Tolerance of *Phytophthora megasperma* Isolates to Metalaxyl

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ABSTRACT

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Thirty-five isolates of *Phytophthora megasperma* were tested in vitro for tolerance to the systemic fungicide metalaxyl (CGA 48988). 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, eight were moderately tolerant, and 12 were highly tolerant of 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 that formed 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 the fungicide may select for naturally occurring tolerant strains, which may in turn lead to loss of disease control.

Commercial applications of systemic fungicides can favor selection of tolerant strains of fungi. As a result, higher rates or numerous applications may be required for disease control. Eventually, fungicidal effectiveness may be lost as tolerant strains multiply, spread, and replace the initially susceptible population. This phenomenon has been well documented (1,5,13) and is of major concern to growers who sustain reduced crop yields and agricultural chemical companies who lose revenue by reduced sales. Many factors determine the speed at which tolerance to systemic fungicides develops, including the number of genes regulating tolerance, gene mutability, the involvement of a polycyclic or monocyclic pathogen, and whether or not tolerant strains existed before fungicide applications began (5,6).

Few studies regarding the prior existence of tolerant strains have been reported. Wuest et al (16) evaluated three *Verticillium malthousei* Ware isolates in vitro for tolerance to benomyl and concluded that one isolate was tolerant, based on linear extension of hyphae, sporulation, and spore germinability. These isolates had been collected before benomyl was released as a commercial fungicide. Other researchers (2,4,8,11,15) have reported tolerance to fungicides, but preexisting tolerance in the natural population could not be determined because the chemicals had been used before isolates were collected.

To increase our understanding of this phenomenon, we initiated this study to determine 1) if in vitro tolerance to the systemic fungicide metalaxyl existed in 35 *Phytophthora megasperma* Drechs. isolates collected before the commercial release of metalaxyl, 2) to what degree isolates were tolerant, and 3) if isolates obtained from asexual spores (zoospores) differed in tolerance from their parents.

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MATERIALS AND METHODS

Thirty-five isolates of *P. megasperma* collected before the commercial release of metalaxyl were obtained from four continents and 14 hosts (Table 1). Eight single-zoospore isolates (SSI) were obtained from each parent isolate (PI) as described elsewhere (9). Effect in vitro of metalaxyl on growth and on oogonia and sporangia formation was determined. Results were expressed as percentage of inhibition as compared with controls. A 2EC formulation of metalaxyl (Ridomil, Ciba-Geigy Corp., Greensboro, NC 27409) was used in all studies.

Growth. Growth inhibition was determined on Difco cornmeal agar (CMA) with additional Bacto agar added to make 2% CMA, amended with 0, 1, and 10 μg/ml metalaxyl. The fungicide was sterilized with a Seitz bacterial and fungal filter. CMA was prepared and cooled to 45 C before the fungicide was added to obtain the specified metalaxyl concentrations. Aliquots of amended CMA (20 ml) were pipetted aseptically into sterile petri plates, and the medium was inoculated with small blocks (3 × 5 mm) cut from the margins of actively growing colonies cultured on CMA plus 20 μg/ml pimaricin. 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 eight SSI) at 1 and 10 μg/ml metalaxyl was used to compare tolerance among the 35 PI.

Results from this initial study were used to separate isolates for two additional growth tests. For the first test, PI were separated into three groups—highly sensitive, moderately tolerant, or highly tolerant to metalaxyl—to facilitate evaluation of tolerance levels. Representative PI from each sensitivity group 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 of incubation at room temperature. The average growth inhibition at each concentration was used to compare tolerance groups.

The second test compared growth of PI with that of their SSI. Growth of two SSI, one more and one less inhibited than the parent, was compared on CMA amended with 0, 1, and 10 μg/ml metalaxyl. Four replications per isolate per concentration were used. CMA was prepared and inoculated as in the previous studies. Average percentage 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. PI and those SSI differing 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. After 4 wk, the area of each colony containing oogonia was delineated. Three random samples (2 mm in diameter) were selected from this area, and the numbers 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 with controls (0 μg/ml) to determine percentage inhibition.

Sporangia formation. Two methods were used to determine inhibition of sporangia formation by PI and selected SSI. Both methods used colonies grown for 7 days in pea broth (14) at room temperature. 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. In the second method, colonies were rinsed with autoclaved distilled water and incubated in autoclaved soil extract water. Empty and full sporangia were counted in three randomly selected fields of view at ×100. The presence of released zoospores was noted for each isolate. Inhibition of sporangia formation was calculated by comparing the average number of sporangia formed in metalaxyl treatments to controls.

RESULTS

Growth. Fifteen PI were highly sensitive (more than 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. 1A).

Tolerance levels observed for all PI in the initial study were confirmed by extensive tests of selected PI on amended CMA (Fig. 2). Growth of PI (numbers 2, 5, 22, and 23) from the highly sensitive group was inhibited 24.8 and 98.9% at 0.01 and 10 μg/ml, respectively, and was prevented at concentrations of 50 μg/ml or more. Growth of PI (numbers 15, 16, 17, and 20) from the moderately tolerant group showed less than 2% inhibition at 0.01 μg/ml and 82% inhibition at 10 μg/ml. Growth of this group was inhibited 80–90% between 10 and 100 μg/ml metalaxyl. Growth of PI (numbers 3, 8, 11, 18, 19, and 32) from the highly tolerant group was inhibited less than 2% at 0.01 μg/ml and never exceeded 50% inhibition at any concentration tested.

Growth inhibition of isolates always increased with increasing fungicide concentration when colony morphology appeared normal. However, three of four moderately tolerant PI and five of six highly tolerant PI showed an anomalous growth pattern characterized by sparse, elongate hyphae, with reduced layering and branching (Fig. 3). Occurrence of this anomalous growth pattern corresponded with the plateau of inhibition of the moderately and highly tolerant groups reached between 10 and 50 μg/ml metalaxyl (Fig. 2).

Oogonia formation. Oogonia formation by 25 PI was inhibited 96–100% at 1 μg/ml, and that of seven other PI was inhibited 56–90% at this fungicide concentration (Fig. 1B). Three PI (25, 29, and 30) failed to form oogonia on the unamended medium. Nineteen of the 25 PI that did not form oogonia at 1 μg/ml had produced mycelia on amended medium. At 10 μg/ml, six PI formed oogonia; only one PI formed oogonia at 100 μg/ml.

No PI classified as highly sensitive on the basis of growth inhibition formed oogonia at 1 μg/ml metalaxyl, although 10 of the 15 PI in this group had grown after 4 wk. Nearly equal numbers of PI from the moderately and highly tolerant groups produced oogonia at 1 μg/ml (six of eight PI and eight of 12 PI, respectively). At 10 μg/ml, one of eight PI in the moderately tolerant group and five of 12 isolates from the highly tolerant group formed oogonia. Only isolate 7 from the highly tolerant group formed oogonia at 100 μg/ml. The percentage of aborted oogonia rose 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. Metalaxyl greatly affected formation of sporangia. Similar results were obtained after 24 and 48 hr from both sterile and unsterile wash treatments. Because more sporangia formed after unsterile treatments after 48 hr, only these results are reported.

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

No relationships were found among our tolerance groups in 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 2).

Comparison of SSI. Two isolate sets (13 and 35) were observed to have one SSI more sensitive and one more tolerant than their parent after extensive testing at 1 μg/ml metalaxyl (Fig. 4). Inhibition of oogonia and sporangia formation also differed between these PI and their SSI.

Table 1. Sources of *Phytophthora megasperma* isolates

Isolate No.	Isolate Designation	Host	Location	Source[a]
1	W1	Alfalfa	Washington	Christen
2	S1	Alfalfa	Salem, OR	OSU
3	P1	Alfalfa	Corvallis, OR	OSU
4	M1	Alfalfa	Medford, OR	OSU
5	PC3	Alfalfa	Princeton, OR	OSU
6	PC5	Alfalfa	Klamath Falls, OR	OSU
7	S2	Alfalfa	Salem, OR	OSU
8	P3	Alfalfa	Corvallis, OR	OSU
9	5b	Alfalfa	Wisconsin	Maxwell
10	DA	Alfalfa	Wisconsin	Maxwell
11	K2	Almond	Red Bluff, CA	Mircetich
12	K3	Almond	Red Bluff, CA	Mircetich
13	K10	Grape soil	Napa County, CA	Mircetich
14	K11	Grape soil	Napa County, CA	Mircetich
15	B3A	Douglas fir	Brownsville, OR	OSU
16	B217	Douglas fir	Brownsville, OR	OSU
17	345	Douglas fir	Brownsville, OR	OSU
18	336	Douglas fir	Toledo, WA	OSU
19	C17	Douglas fir	Elkton, OR	OSU
20	520	Douglas fir	Brownsville, OR	OSU
21	NF1	Noble fir	Corvallis, OR	OSU
22	908	Soybean (race 1)	Wisconsin	Grau
23	909	Soybean (race 3)	Wisconsin	Grau
24	105	Clover	Mississippi	Pratt
25	117	Clover	Mississippi	Pratt
26	102	Clover	Mississippi	Pratt
27	T14	Apple	New Zealand	CMI 144023
28	T47	Apple	New Zealand	CMI 147131
29	PA	Rose	Japan	Nagai
30	PB	Rose	Japan	Nagai
31	K1	Cherry	Stockton, CA	Mircetich
32	K8	Pear	Walnut Grove, CA	Mircetich
33	K9	Juniper	Davis, CA	Mircetich
34	T56	*Brassica* sp.	United Kingdom	CMI 56348
35	T28	*Populus robusta*	United States	ATCC 28765

[a] Isolates were obtained from A. Christen, Irrigated Agriculture Research and Extension Center, Prosser, WA (Christen); P. B. Hamm, Oregon State University (OSU); D. P. Maxwell, University of Wisconsin, Madison (Maxwell); S. Mircetich, USDA, University of California, Davis (Mircetich); C. R. Grau, University of Wisconsin, Madison (Grau); R. G. Pratt, USDA, Mississippi State University, Mississippi State (Pratt); Commonwealth Mycological Institute, Kew, Surrey, England (CMI); Y. Nagai, Laboratory of Plant Pathology, Chiba, Japan (Nagai); and American Type Culture Collection, Rockville, MD (ATCC).

In general, SSI more sensitive to metalaxyl in growth also formed fewer oogonia and sporangia than their parents, and 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 yr have dealt with tolerance to fungicides. Most have involved training fungi to tolerate higher fungicide doses, using mutagens to induce tolerance, or determining natural tolerance in fungal strains. However, most studies of natural tolerance have used strains isolated after a fungicide was introduced, and it is difficult to conclude whether tolerance existed in the population before the first application of fungicide. Webster et al (15) reported tolerance in *Botrytis cinerea* Fr. to 2,6-dichloro-4-nitroaniline (DCNA) but could not determine whether exposure to the chemical had occurred in vivo before their isolations were made. Harding (10) reported that strains of *Penicillium italicum* Wehmer and *Penicillium digitatum* Sacc. tolerant to thiabendazole occurred naturally because such strains could be isolated from orchards and packinghouses where the fungicide had not been used. Bolton (2) reported in vitro tolerance to benomyl, dicloran, and triadimefon in *B. 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 (8) isolated strains of *Penicillium digitatum*

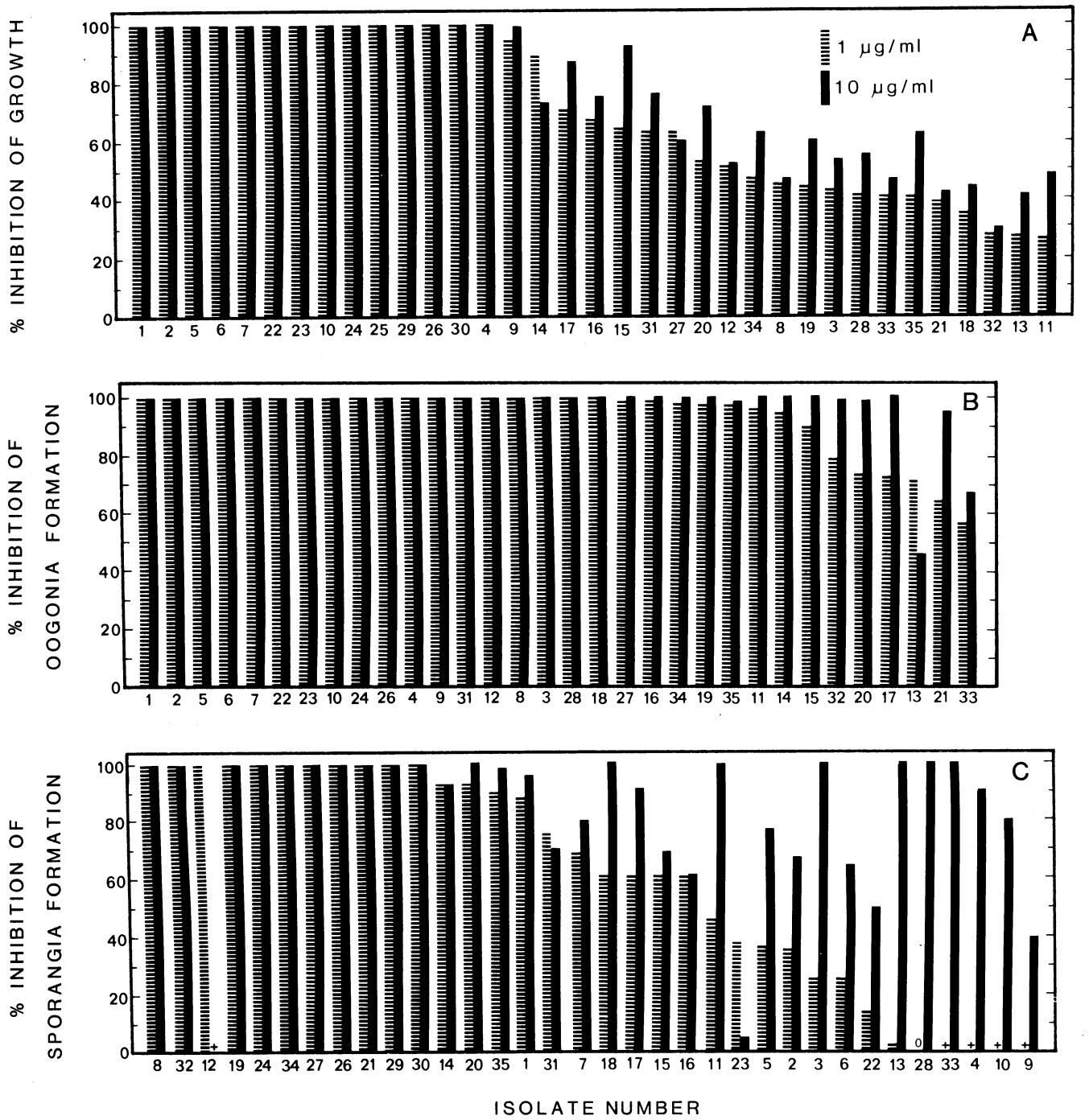

Fig. 1. Inhibition of *Phytophthora megasperma* isolates by two concentrations of metalaxyl, compared with controls: **(A)** Inhibition of growth after 5 days on cornmeal agar at room temperature (22–25C). **(B)** Inhibition of oogonia formation on clarified V-8 juice agar after incubation for 4 wk at room temperature. **(C)** Inhibition of sporangia formation by colonies grown in pea broth for 7 days at room temperature, washed with distilled water, and incubated for 48 hr in soil extract water amended with metalaxyl. A plus sign (+) means that the isolate formed more sporangia in metalaxyl-amended soil extract water than in the control.

Table 2. Effect of metalaxyl on sporangia formation[a] and zoospore release by 35 isolates of *Phytophthora megasperma*

Metalaxyl concentration (μg/ml)	Isolates forming sporangia (%)	Isolates with empty sporangia and/or zoospores (%)	Number of sporangia observed per isolate that formed sporangia[b]
0	97	95	28
1	66	86	13
10	57	57	6
100	6	0	0.7

[a]Sporangia were observed in colonies on pea broth incubated in soil extract water for 48 hr.
[b]Mean of three random observations per isolate.

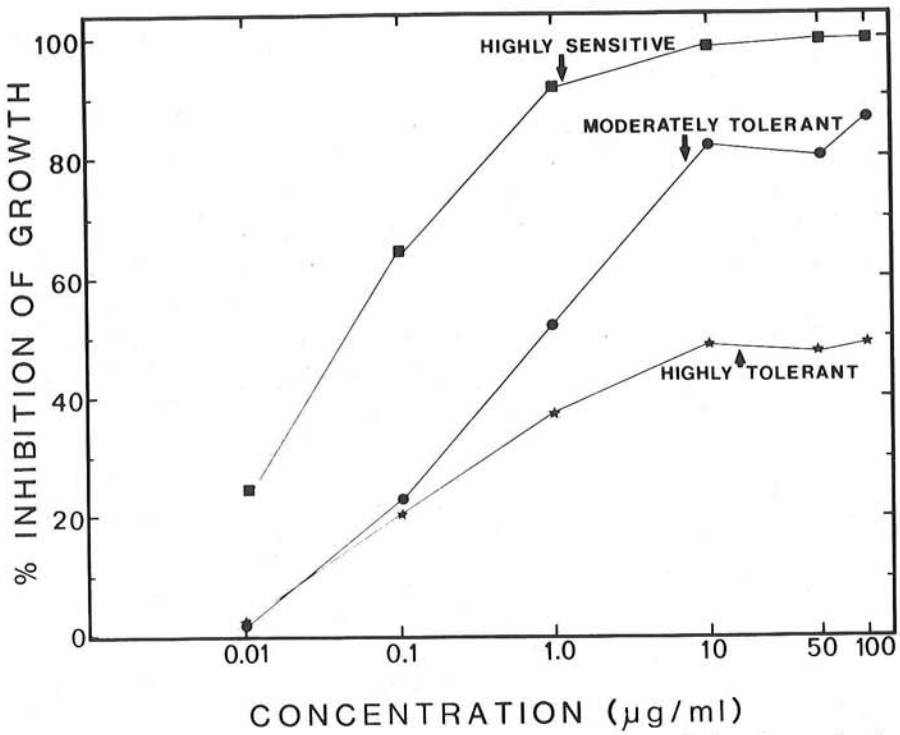

Fig. 2. Differential inhibition of growth of *Phytophthora megasperma* isolates by metalaxyl. Isolates were initially grouped as highly sensitive, moderately tolerant, or highly tolerant to metalaxyl on the basis of growth inhibition by 1 and 10 μg/ml metalaxyl. Inhibition of growth of representative isolates from each group by seven concentrations of metalaxyl was compared on cornmeal agar after incubation for 5 days at room temperature (22–25 C).

and *Penicillium italicum* tolerant to benzimidazole fungicides from citrus orchards in Israel where benzimidazoles had never been applied. They 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 (12) 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 wk, respectively, after metalaxyl (Ridomil 2EC) was applied. These levels were associated with complete control of hop downy mildew caused by *Pseudoperonospora humuli* (Miy. & Tak.) G. W. Wils. Levels fell to 0.25–0.75 mg/kg in leaves 4–10 wk after 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 after applications of metalaxyl.

Differential tolerances to fungicides among isolates and subsequent single-spore cultures have been reported. Webster et al (15) identified resistant and sensitive single asexual spore cultures from strains of *B. cinerea* resistant to DCNA. Bruin and Edgington (3) reported large differences in sensitivity of monozoospore cultures of *Phytophthora* and *Pythium* species to metalaxyl. These and other researchers (7) suggest that heterokaryosis is a mechanism that could maintain low levels of fungicide tolerance in multinucleate fungi and that tolerant strains emerge as a result of selection by the fungicide. In addition, Davidse (4), using one isolate of *P. megasperma* f. sp.

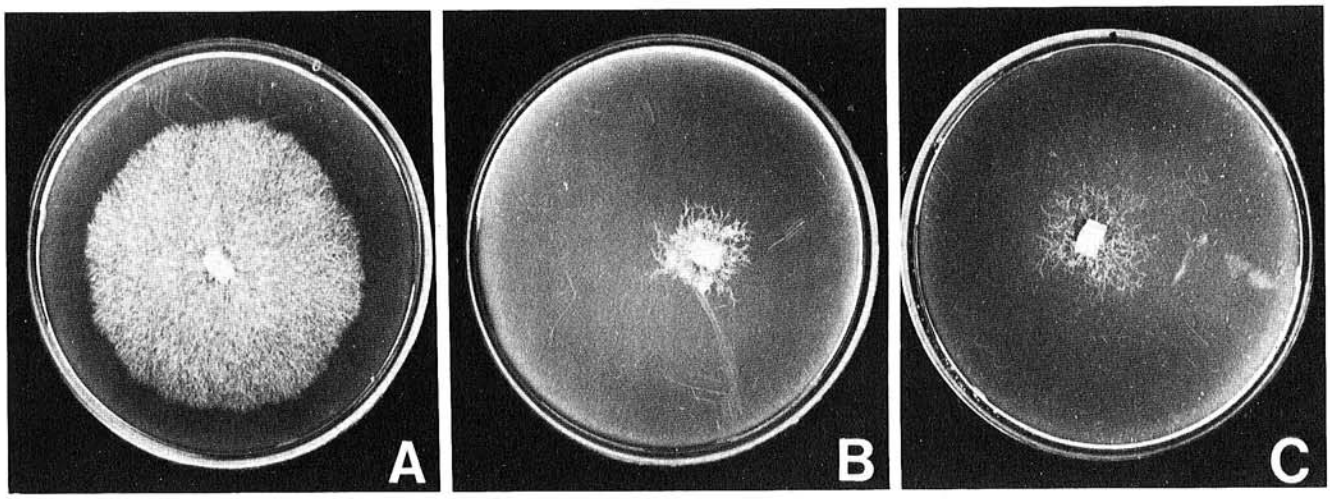

Fig. 3. Anomalous growth pattern of certain *Phytophthora megasperma* isolates cultured on cornmeal agar for 5 days at room temperature (22–25 C): **(A)** Hyphal growth on unamended media. **(B)** Growth at 10 μg/ml metalaxyl. **(C)** Growth at 50 μg/ml metalaxyl. Note the abnormally elongate hyphal growth (C), which was correlated with a plateau of growth inhibition of the moderately and highly tolerant groups by metalaxyl between concentrations of 10 and 50 μg/ml.

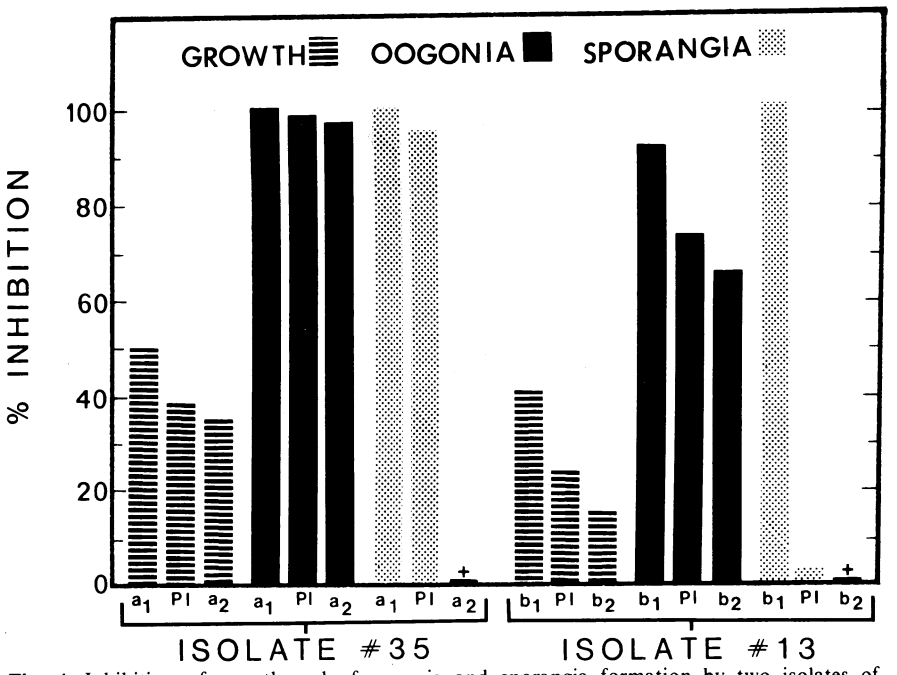

Fig. 4. Inhibition of growth and of oogonia and sporangia formation by two isolates of *Phytophthora megasperma* (PI) and two single-zoospore isolates from each parent (a_1, a_2, b_1, b_2) by 1 μg/ml metalaxyl. Percentage inhibition was determined by comparison with controls. A plus sign (+) means that the isolate formed more sporangia in metalaxyl-amended soil extract water than in the control.

medicaginis, found single-zoospore isolates more tolerant to metalaxyl than their parent in vitro. These observations are supported by our study, in which tolerance to metalaxyl varied greatly between some parents and their SSI. At least two of 280 SSI tested were consistently more tolerant to metalaxyl than their parents. Thus, production of asexual spores (eg, zoospores) in vivo may contribute to the variation in tolerance observed among our isolates.

The wide tolerance range we observed among isolate sets suggests that tolerance values (eg, ED_{50}, LD_{50}, etc.) also vary greatly. Papavizas and Bowers (11) 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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