Competition Between Metalaxyl-Resistant and -Sensitive Strains of *Pseudoperonospora cubensis* on Cucumber Plants

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ABSTRACT

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The infectivity of two pairs of metalaxyl-resistant and -sensitive strains of *Pseudoperonospora cubensis* was compared in the absence of metalaxyl selection pressure by single strain inoculations, passing mixed (1:1, 1:6, and 1:20 resistant:sensitive in initial sporangial suspension inoculum) strains through two inoculation cycles on intact cucumber cotyledons in growth chambers, and exposing cucumbers growing in plastic houses to both strains (at a ratio of 1:1 or 1:4). With single strain inoculation, strains were equally infective to cucumbers. In mixed strain inoculations in growth chambers, after one sporulation cycle (two successive inoculations) the original 1:1, 1:6, and 1:20 ratio of resistant:sensitive components changed essentially to 1:0. In mixed strain inoculations in plastic houses, the original 1:1 and 1:4 ratio (resistant:sensitive) changed essentially to 1:0 after 12–17 and 23 days, respectively. The results indicate that, with the technique used to monitor strain frequency, the two metalaxyl-resistant strains of the fungus compete favorably with the two metalaxyl-sensitive strains in the absence of the fungicide.

Additional key words: *Cucumis sativus*.

The failure of metalaxyl (DL-methyl-*N*[2,6-dimethylphenyl]-*N*-[methoxyacetyl] alanine methyl ester) to control plant diseases incited by fungal pathogens of the Peronosporales was first reported from Israel in 1979 for *Pseudoperonospora cubensis* in plastic-house-grown cucumbers (15). This failure was then reported to occur in Greece (10), and was followed by similar reports for *Peronospora hyoscyami* in the U.S. (1), *Phytophthora infestans* in Holland (8), Northern Ireland (7), and Israel (5) and for *Plasmopara viticola* in France (3).

In Israel, resistant strains of *P. cubensis* predominated during 1980 in both plastic houses and in the open field, some of which were not treated with metalaxyl. Predominance of the wild-type sensitive strains did not return before mid-1981, about half a year after the use of metalaxyl in cucurbits was abandoned.

The present study was conducted to determine the infectivity to cucumbers and the competitive capacity of two metalaxyl-sensitive and two metalaxyl-resistant strains of *P. cubensis* in the absence of metalaxyl under growth chamber conditions and in plastic houses. A preliminary report was published (14).

MATERIALS AND METHODS

Plants and pathogen. Cucumber (*Cucumis sativus* L. 'Bet-Alpha') highly susceptible to downy mildew was used in all studies. For inoculum increase, trap plants, and mixed strain inoculations in the laboratory, plants were grown in 10-cm-diameter plastic pots, 12–15 plants per pot, in the greenhouse (20–34 C) and used about 10 days after sowing when cotyledonary leaves were 2–3 cm long. The metalaxyl-sensitive (wild-type) strains MS_1 and MS_2 of *P. cubensis* (Berk. et Curt.) Rost. were collected from field-grown cucumber plants in June 1978 and September 1981 on campus and in Zikim, respectively. The metalaxyl-resistant strains MR_1 and MR_2 were collected from plastic-house-grown cucumbers in Hadera district in December 1979, and in Ramat-Hasharon in January 1982, respectively. The four strains were maintained on cucumber plants in separate growth chambers. Strict measures were employed to avoid cross contamination of the strains. The MS strains were maintained and increased on fungicide-free plants, but periodic parallel inoculations of metalaxyl-treated plants (soil drenched with 0.1 mg a.i. of metalaxyl per pot) were conducted to check sensitivity to metalaxyl. The MR strains were maintained and increased on metalaxyl-treated plants (a soil drench of 5 mg a.i. of metalaxyl per pot given 1 hr before inoculation).

Epidemics in plastic houses. Eight commercial walk-in plastic houses (5 × 12 m) were constructed on campus between December 1980 and December 1982 (Table 1). One hundred and twenty cucumber plants were grown in each house. When they reached the 8- to 10-leaf stage (about 4–5 wk after sowing), plants were exposed to *P. cubensis* of either an MS strain, an MR strain, or both. Exposure was done by introducing about 40 sporulating intact cotyledons into the plastic houses for 48 hr. When exposure to both MS and MR strains had to be done at a ratio of 1:1 or 1:4 (MR:MS) 20 cotyledons infected with the MR strain, and 20 cotyledons infected with the MS strain, were introduced, or 8 and 32, respectively. Except for houses 1 and 3, plants growing in houses were not chemically treated against downy mildew. Occasionally, plants were treated with a protectant fungicide against powdery mildew. Plants in houses 1 and 3 were sprayed to run-off with metalaxyl (25 WP, 250 μg a.i./ml) at 10-day intervals, starting from 3 wk after sowing. Disease development on plants growing in houses was recorded visually (0–5 scale) at 2- to 6-day intervals according to the method described before (6). To monitor the frequency of the MS and the MR strains in houses, six groups of 50 intact cucumber plants each (at the cotyledonary stage) were placed as traps inside houses at 2- to 6-day intervals. Half of the trap plants in each group were treated with a soil drench of 5 mg a.i. of metalaxyl per pot and half with water. Trap plants were removed (usually after 24 hr unless otherwise stated), sprayed with water, placed in a moist chamber at 17 C in the dark for 20 hr, and then incubated for 7 days in a 25 C cabinet for symptom production.

A hygrothermograph was placed in the middle of each plastic house at 0.5 m above ground level.

Mixed strain inoculation in the laboratory. Sporangial suspensions of an MS and an MR strain were prepared separately in ice-cooled double distilled water and calibrated to either 5 or 2.5 (±10) sporangia per 10-μl droplet. Droplets were produced using a Nichiryo syringe dispenser model 8100 (Nichiryo Co., Chiyoda-Ku, Tokyo). The MS and the MR strain suspensions were mixed at ratios (MR:MS) of 1:1, 1:6, and 1:20 by volume. Each inoculum suspension was used to inoculate about 100 intact metalaxyl-treated (5 mg a.i. per pot) and 100 fungicide-free cotyledons (four

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TABLE 1. Experimental set-up and relative frequency of fungal strains in cucumbers grown in walk-in plastic houses mixed-inoculated with metalaxyl-sensitive and metalaxyl-resistant strains of *Pseudoperonospora cubensis*

Plastic house	Growing season	Fungal strains introduced[a]	Fungicide treatment	Relative frequency[b] (%) of metalaxyl-sensitive	Relative frequency[b] (%) of metalaxyl-resistant	Frequency determination time (days)
1	Dec. 80–Jan. 81	MS_1	Metalaxyl[c]	0	0	32
2	Dec. 80–Jan. 81	MS_1	None	100	0	32
3	Dec. 80–Jan. 81	MR_1	Metalaxyl[d]	0	100	32
4	Dec. 80–Jan. 81	MR_1	None	0	100	32
5	Mar. 81–Apr. 81	$MR_1 + MS_1$ (1:1)	None	0	100	12 onward
6	Jan. 82–Feb. 82	$MR_2 + MS_2$ (1:1)	None	0	100	12 onward
7	Nov. 82–Dec. 82	$MR_2 + MS_2$ (1:1)	None	0	100	17 onward
8	Nov. 82–Dec. 82	$MR_2 + MS_2$ (1:4)	None	0	100	23 onward

[a] MS_1 and MS_2 = metalaxyl-sensitive strains collected in June 1978 on campus, and in September 1981 from Zikim, respectively.
[b] Metalaxyl (25WP) suspension (250 μg a.i./ml) was applied to run-off every 10 days starting at about 10 days before inoculum was introduced. Relative frequency of strains was determined on either trap plants or in sporangia collected manually from plants growing in houses.
[c] MS strains were controlled in plants treated with a soil drench of 0.1 mg a.i. per pot (0.3 kg of air-dried soil per pot).
[d] MR strains sporulated abundantly on plants drenched with 5 mg a.i. per pot.

TABLE 2. Percent infection by *Pseudoperonospora cubensis* on intact cucumber cotyledons either treated or untreated with metalaxyl and exposed for 1 hr to infectious plants in plastic house 6 at day 17

Exposure period of trap plants	Cotyledons infected (%)	
	Untreated	Metalaxyl-treated
0900–1000	29	36
1000–1100	45	53
1100–1200	79	57
1300–1400	65	69
1400–1500	59	55
1500–1600	9	14
1600–0800	63	62
0800–0800	97	97

pots per treatment). Inoculation was done by placing one 10-μl inoculum droplet in the middle of the upper leaf surface of each leaf. Inoculated plants were kept in a dew chamber (model I-60D; Percival Refrigeration and Mfg. Co., Boone, IA 50036) at 17 C for 20 hr, and then transferred to a 25 C cabinet (40–60% RH) illuminated 12 hr/day with VHO fluorescent lamps at an intensity of about 150 μEinsteins·m^{-2}·sec^{-1}. Percentage infected cotyledons was visually determined at 7 days after inoculation. Infected metalaxyl-free plants were then placed in a dew chamber at 17 C in the dark for 24 hr to allow fungal sporulation. Sporangia produced were harvested from leaves of each treatment separately and used to inoculate (in the manner described) other plants treated or untreated with metalaxyl. The percentage of cotyledons that became infected as a result of the second inoculation was used to estimate the relative frequency of the two strains in the sporangial suspension. Mixed strain inoculations in the laboratory were repeated every 2 mo during a 2-yr period.

RESULTS

Mixed strain inoculations in plastic houses. Table 1 summarizes the experimental set-up and some results obtained in eight plastic houses during December 1980–December 1982.

In house 1, the MS_1 strain was totally inhibited by metalaxyl but it was not inhibited in house 2 in which plants remained untreated (Fig. 1A). Similar results (not presented) were obtained for strain MS_2. The MR_1 strain developed equally well in house 3 on plants treated with metalaxyl as in house 4 on plants free of the fungicide (Fig. 1B). Similar results (not presented) were obtained for MR_2.

No significant differences were recorded between rates of epidemic development in houses 2 and 4, indicating that in the absence of metalaxyl MS_1 and MR_1 had equal infectivity to cucumber plants. In a similar manner we noted (data not presented) that MS_2 and MR_2 were equally infective to cucumbers in the absence of metalaxyl.

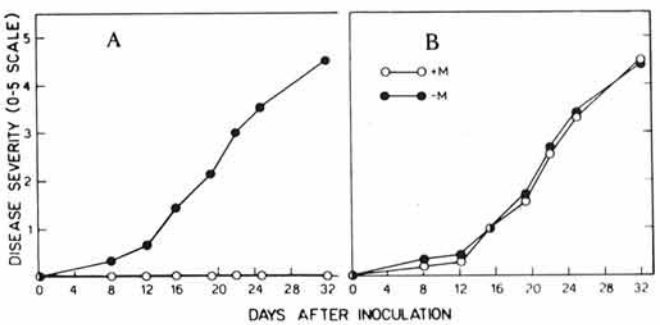

Fig. 1. Disease progress curves of downy mildew incited by *Pseudoperonospora cubensis* on cucumber plants growing in walk-in plastic houses 1–4. **A,** Epidemics incited by the MS_1 strain (metalaxyl-sensitive) of the fungus. Open circles—plants treated with metalaxyl (house 1). Closed circles—plants not treated with metalaxyl (house 2). **B,** Epidemics incited by the MR_1 strain of the fungus (metalaxyl-resistant). Open circles—plants treated with metalaxyl (house 3). Closed circles—plants not treated with metalaxyl (house 4).

In houses 5, 6, and 7, in which MS and MR strains were introduced at a ratio of 1:1, a gradual increase in the infectivity of fungal populations to metalaxyl-treated trap plants was recorded (Fig. 2 A to C). At 12–17 days after introducing inoculum into the houses, trap plants (whether treated or untreated with metalaxyl) became equally infected. In house 8, in which MS and MR strains were introduced at a ratio of 1:4 (MR_2:MS_2), equal infectivity to trap plants treated or not treated with metalaxyl was determined at 23 days after inoculum was introduced (Fig. 2D). It is interesting to note that, in spite of the high temperature prevailing in some of the houses, disease progressed very rapidly (see house 6) probably due to the prolonged periods of high relative humidity.

Table 2 shows that the predominance of the MR_2 strain in house 6 at day 17 was exhibited also in trap plants exposed to sporangial deposition for only 1 hr. Tables 3 and 4 show that sporangia manually collected from plants in house 6 at day 17 were equally infective to metalaxyl-treated and metalaxyl-free plants, indicating that predominance of the MR strain did not result from differential sporangial dispersal or viability but from predominance on the sporulating leaves.

Mixed strain inoculations in growth chambers. Results of a typical experiment (out of a dozen conducted during 1980–1982) are presented in Table 5. They show that in the first inoculation, the percent of cotyledons infected among metalaxyl-treated plants decreased with decreasing proportion of MR in the inoculum mixture. The decrease, however, did not reach the proportion of MR in the mixture. The results also show that sporangia produced as a result of the first inoculation on the metalaxyl-free plants were equally infective (second inoculation) to metalaxyl-treated and

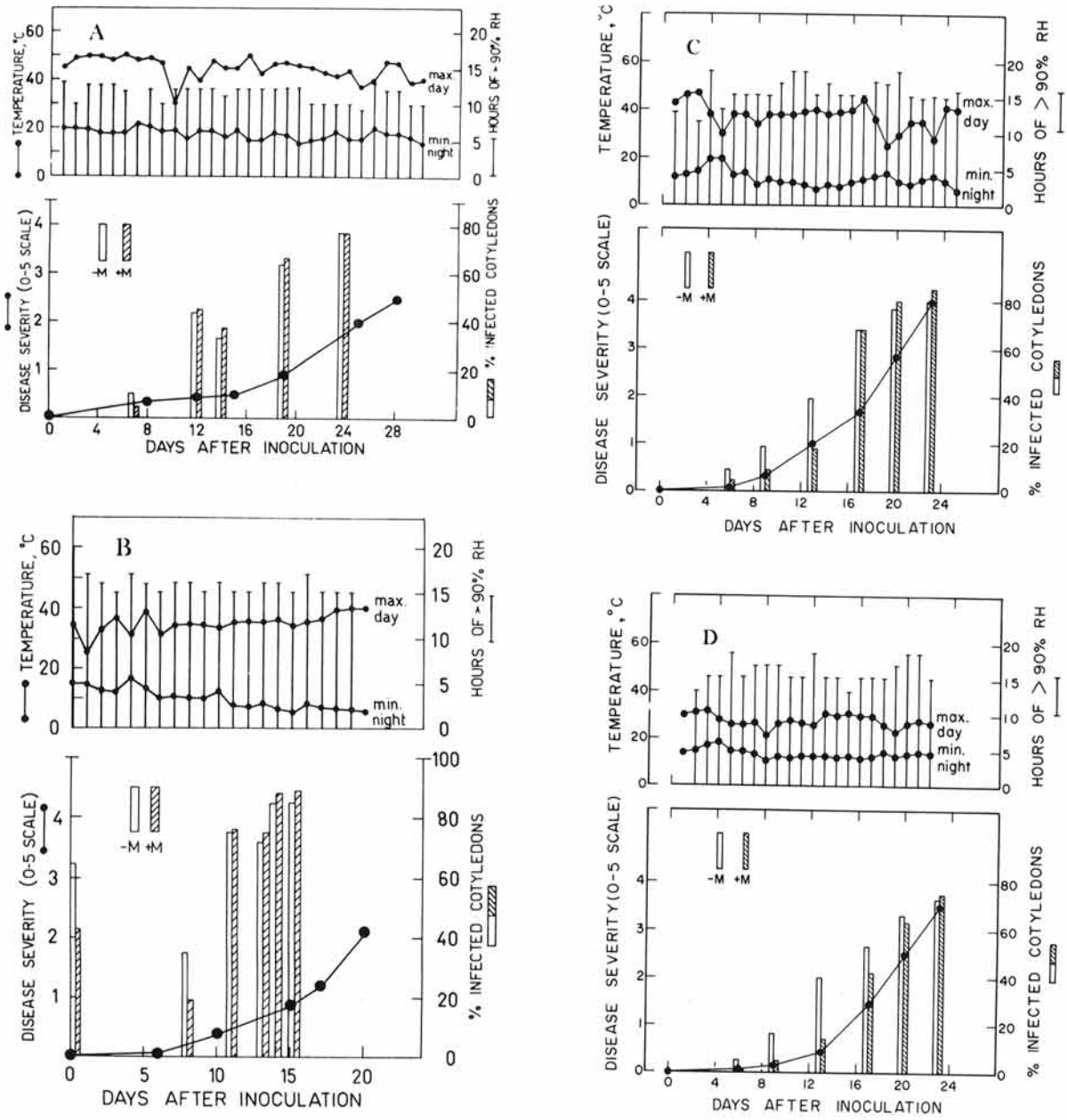

Fig. 2. Competition between metalaxyl-resistant and metalaxyl-sensitive strains of *Pseudoperonospora cubensis* in plastic houses in the absence of metalaxyl. **A,** Disease incited by the MR_1 and MS_1 strains at a ratio of 1:1 (house 5, March 1981). **B,** Disease incited by the MR_2 and MS_2 strains at a ratio of 1:1 (house 6, January 1982). **C,** Disease incited by the MR_2 and MS_2 strains at a ratio of 1:1 (house 7, November 1982). **D,** As in C, but at a ratio of 1:4. Disease progress curves represent disease severity in plants growing in plastic houses. Bars represent infectivity of sporangia produced in houses to trap plants placed in houses for only 24 hr. Half of the trap plants were treated and half not treated with metalaxyl.

TABLE 3. Infectivity to metalaxyl-treated and metalaxyl-free cucumber plants of sporangia of *Pseudoperonospora cubensis* collected at various times of day 17 from nontreated cucumber plants growing in plastic house 6

Collection time of sporangia[a]	Disease severity (0–5 scale) ± S.D.	
	Metalaxyl-treated plants	Metalaxyl-free plants
0930	3.9 ± 0.7	3.8 ± 0.6
1200	3.4 ± 0.9	3.6 ± 0.4
1500	1.8 ± 1.0	1.8 ± 0.8

[a] Sporangia were collected at day 17 from leaves of plants in plastic house 6. The plants had been exposed to the MS_2 and MR_2 strains of the fungus at a ratio of 1:1 (Table 1 and Fig. 2B). Sporangia, collected by brushing into water, were inoculated onto two-leaf plants using a quantitative inoculator (10^5 sporangia per milliliter). For detailed description of inoculation technique and disease severity evaluation, see (4).

TABLE 4. Infectivity to metalaxyl-treated and metalaxyl-free cucumber plants of sporangia of *Pseudoperonospora cubensis* at various concentrations collected from nontreated cucumber plants growing in plastic house 6

Inoculum concentration[a] (sporangia/ml ×1000)	Disease severity (0–5 scale) ± S.D.	
	Metalaxyl-treated plants	Metalaxyl-free plants
84	3.3 ± 0.5	3.3 ± 0.6
42	1.6 ± 0.7	1.9 ± 0.4
21	0.8 ± 0.4	0.8 ± 0.2
10.5	0.4 ± 0.1	0.4 ± 0.2

[a] Sporangia were collected on day 17 at 1200 hours (see Table 1, Fig. 2B, and reference [4] for details).

TABLE 5. Competition between a metalaxyl-sensitive strain (MS_2) and a metalaxyl-resistant strain (MR_2) of *Pseudoperonospora cubensis* under laboratory conditions. Strains were inoculated singly or in mixtures of various proportions to cucumber cotyledons either treated or untreated with metalaxyl at two successive inoculations

Composition of original inoculum mixture (%)		Percent infected cotyledons[a] ± S.D.							
		5 sporangia/cotyledon				2.5 sporangia/cotyledon			
		First inoculation		Second inoculation		First inoculation		Second inoculation	
MR_2	MS_2	Without metalaxyl	With metalaxyl[b]	Without metalaxyl	With metalaxyl	Without metalaxyl	With metalaxyl	Without metalaxyl	With metalaxyl
100	0	90 ± 5	95 ± 4	93 ± 6	90 ± 9	83 ± 5	79 ± 4	81 ± 7	80 ± 9
50	50	89 ± 4	45 ± 9	96 ± 9	90 ± 10	80 ± 10	45 ± 9	80 ± 6	77 ± 18
14	86	90 ± 6	24 ± 9	93 ± 8	91 ± 11	82 ± 4	21 ± 7	76 ± 6	75 ± 12
5	95	91 ± 3	24 ± 12	85 ± 9	82 ± 13	81 ± 3	13 ± 3	78 ± 4	74 ± 4
0	100	89 ± 5	0	90 ± 5	0	79 ± 5	0	79 ± 5	0

[a] Eight pots with 12–15 plants per pot were inoculated in each treatment. Sporangia produced on metalaxyl-free plants in the first inoculation were used for the second inoculation.
[b] Metalaxyl (25 WP) was applied as a soil drench at 5 mg a.i. per pot.

metalaxyl-free plants. These results indicated predominance of the MR strain in leaves that had been inoculated with a mixture of a resistant and a sensitive strain.

DISCUSSION

Results presented in this paper demonstrate that in mixed strain inoculations in the absence of metalaxyl, two metalaxyl-resistant strains of *P. cubensis* had a high competitive capacity compared to two metalaxyl-sensitive strains. This capacity could not be attributed to differential fitness to cucumber plants, as both resistant and sensitive strains exhibited similar pathogenicity to cucumbers when inoculated singly in the absence of the fungicide.

In either growth chamber or plastic house mixed strain inoculations a full predominance of the resistant strains occurred within about two or three disease cycles. Predominance occurred even when the proportion of the resistant strain in the original inoculum mixture was 5%. This stands in accord with the findings of Brown and Sharp (2) and Ogle and Brown (13), who reported that in mixed race inoculations of rusts one race predominated regardless of its proportion in the inoculum mixture. To determine the relative frequency of the two strains in a population of the pathogen, cucumber plants, either treated or untreated with metalaxyl, were inoculated. Resultant infection on treated plants reflected the frequency of an MR strain in the population, whereas that on untreated plants reflected both the MR and the MS strain frequencies. Subtracting the treated plant value from the untreated plant value gave us an indirect estimate of the frequency of the MS strain. The data in Table 1, for example, which show 100% frequency of an MR strain in some plastic houses, means that equal percent infections were observed on treated and untreated trap plants as a result of 24 hr of exposure to fungal populations in plastic houses. Although indirect, this was the only method available to estimate frequency of MS strains due to the obligate nature of the fungus, which makes in vitro tests of fungal growth impossible. There is a remote possibility, however, that if other (more laborious) methods of recovery were attempted, for example, isolation of individual sporangia followed by characterization of each sporangial population, there would remain a small proportion of the metalaxyl-sensitive strains in houses 5–8.

The mechanism by which MR strains dominate subsequent to mixed strain inoculations in the absence of metalaxyl is not understood. Thurston (16), who experimented with mixed race inoculations of *Phytophthora infestans*, found that within two-to-nine generations race 0 predominated or entirely displaced the other races with which it was mixed due to its higher infectivity and antagonism to other races. Dovas et al (9), who conducted experimental comparisons between benomyl-sensitive and benomyl-resistant strains of *Cercospora beticola* found that the former strain had a lower fitness to sugar beets, and as a result the proportion of resistant conidia increased even in control fields not sprayed with benomyl. McGee and Zuck (12) compared the fitness of benomyl-resistant and sensitive populations of *Venturia inaequalis* by passing mixed (1:1) populations through several sporulation cycles on apple seedlings in the greenhouse. They found no change in the original 1:1 ratio after eight such cycles.

Wild and Eckert (17) showed that a benzimidazole-sensitive isolate of *Penicillium digitatum* increased the infectivity of a benzimidazole-resistant isolate when mixtures of the two were inoculated into oranges subsequently treated with benomyl. They suggested that this synergy resulted from pectinases secreted by germinating spores of the fungicide-sensitive isolate. Such a mechanism of synergy may explain our results presented in Table 5, in which infectivity of the original mixed strain inoculum to metalaxyl-treated plants was higher than the proportion of MR in the mixture. As metalaxyl inhibits sensitive phycomycetes at the stage of haustorial formation (11), it may well be that the presence of the sensitive strain in the mixture increases the infectivity of the resistant strain.

We propose the following hypothesis to explain dominance of MR over MS strains: in the presence of MS, MR gains higher infectivity; and hyphal growth and/or sporangial production of MS in infected leaves are suppressed in the presence of MR hyphae.

The results explain the occurrence, and sometimes the dominance, of resistant strains of *P. cubensis* in plastic houses in winter 1979–1980, and in fields during summer and autumn 1980, even in some locations not treated with the fungicide (Reuveni and Cohen, *unpublished*). We assume that resistant strains build up in treated plants in sufficient quantities to compete favorably with sensitive strains in the absence of the fungicide. The use of metalaxyl in cucurbits was abandoned in mid-1981, as a result of which the frequency of resistant strains gradually declined until, except for a very few locations, sensitive populations were usually isolated in late 1981 and during 1982. This recovery of the sensitive wild-type strains shows clearly that they are better adapted in nature (overwintering, etc.) than resistant strains, but the latter are highly competitive when they reach a certain proportion in the population.

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