ml, and never exceeded 50% inhibition at any concentration.

Growth inhibition of isolates always increased with increasing fungicide concentration when colony morphology appeared normal. However, 3 of 4 moderately tolerant PI and 5 of 6 highly tolerant PI showed an anomalous growth pattern characterized by sparse, elongate hyphae, with reduced layering and branching (Fig. 4). Occurrence of this anomalous growth pattern correlated with the plateau of inhibition between 10 and 50 µg/ml of the moderately and highly tolerant groups (Fig. 3).

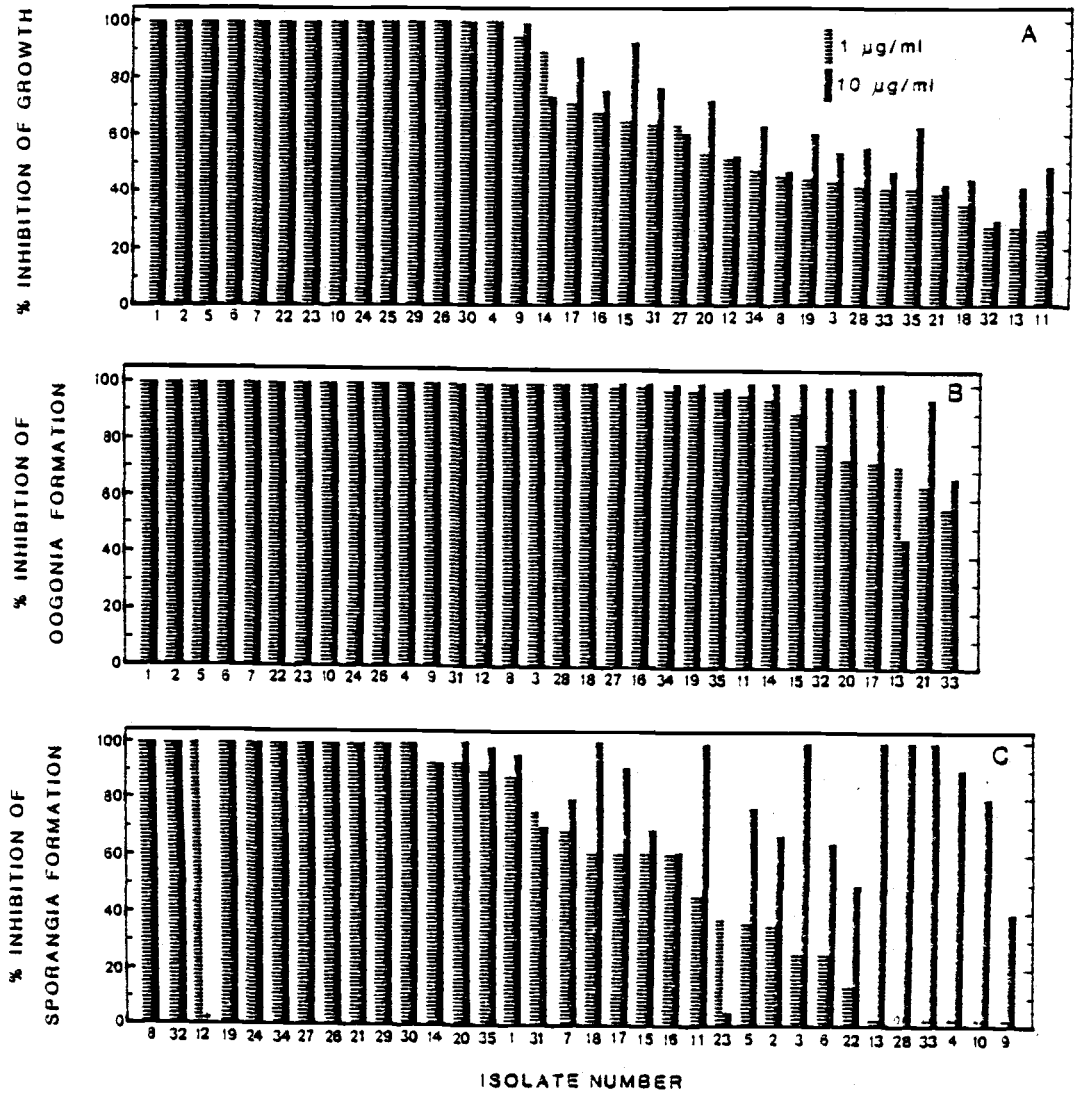

Fig. 2. Inhibition of Phytophthora megasperma isolates at two concentrations of metalaxyl as compared to controls: (A) Inhibition of growth after 5 days on corn meal agar at room temperature (22-25 C). (B) Inhibition of oogonia formation on clarified V-8 juice agar after 1 month incubation at room temperature (22-25 C). (C) Inhibition of sporangia formation by colonies grown in pea broth for 7 days at room temperature (22-25 C), washed with distilled water and incubated for 48 hours with soil extract water amended with metalaxyl. + = more sporangia formed by isolate in metalaxyl amended soil extract water than in the control.

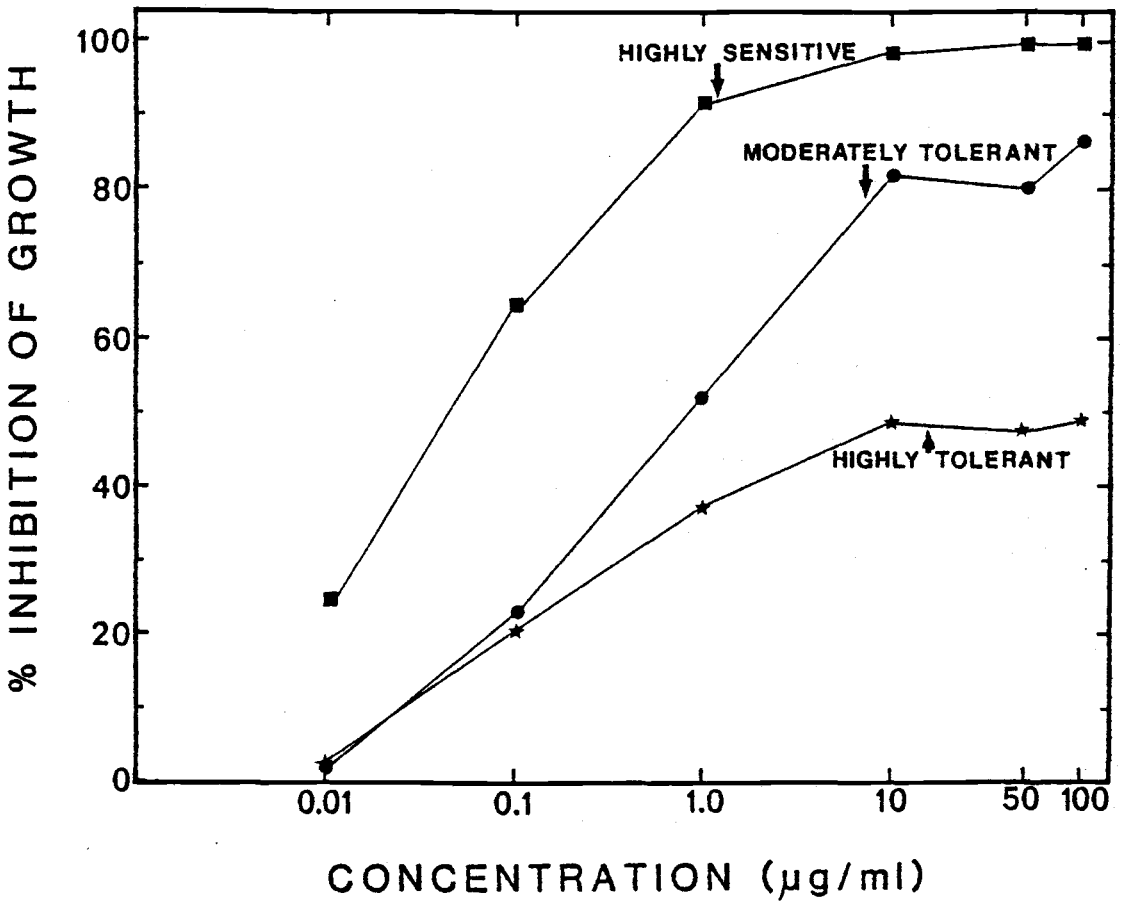

Fig. 3. Differential growth inhibition of Phytophthora megasperma isolates to metalaxyl. Isolates were initially grouped as highly sensitive, moderately tolerant, or highly tolerant to metalaxyl based on growth inhibition at 1 and 10 µg/ml metalaxyl. Growth inhibition of representative isolates from each group was compared on corn meal agar after 5 days incubation at room temperature (22-25 C) at seven concentrations of metalaxyl.

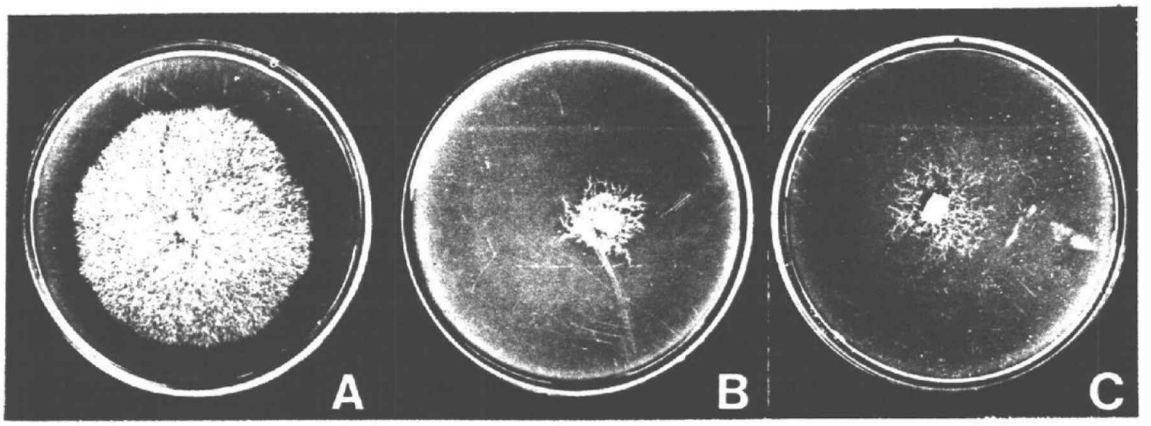

Fig. 4. Anomalous growth pattern by certain Phytophthora megasperma isolates when cultured on corn meal agar for 5 days at room temperature (22-25 C): (A) Hyphal growth on unamended media. (B) Growth at 10 µg/ml metalaxyl. (C) Growth at 50 µg/ml metalaxyl. Note the elongate hyphal growth (C) which correlated with the plateau of inhibition between 10 and 50 µg/ml metalaxyl of the moderately and highly tolerant groups.

Oogonia Formation. At 1 µg/ml oogonia formation by 25 PI was inhibited 96-100% and seven PI were inhibited 56-90% (Fig. 2B). Three PI (#'s 25, 29, 30) failed to form oogonia on the unamended medium. Nineteen of the 25 PI not forming oogonia at 1 µg/ml had produced mycelia on amended medium. At 10 µg/ml, six PI formed oogonia, and only one PI formed oogonia at 100 µg/ml.

No PI in the highly sensitive group identified by growth inhibition formed oogonia at 1 µg/ml metalaxyl, although 10 of the 15 PI in this group had grown after 4 wk. A nearly equal number of PI from the moderately and highly tolerant groups produced oogonia at 1 µg/ml (6 of 8 PI and 8 of 12 PI, respectively). At 10 µg/ml, one of eight PI in the moderately tolerant group and 5 of 12 isolates from the highly tolerant group formed oogonia. Only isolate #7, belonging to the highly tolerant group formed oogonia at 100 µg/ml. The percentage of aborted oogonia increased slightly with increasing fungicide concentration (30.4, 37.3, 38.2, and 46.0% at 0, 1, 10, and 100 µg/ml, respectively).

Sporangia Formation. Formation of sporangia was affected greatly by metalaxyl. Similar results were obtained after 24 and 48 hours from both sterile and non-sterile wash treatments. Since larger numbers of sporangia formed following non-sterile treatments after 48 hours, only these results are reported.

Ten PI failed to form sporangia in soil water containing 1 or 10 µg/ml metalaxyl (Fig. 2C). Seven PI formed sporangia at 1 µg/ml, but were completely inhibited at 10 µg/ml. One PI (#25) did not form sporangia in amended or control (0 µg/ml) soil extract water.

No relationships were found between tolerance groups noted in the growth studies and inhibition of sporangia formation. However, the percentage of isolates forming sporangia, the percentage of isolates with empty sporangia and/or zoospores, and the mean number of sporangia observed per isolate that formed sporangia decreased with increasing fungicide concentration (Table 7).

Comparison of SSI. Two isolate sets (#13 and 35) were observed to have one SSI more sensitive and one more tolerant than their parent following extensive testing at 1 µg/ml metalaxyl (Fig. 5). Inhibition of oogonia and sporangia formation also differed between these PI and their SSI. Generally, SSI more sensitive to metalaxyl in growth also formed fewer oogonia and sporangia than their parents. SSI more tolerant than their parents formed more oogonia and sporangia. Similar results were obtained at 10 µg/ml.

DISCUSSION

Many studies during the last 25 years have dealt with tolerance to fungicides. Most have involved (i) training fungi to tolerate higher fungicide doses, (ii) use of mutagens to induce tolerance, or (iii) natural tolerance in fungal strains. However, most studies on natural tolerance have used strains isolated after a fungicide was introduced and it is difficult to conclude whether tolerance existed in the population prior to first application. Webster et al. (49), reported tolerance in Botrytis cinerea Fr. to 2,6-dichloro-4-nitroaniline (DCNA), but they could not determine whether exposure to the chemical had occurred in vivo before their isolations were

Table 7. Effect of metalaxyl on sporangia formation and zoospore release by 35 isolates of *Phytophthora megasperma*[1]

	Metalaxyl Concentration (µg/ml)			
	0	1	10	100
Percentage of isolates forming sporangia	97	66	57	6
Percentage of isolates with empty sporangia and/or zoospores	95	86	57	0.0
Mean number of sporangia observed per isolate that formed sporangia[2]	28	13	6	0.7

[1] Sporangia observed in pea broth colonies incubated in soil extract water for 48 hours.

[2] Mean of 3 random observations per isolate

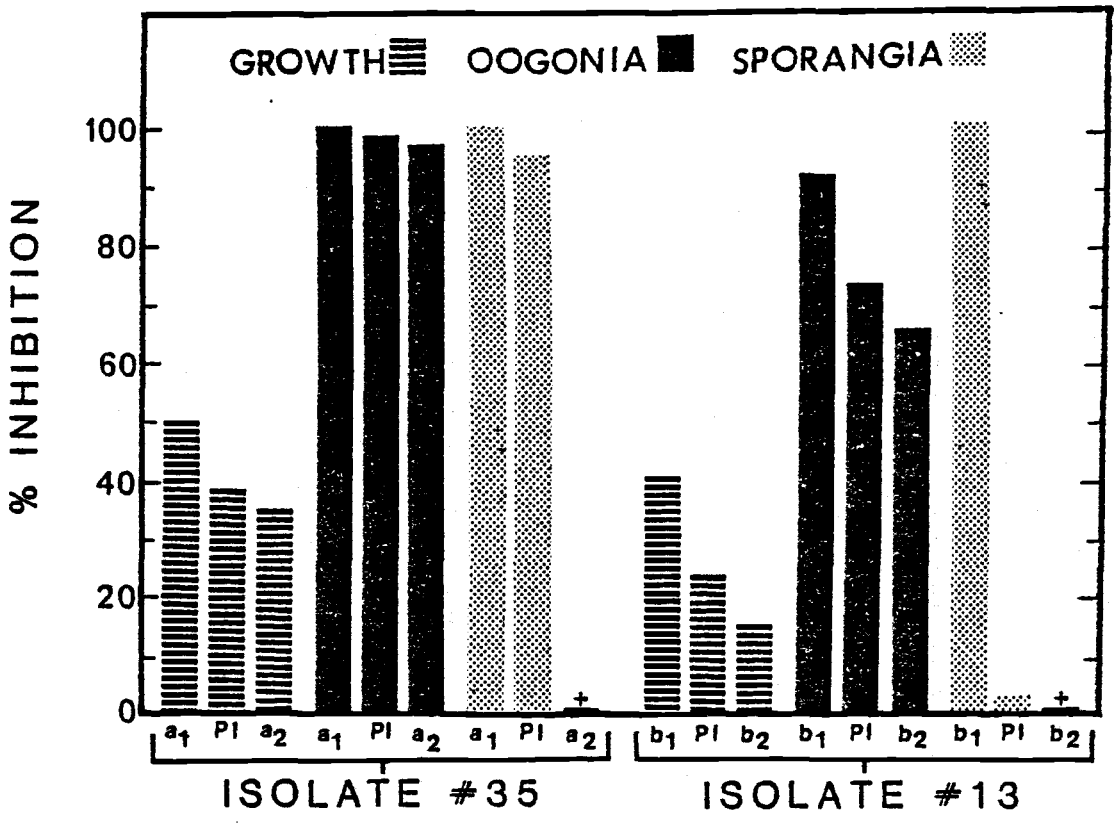

Fig. 5. Growth inhibition, and inhibition of oogonia and sporangia formation at 1 µg/ml metalaxyl by two isolates of Phytophthora megasperma (PI) and two single zoospore isolates from each parent (a_1, a_2, b_1, b_2). Percent inhibition was determined by comparison to controls. + = more sporangia formed by isolate in metalaxyl amended soil extract water than in the control.

made. Harding (27) reported that Penicillium italicum Wehmer and P. digitatum Sacc. strains tolerant to thiabendazole occurred naturally because such strains could be isolated from orchards and packinghouses where the fungicide had not been used. Bolton (6) reported in vitro tolerance to benomyl, dichloran, and triadimefon in Botrytis cinerea Pers. isolates not previously exposed to these fungicides. However, isolation of tolerant strains from areas or hosts not previously treated with a fungicide does not unequivocally demonstrate that tolerant strains occur naturally. Gutter et al. (23) isolated strains of Penicillium digitatum and P. italicum tolerant to benzimidazole fungicides from citrus orchards in Israel although benzimidazoles had never been applied to these orchards. These authors attributed this tolerance to spores from packinghouses where benzimidazoles were used. In our study, we used P. megasperma isolates collected before metalaxyl had been released. Thus, the in vitro tolerance we observed must have occurred naturally in the fungus.

Royle (40) reported metalaxyl concentrations of 13-29 mg/kg in basal hop shoots, and 2 mg/kg in leaves of climbing hop vines 1 and 4 weeks after application of metalaxyl (Ridomil 2 EC), respectively. These levels were associated with complete control of hop downy mildew caused by Pseudoperonospora humuli (Miy. et Tak.) Wils. Levels declined to 0.25 - 0.75 mg/kg in leaves during 4 to 10 weeks following application. These lower levels of metalaxyl were associated with erratic disease control. Our study identified isolates of another oomycete that were highly tolerant in vitro to similar and higher metalaxyl concentrations. This tolerance may give these isolates

a selective advantage in the field following applications of metalaxyl.

Differential tolerances to fungicides between isolates and subsequent single spore cultures have been reported. Webster et al. (49) identified resistant and sensitive single asexual spore cultures from strains of Botrytis cinerea resistant to 2,6-dichloro-4-nitroaniline (DCNA). Bruin and Edgington (7) reported large differences in metalaxyl sensitivity between mono-zoospore cultures of Phytophthora and Pythium species. These and other researchers (18) suggest heterokaryosis as a mechanism that could maintain low levels of fungicide tolerance in multinucleate fungi, and that tolerant strains emerge due to selection by the fungicide. In addition, Davidse (11) using one isolate of Phytophthora megasperma f. sp. medicaginis found single zoospore isolates more tolerant to metalaxyl than their parent in vitro. These observations are supported by our study in which a large variation in tolerance to metalaxyl was observed between some parents and their SSI. At least two SSI of 280 tested were consistently more tolerant to metalaxyl than their parents. Thus, production of asexual spores (e.g. zoospores) in vivo may contribute to the variation in tolerance observed between our isolates.

The wide tolerance range we observed between isolate sets suggests that tolerance values (e.g. ED_{50}, LD_{50}, etc.) are also highly variable. Papavizas and Bowers (37) suggested that P. megasperma was more sensitive to metalaxyl than P. capsici Leonian based on a comparison of ED_{50} values obtained from one P. megasperma isolate and four P. capsici isolates. Our data suggest that P. megasperma could

be judged more sensitive, as sensitive, or less sensitive to metalaxyl depending on the *P. megasperma* isolate used. Thus, we suggest that variation in ED_{50} values obtained from many PI and SSI may more adequately reflect the potential tolerance of a species.

Our study indicates that *in vitro* tolerance to metalaxyl exists naturally within *P. megasperma*. If similar tolerance occurs *in vivo*, selection for tolerant strains may proceed rapidly and disease control with metalaxyl may be quickly lost.

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CHAPTER V

Response of Three *Phytophthora* Species to Metalaxyl

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ABSTRACT

Hunger, R. M., and C. E. Horner. 1982. Response of three *Phytophthora* species to metalaxyl.

Response to the systemic fungicide metalaxyl by 16 *Phytophthora* isolates was examined using ED_{50} values and slopes of dosage-response curves. Percent inhibition of growth determined by comparing hyphal extension on metalaxyl amended and unamended agar was used to generate the dosage-response curves. One isolate each of *P. cinnamomi* and *P. drechsleri*, and 14 isolates of *P. megasperma* were examined. *P. cinnamomi* and *P. drechsleri* isolates were highly sensitive to metalaxyl (ED_{50} values < 0.1 µg metalaxyl/ml, and slope values > 1.0). *P. megasperma* isolates ranged from highly sensitive (ED_{50} values < 0.1 µg metalaxyl/ml, and slope values > 1.0) to highly tolerant (ED_{50} values > 30 µg metalaxyl/ml, and slope values < 0.65). Response to metalaxyl

would not be helpful in identifying isolates to species, but may be useful in separating P. megasperma isolates into morphologically distinct groups if used in addition to morphological traits. The possibility of metalaxyl applications selecting for tolerant strains already present in nature, as well as tolerant strains resulting from mutations, is discussed.

Numerous studies have examined the response of fungi to the systemic fungicide metalaxyl [N-(2,6,-dimethyl phenyl)-N-(methoxyacetyl)-alanine methyl ester](2, 11, 19, 28, 31, 32, 37, 45). Usually growth inhibition was calculated by comparing hyphal extension on amended and unamended media. Dosage-response (DR) curves were generated by plotting percent inhibition of growth against the log of metalaxyl concentrations. ED_{50} values interpolated from the DR curves were used to indicate toxicity of metalaxyl to the fungus, or sensitivity of the fungus to metalaxyl.

Many factors affect ED_{50} values interpolated from DR curves. One factor is the difference in sensitivity to a fungicide between fungal species, and between isolates of a single species (22, 28, 34). Although many studies have measured the toxicity of metalaxyl to different species, few have examined the difference in sensitivity between multiple isolates of the same species (11, 28, 37). Therefore, due to the lack of information in this area, our study used results from DR curves to compare the sensitivity to metalaxyl of one isolate each of Phytophthora cinnamomi Rands and P. drechsleri Tuckers, and 14 isolates

of P. megasperma Drech.

MATERIALS AND METHODS

Test organisms. Single field isolates of P. cinnamomi (PC) and P. drechsleri (PD), and 14 field isolates of P. megasperma (PM) were used (Table 8). All isolates had been collected before Ciba-Geigy Corp. released metalaxyl for commercial use. Thus, these isolates had never been exposed to metalaxyl. Cultures were stored at 5 C on Difco corn meal agar (CMA) plus 20 mg pimaricin/liter.

Growth inhibition tests. An emulsifiable concentrate of metalaxyl (239 gm active ingredient/liter; Ridomil; Ciba-Geigy Corp.) was used in all tests. Appropriate amounts of fungicide sterilized with a Seitz fungal and bacterial filter were added to CMA (containing 2% agar) at 45 C to obtain the desired metalaxyl concentrations. Aliquots (20 ml) of amended CMA were pipetted aseptically into sterile petri plates. Small blocks (3 x 5 mm) cut from actively growing colony margins were used to inoculate amended and unamended (control) medium. Three plates each were inoculated with PC and PD isolates at 0.0, 0.001, 0.01, 0.1, and 1 µg metalaxyl/ml. Four and eight plates were inoculated with PM isolates 1 - 8 and 9 - 14, respectively at 0.0, 0.01, 0.1, 1, 10, and 100 µg metalaxyl/ml. Hyphal extension along the largest radius of each culture was measured after 4, 4, and 5 days incubation at room temperature (22 - 25 C) for PC, PD, and PM isolates, respectively. Percent inhibition of growth was determined by comparing the average hyphal extension on amended and unamended medium. Percent growth inhibition values were transformed to probits and

Table 8. Sources of *Phytophthora* Isolates.

Species	Isolate Number	Host	Location	Source
P. cinnamomi	PC	Douglas fir	Oregon	OSU[a]
P. drechsleri	PD	Douglas fir	Oregon	OSU
P. megasperma	1 (PC3)	Alfalfa	Oregon	OSU
	2 (S1)	Alfalfa	Oregon	OSU
	3 (909)	Soybean (race 3)	Wisconsin	Grau[b]
	4 (908)	Soybean (race 1)	Wisconsin	Grau
	5 (345)	Douglas fir	Oregon	OSU
	6 (B3A)	Douglas fir	Oregon	OSU
	7 (B217)	Douglas fir	Oregon	OSU
	8 (520)	Douglas fir	Oregon	OSU
	9 (C17)	Douglas fir	Oregon	OSU
	10 (336)	Douglas fir	Washington	OSU
	11 (P3)	Alfalfa	Oregon	OSU
	12 (P1)	Alfalfa	Oregon	OSU
	13 (K8)	Pear	California	Mircetich[c]
	14 (K2)	Almond	California	Mircetich

[a]OSU = P. B. Hamm, Oregon State University, Corvallis, OR 97331.

[b]C. R. Grau, University of Wisconsin, Madison, WI 53706.

[c]S. Mircetich, USDA, University of California, Davis, CA 95616.

plotted against the corresponding metalaxyl concentrations on a log scale (3). A simple regression line was fitted to the points and an ED_{50} and slope value were calculated from each DR curve (15).

RESULTS AND DISCUSSION

DR curves of the 16 Phytophthora isolates are presented (Fig. 6-14). The ED_{50} and slope values of the DR curves are presented in Table 9. Coefficients of correlation (r) were significant (P = 0.05) except for PM #11 (r = 0.81) and #12 (r = 0.77).

Response to metalaxyl for all isolates. Kerkenaar and Kaars Sijpesteijn (32) reported an ED_{50} value of 1 µg metalaxyl/ml for two P. cinnamomi isolates (designated CBS and CG). These isolates were cultured on glucose mineral salts agar plus 100 µg vitamin B_1/ml for one to three weeks at 24 C. Benson (2) reported an ED_{50} value of 0.11 µg metalaxyl/ml for P. cinnamomi (designated isolate 101 cultured on CMA for five days at 25 C. These two ED_{50} values are greater than the value (0.01 µg metalaxyl/ml) we obtained for the PC isolate we cultured on CMA for four days at 25 C. Different incubation conditions and/or natural variation in response to metalaxyl between the isolates may account for the different ED_{50} value obtained in each study.

The ED_{50} value we obtained with our PD isolate (0.04 µg metalaxyl/ml) is believed the first reported for this species. Kelley (31) reported that radial growth of the P. drechsleri isolate he tested was inhibited 71.8% after five days on V-8 juice agar amended at 1 µg metalaxyl/ml. In our study, growth was completely inhibited after four days on CMA at the same concentration.

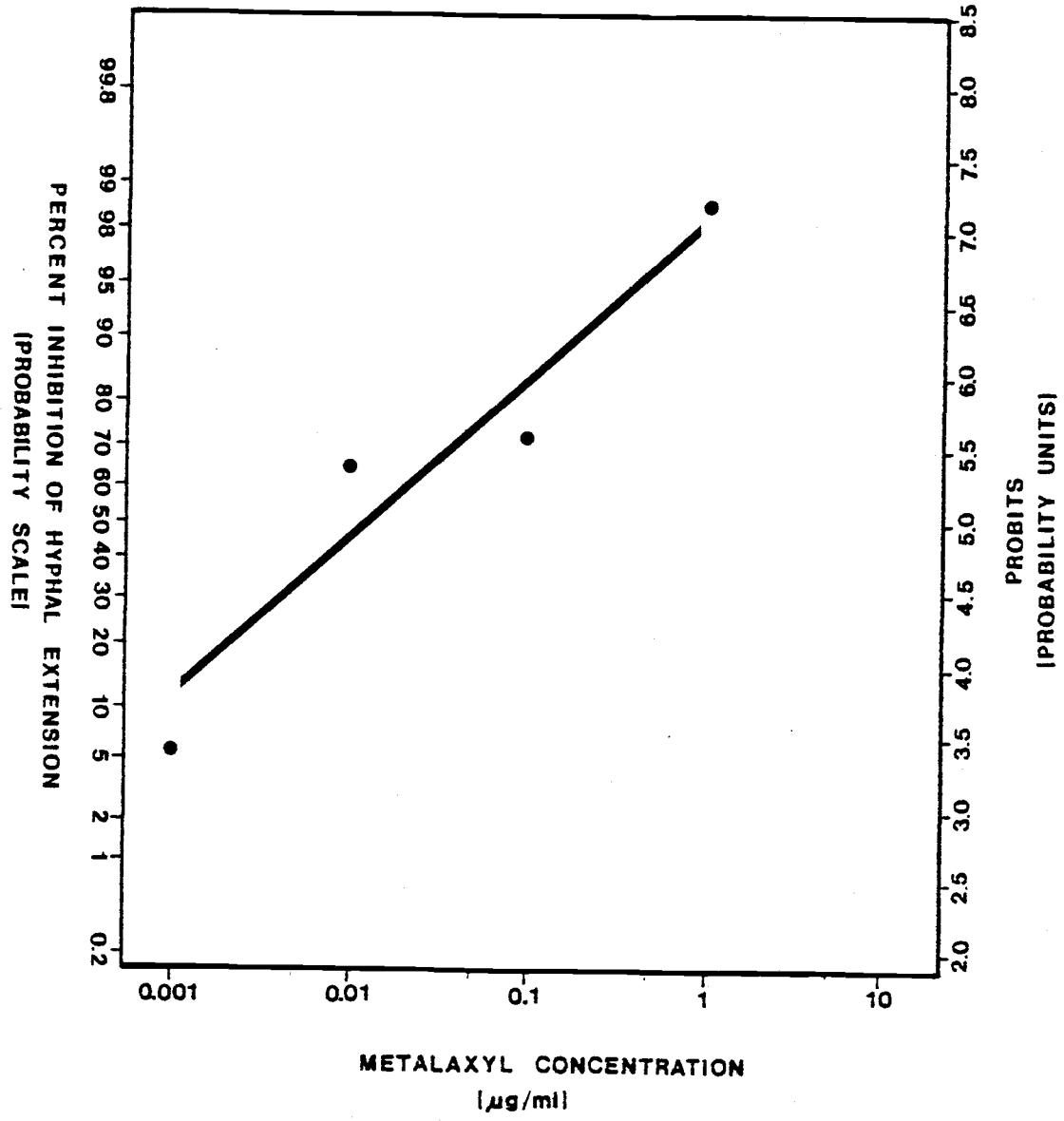

Fig. 6. Dosage-response curve of Phytophthora cinnamomi. Percent inhibition of hyphal extension was determined by comparing extension on metalaxyl amended and unamended media. r = .968 and is significant at P = 0.05.

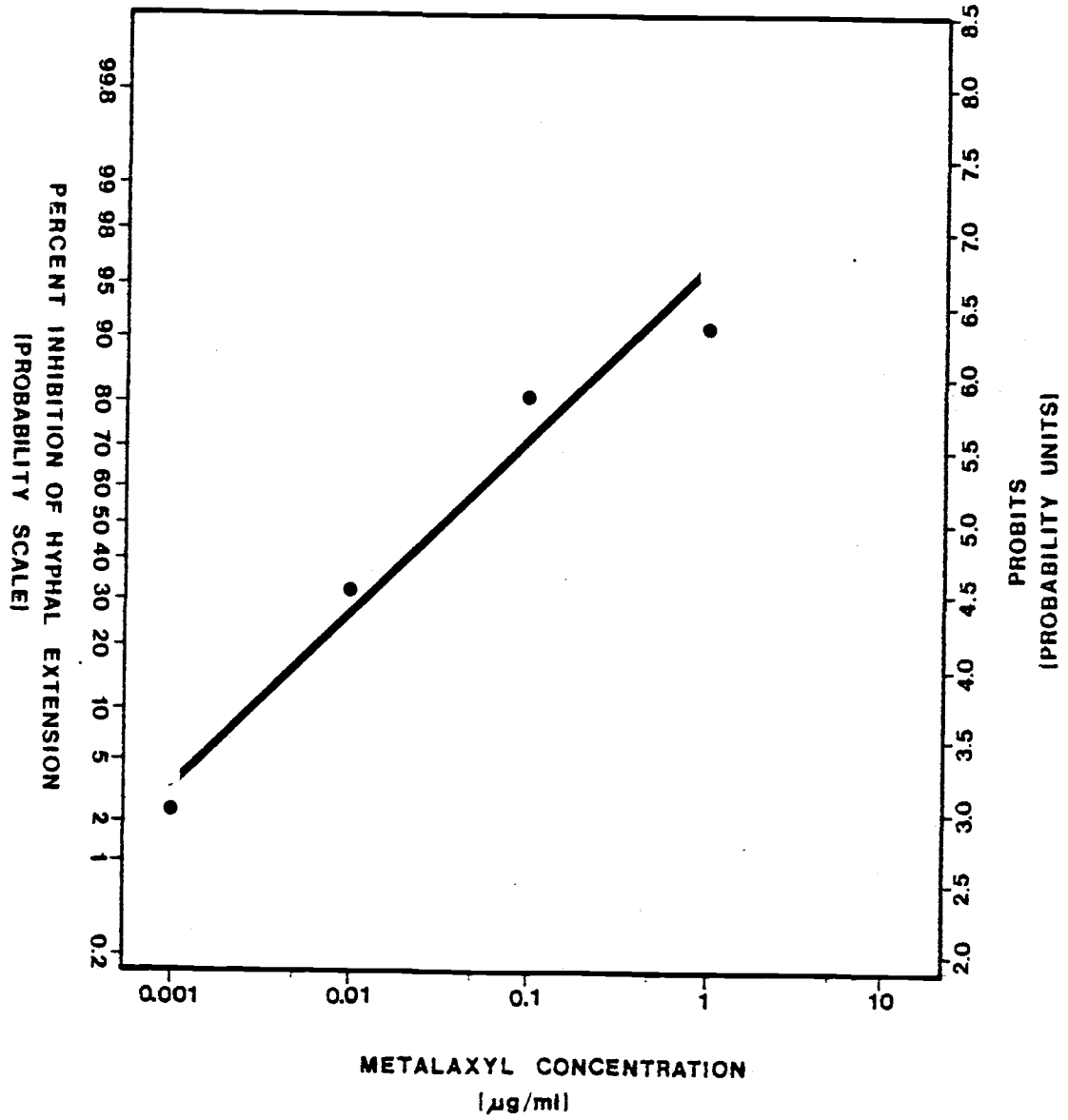

Fig. 7. Dosage-response curve of Phytophthora drechsleri. Percent inhibition of hyphal extension was determined by comparing extension on metalaxyl amended to unamended media. r = .985 and is significant at P = 0.05.

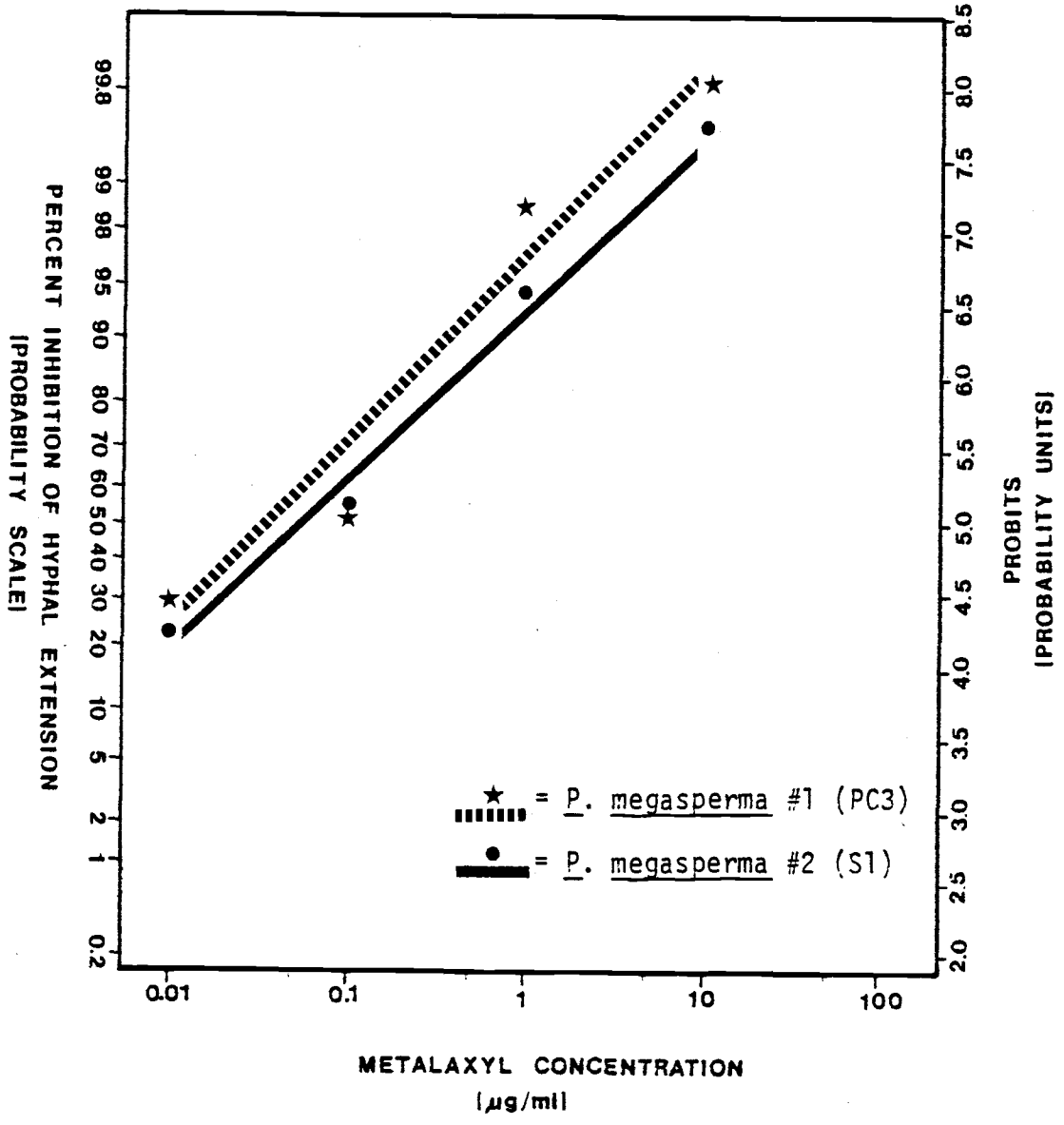

Fig. 8. Dosage-response curve of Phytophthora megasperma isolates #1 (PC3) and #2 (S1). Percent inhibition of hyphal extension was determined by comparing extension on metalaxyl amended to unamended media. r = .977 and .998 for isolate #1 and #2, respectively, and are significant at P = 0.05.

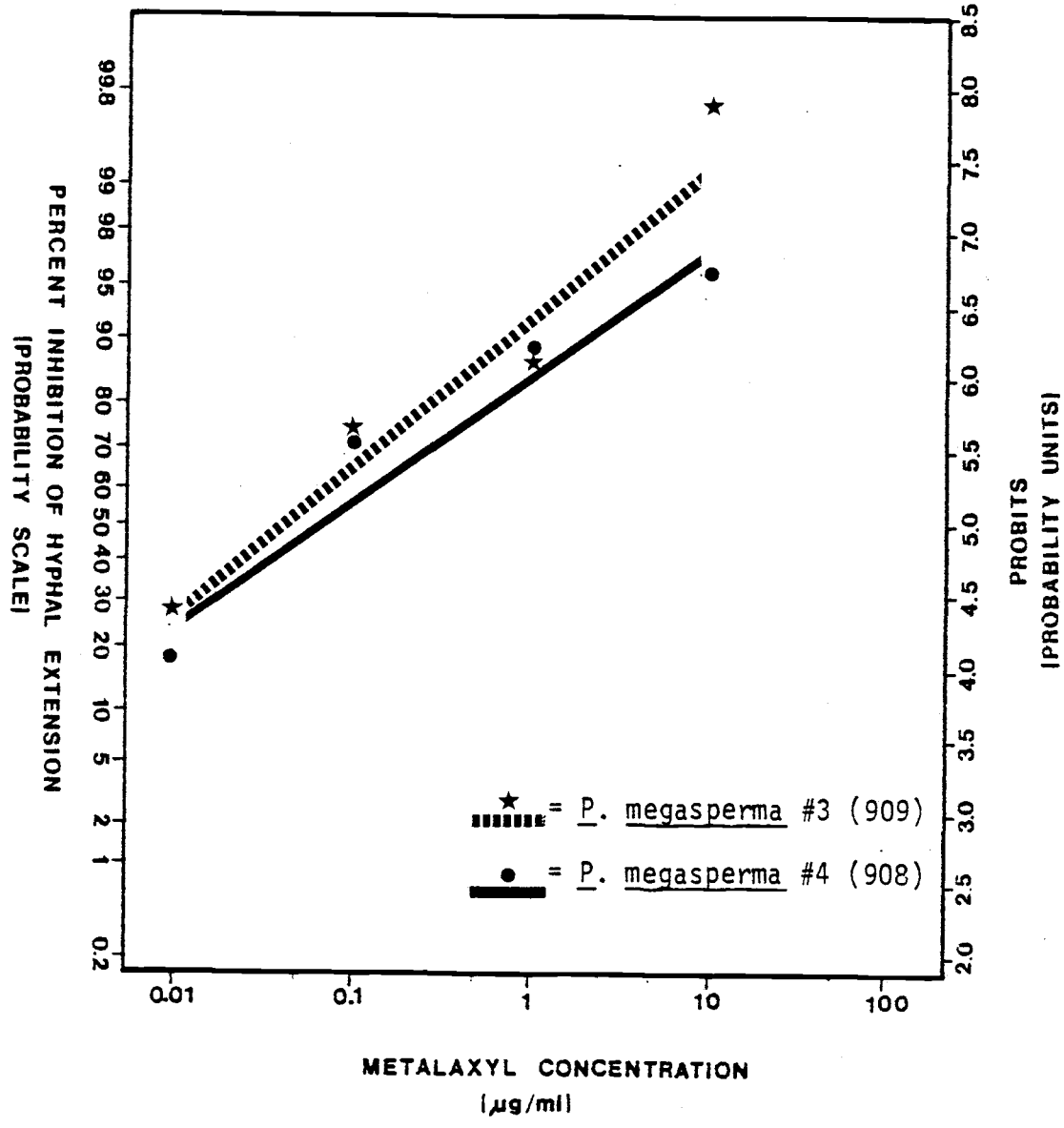

Fig. 9. Dosage-response curves of Phytophthora megasperma isolates #3 (909) and #4 (908). Percent inhibition of hyphal extension was determined by comparing extension on metalaxyl amended to unamended media. r = .987 and .970 for isolate #3 and #4, respectively, and are significant at P = 0.05.

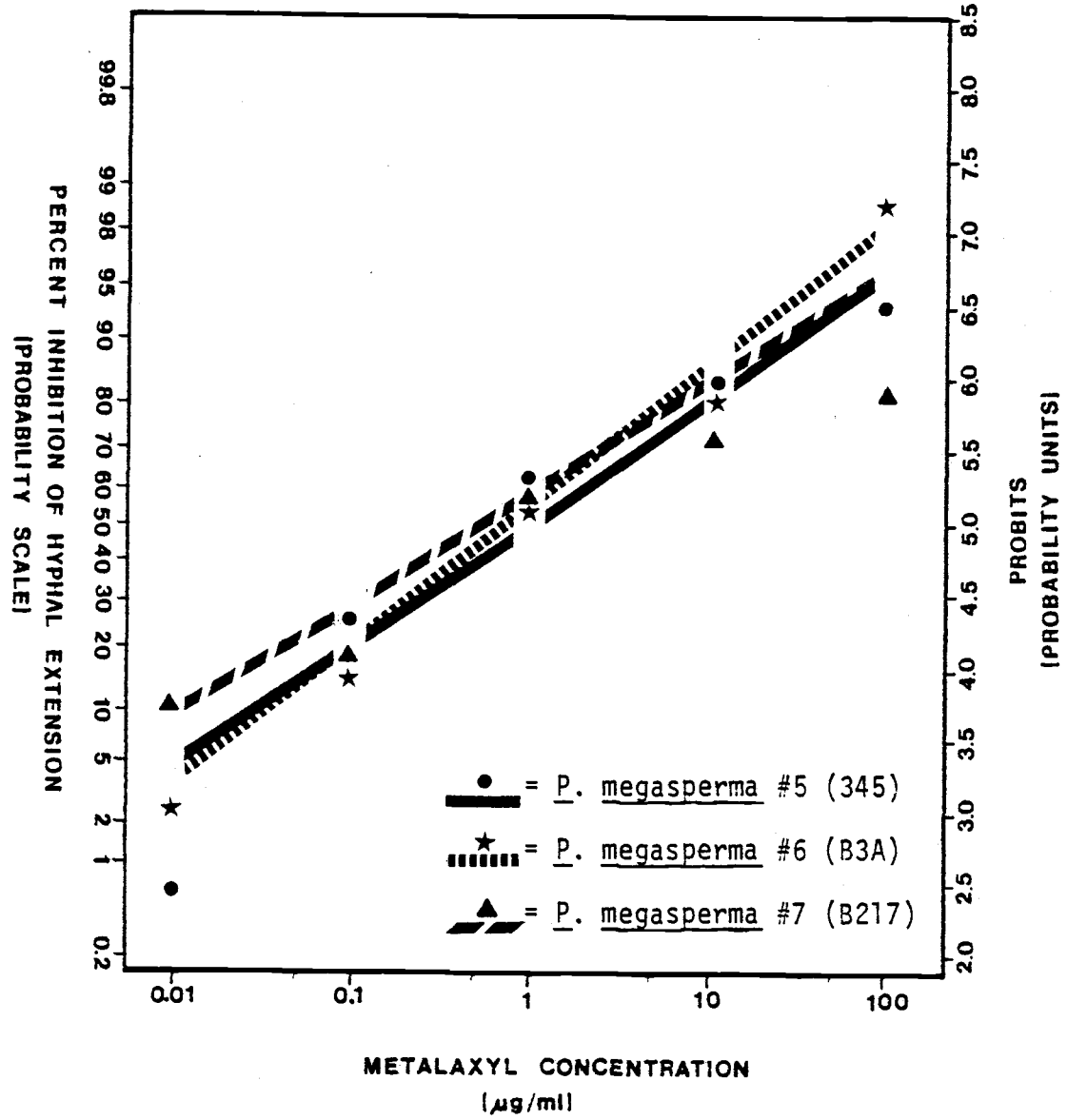

Fig. 10. Dosage-response curves of Phytophthora megasperma isolates #5 (345), #6 (B3A), and #7 (B217). Percent inhibition of hyphal extension was determined by comparing extension on metalaxyl amended to unamended media. r = .963, .998, and .977 for isolates #5, #6, and #7, respectively, and are significant at P = 0.05.

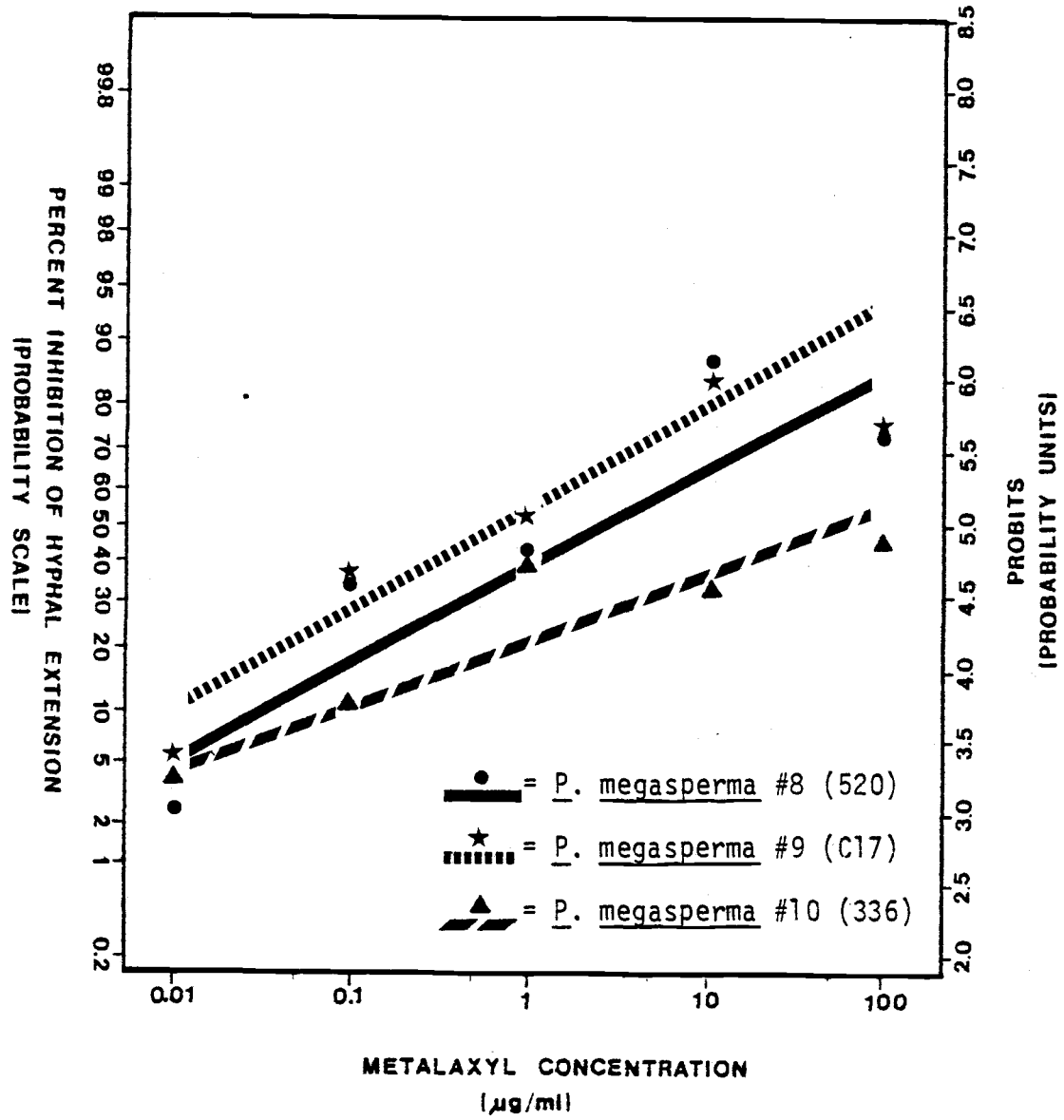

Fig. 11. Dosage-response curves of Phytophthora megasperma isolates #8 (520), #9 (C17), and #10 (336). Percent inhibition of hyphal extension was determined by comparing extension on metalaxyl amended to unamended media. r = .891, .902, and .918 for isolates #8, #9, and #10, respectively, and are significant at P = 0.05.

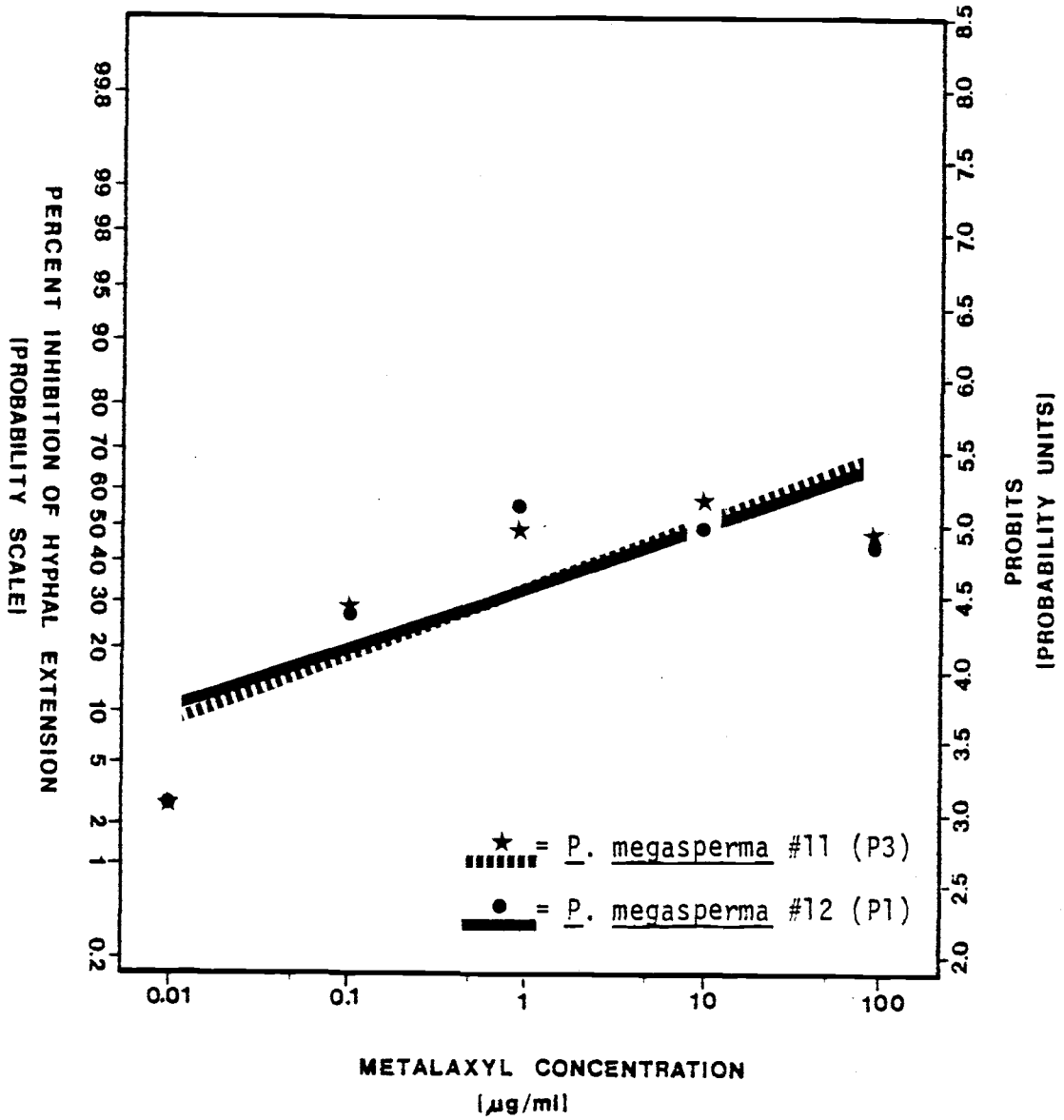

Fig. 12. Dosage-response curves of Phytophthora megasperma isolates #11 (P3) and #12 (P1). Percent inhibition of hyphal extension was determined by comparing extension on metalaxyl amended to unamended media. r = .813 and .775 for isolates #11 and #12, respectively, and are not significant at P = 0.05.

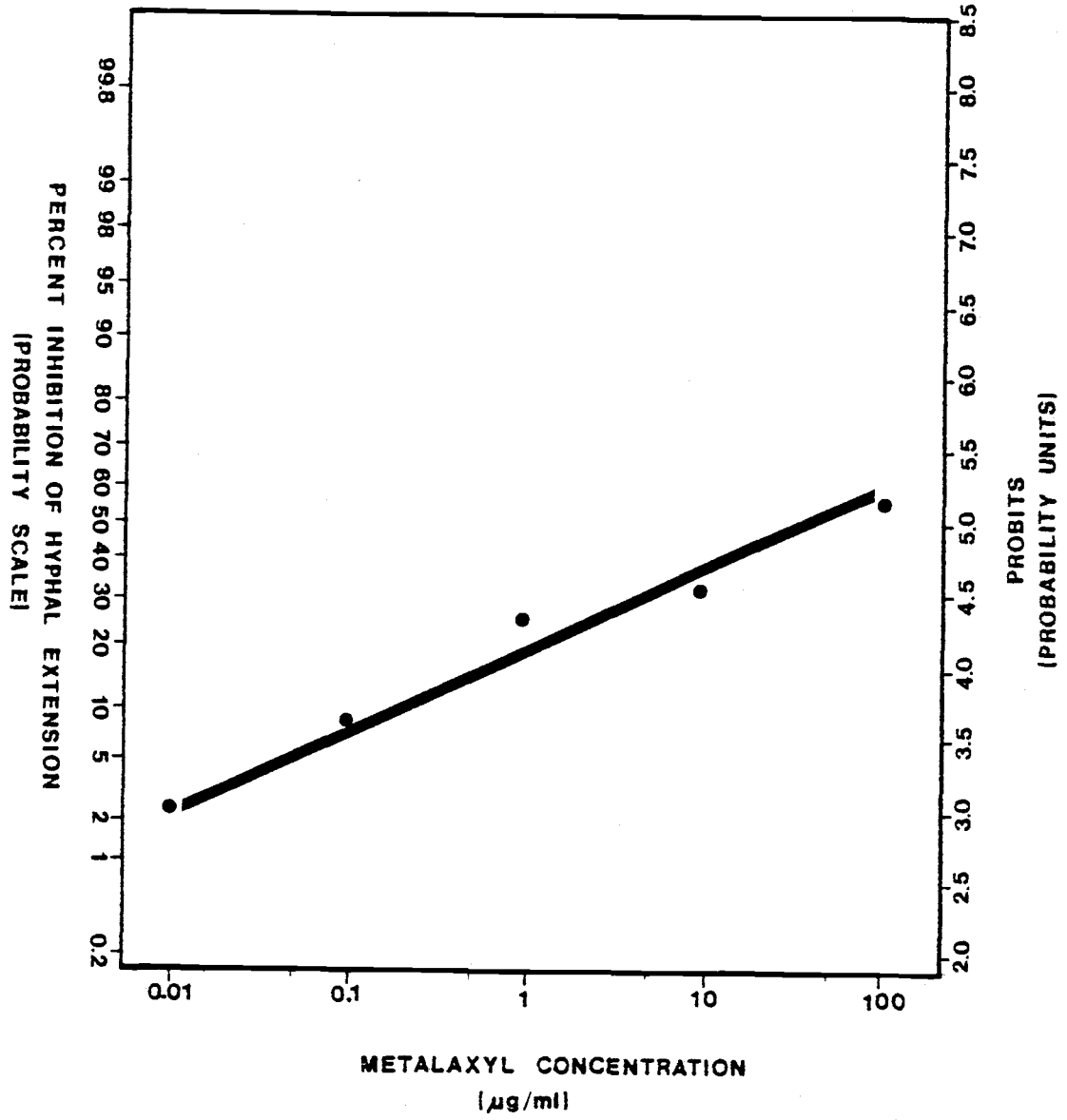

Fig. 13. Dosage-response curve of Phytophthora megasperma isolate #13 (K2). Percent inhibition of hyphal extension was determined by comparing extension on metalaxyl amended to unamended media. r = .926 and is significant at P = 0.05.

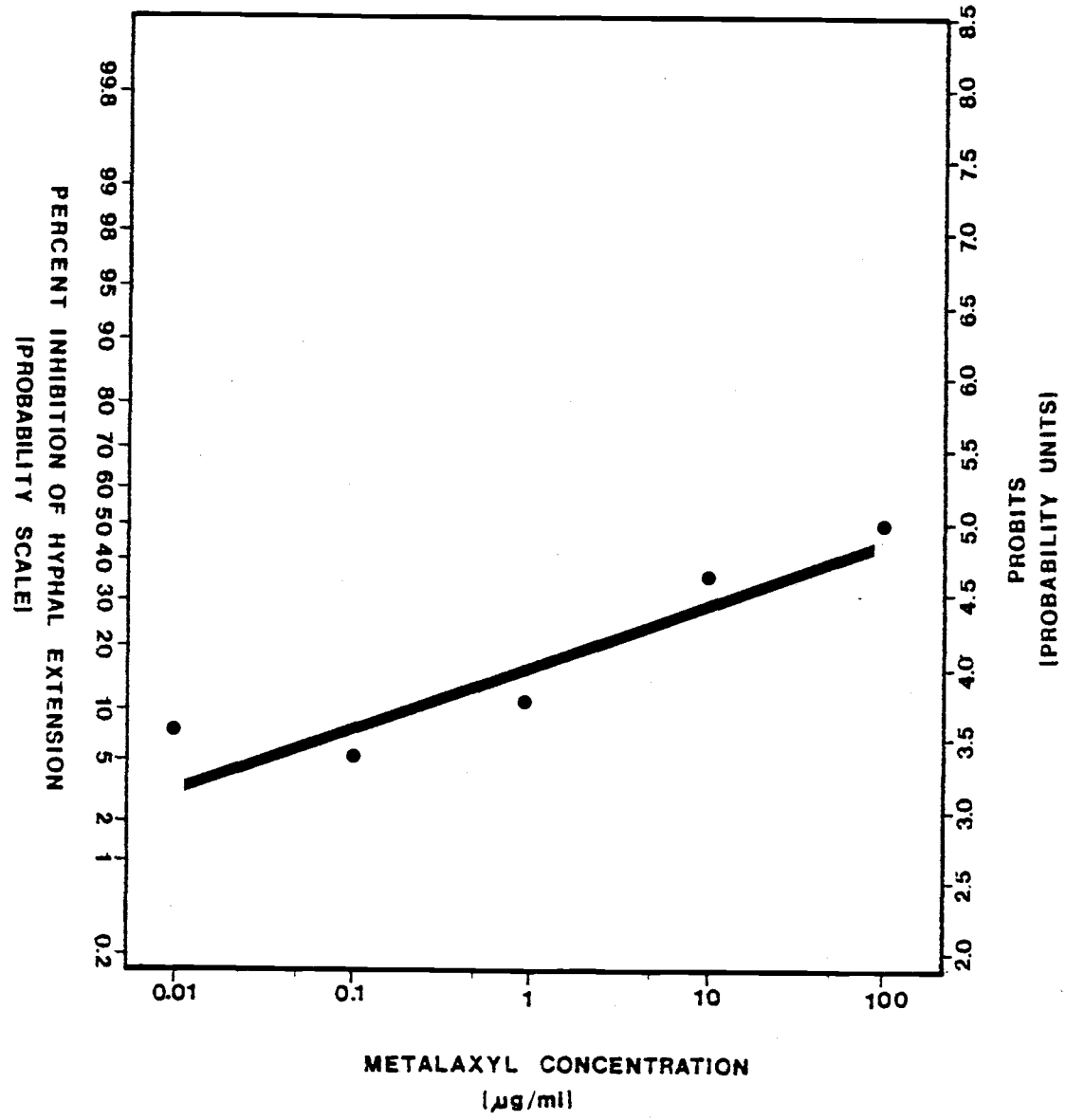

Fig. 14. Dosage-response curve of Phytophthora megasperma isolate #14 (K8). Percent inhibition of hyphal extension was determined by comparing extension on metalaxyl amended to unamended media. r = .985 and is significant at P = 0.05.

Table 9. Response of *Phytophthora* isolates to the systemic fungicide metalaxyl.

Species[w]	Response to metalaxyl: ED_{50} value[x] (µg/ml)	$\bar{X} \pm$ s.e.	Slope value[x]	Inhibition by metalaxyl (%)[y] $\bar{X} \pm$ s.e.
P. cinnamomi	0.01	———	1.16*	———
P. dreschleri	0.04	———	1.18*	———
P. megasperma				
1 Alfalfa (AL1)	0.04	.05±.01	1.28*	99±.79
2 Alfalfa (AL1)	0.06		1.19*	
3 Soybean (SB)	0.04	.05±.01	1.00*	100±0.0
4 Soybean (SB)	0.06		0.85*	
5 Douglas fir (D1)	1.10	1.2±.22	0.94*	67±1.7
6 Douglas fir (D1)	0.85		1.03*	
7 Douglas fir (D1)	1.60		0.57*	
8 Douglas fir (D2)	1.70	17±16	0.65*	44±5.1
9 Douglas fir (D2)	0.50		0.58*	
10 Douglas fir (D2)	50.00		0.42*	
11 Alfalfa (AL2)	13.00	13±0	0.41	44±1.3
12 Alfalfa (AL2)	13.00		0.43	
13 ———	35.00	———	0.54*	———
14 ———	130.00[z]	———	0.41*	———

[w] *P. megasperma* isolates are separated into morphologically distinct groups and designated (AL1, SB, etc.) according to Hansen and Hamm.[26]

[x] Values are from dosage-response curves based on growth inhibition determined by comparing hyphal extension on corn meal agar amended with metalaxyl to extension on unamended agar. Slope values (b) are from the regression formula of each dosage-response curve. *indicates that the correlation coefficient (r) is significant at P=0.05.

[y] Based on 8, 2, 3, 3, and 2 isolates for AL1, SB, D1, D2, and AL2, respectively. Inhibiton was determined by measuring hyphal extension on corn meal agar amended and unamended with 1 µg metalaxyl/ml. Taken by permission from Hansen and Hamm.[26]

[z] Extrapolated value. All other ED_{50} values were interpolated.

Response to metalaxyl by PM isolates 1-4 was similar to the response by PC and PD isolates (Table 9). The ED_{50} values were low (0.1 µg metalaxyl/ml), and the slopes of DR curves were steep (0.85-1.28). DR curves of these isolates did not include growth inhibition at 100 µg metalaxyl/ml because inhibition was at or near 100% at 10 µg metalaxyl/ml.

ED_{50} values of PM isolates 5-14 ranged from 0.85-130 µg metalaxyl/ml. Slopes of DR curves generated with these isolates were generally flatter than curves of PC, PD, and PM isolates 1-4 (Table 9). Thus, PM isolates 5-14 were relatively more tolerant to metalaxyl than PM isolates 1-4, PC, and PD.

Response to metalaxyl as a taxonomic trait. Response to metalaxyl usually was consistent between PM isolates that were morphologically distinct. Hansen and Hamm (26) extensively studied 54 PM isolates from 14 hosts, including the PM isolates used in our study. If PM isolates 1-14 are separated into the morphologically distinct groups they reported (Table 9), isolates in each group generally have similar ED_{50} values and DR curve slopes. Only the DR curve slope of PM isolate #7 and the ED_{50} and slope value for PM #10 differed considerably from isolates in the same group.

The concept that fungicide sensitivity may be useful as a taxonomic aid was proposed originally by Edgington and Barron (17), and later by Bollen and Fuchs (4) who studied sensitivity of many fungi to oxathiin compounds and to benomyl, respectively. Hall (24) studied the response of Verticillium dahliae, V. albo-atrum, and V. nigrescens isolates to benomyl. He concluded that mean ED_{50} values and mean slope

values of DR curves differed significantly between species. However, he felt that the overlap in ranges of these slope made identification of isolates to species impossible. Our results support this conclusion because PC, PD, and PM isolates 1-4 had nearly identical ED_{50} values and DR curve slopes (Table 9). However, our results, and results presented by Hansen and Hamm (26) (Table 9) do suggest that response to metalaxyl may be helpful in separating PM isolates into morphologically distinct groups, although morphological traits should be considered first.

Tolerance of Metalaxyl by PM isolates. Davidse (11) reported that isolates of P. megasperma f. sp. medicaginis were slightly tolerant (ED_{50} values < 5 µg metalaxyl/ml) or highly tolerant (ED_{50} values > 100 µg metalaxyl/ml) to metalaxyl. In contrast, our results indicate a wide range of metalaxyl tolerance between the PM isolates we tested (Table 9). These differences may be explained by the PM isolates used in each study. Davidse (11) started with one isolate of P. megasperma f. sp. medicaginis that was highly sensitive to metalaxyl. Tolerant isolates were selected from the original isolate by mycelial adaptation and by mass selection from untreated and mutagen treated zoospores. In our study, the degree of metalaxyl tolerance was measured in isolates collected from different locations and hosts. Thus, Davidse's study indicates the potential of a single isolate to become tolerant to metalaxyl. Our results indicate that PM isolates never exposed to metalaxyl and collected from different locations and/or from different hosts vary in their tolerance to metalaxyl. If similar variation in metalaxyl tolerance occurs in vivo, metalaxyl

applications would select for tolerant strains already present within the population as well as select for tolerance following a mutation in the fungus.

CHAPTER VI

CONCLUSIONS

Downy mildew has been the primary disease problem on hops cultivated in Oregon, and a serious intermittent problem in Washington, Idaho, and California. Data collected during 1979-1981 demonstrated that the fungicide metalaxyl effectively controlled this disease (Chapter III). Using these results, an emergency registration of metalaxyl for use on hops in Oregon, Washington, and Idaho was obtained in March 1981. Metalaxyl was applied to susceptible hop varieties in these states during April 1981. Nearly complete control of the disease was obtained and substantial yield losses were avoided. A full-use label is pending with the Environmental Protection Agency and should be approved no later than 1983. For 1982, states will reapply for an emergency use permit.

The other fungicides tested (M 9834, propamocarb, and efosite aluminum) also gave substantial control of downy mildew (Chapter III). The companies that developed these fungicides currently are not interested in pursuing registration; however, if tolerance to metalaxyl develops, this attitude will probably change. Propamocarb and efosite aluminum would be useful alternatives because the structures of these compounds are unrelated to metalaxyl and the occurrence of cross tolerance is unlikely.

Whether or not P. humuli strains tolerant to metalaxyl occur in the future will depend primarily on prudent and proper use of metalaxyl. However, hop growers currently are obtaining excellent control of downy mildew and are optimistic about controlling this disease with metalaxyl in the future.

Loss of disease control following intensive use of metalaxyl has been reported for different oomycete fungi (11, 21, 29). Subsequently, fungal strains were isolated, tested, and found to be tolerant of metalaxyl. Whether tolerant strains occurred naturally before application of metalaxyl, or resulted from mutation and selection is not known. Results of *in vitro* tests presented in this thesis indicate that a wide range of metalaxyl tolerance exists between *Phytophthora* isolates never exposed to metalaxyl (Chapters IV and V). If similar tolerance exists *in vivo*, disease control may be lost quickly following metalaxyl applications.

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