Synergy Between Metalaxyl-Sensitive and Metalaxyl-Resistant Strains of *Pseudoperonospora cubensis*

Yair Samoucha and Yigal Cohen

Graduate student and professor, respectively, Department of Life Sciences, Bar-Ilan University, Ramat-Gan 52100, Israel.
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ABSTRACT

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Metalaxyl-sensitive (MS) strains of *Pseudoperonospora cubensis* increased the infectivity of metalaxyl-resistant (MR) strains when mixtures of the two were inoculated into cucumber plants treated with 5 mg a.i. metalaxyl per pot (0.4 kg of air-dried soil). The synergy factor ([percent infection with MR + MS]/[percent infection with MR + H_2O]) in various experiments ranged from 3 to 6. Synergy was also observed when inoculation with the sensitive strain preceded that of the resistant strain. This phenomenon may have enhanced the spread of resistant strains of the fungus in commercial plastic greenhouses in Israel from 1978 to 1980.

In a previous paper (2) we demonstrated that in mixed-strain inoculations of cucumber plants in the absence of metalaxyl, metalaxyl-resistant strains of *Pseudoperonospora cubensis*, the causal agent of downy mildew in cucurbits, exhibited a higher competitive capacity than metalaxyl-sensitive strains. In either artificial inoculations in growth chambers or in epidemics induced in walk-in plastic greenhouses, mixed-strain inoculations resulted in a full predominance of the resistant strains within two to three disease cycles. This capacity could not be attributed to differential fitness to cucumbers, as both resistant and sensitive strains exhibited similar pathogenicity when inoculated singly on plants in the absence of the fungicide. In mixed inoculations in growth chambers, predominance of the resistant strains occurred even when their proportion in the original inoculum mixture was as low as 5%. We then raised the hypothesis that in the presence of metalaxyl-sensitive strains in the inoculum mixture, metalaxyl-resistant strains exhibit higher infectivity, and/or that hyphal growth and sporangial production of sensitive strains in infected leaves are suppressed by hyphae of resistant strains.

The present study was conducted to test whether the pathogenicity of resistant strains to metalaxyl-treated cucumber plants is affected by the presence of sensitive strains in the inoculum mixture.

MATERIALS AND METHODS

Cucumber (*Cucumis sativus* L.) cultivar Elem, which is highly susceptible to downy mildew, was used in all of these experiments. Plants were grown in 10-cm-diameter plastic pots (0.4 kg of air-dried potting mixture made of sandy loam, peat, vermiculite [2:1:1, v/v]), 10 plants per pot, in the greenhouse (20–34 C) and used for inoculation about 10 days after sowing when cotyledonary leaves were 2–3 cm long, unless otherwise stated. The metalaxyl-sensitive strains MS1 and MS2 and the metalaxyl-resistant strains MR1 and MR2 of *P. cubensis* (Berk. et Curt.) Rost. collected in nature from 1978 to 1982 (2,5) were used for inoculations. Sensitive strains were controlled in plants treated with a soil drench of 0.25 mg a.i. metalaxyl per pot, whereas MR strains sporulated abundantly on plants drenched with 5 mg a.i. of metalaxyl per pot. The four strains were propagated on cucumber plants in separate growth chambers and strict measures were employed to avoid cross contamination.

Sporangial suspensions of MS and MR strains were prepared and kept in ice-cooled double-distilled water and calibrated to 5 ± 1 sporangia per 10-μl droplet. Droplets were produced using a Nichiryo syringe dispenser (model 8100; Nichiryo Co., Chiyoda-Ku, Tokyo). MR suspensions were mixed with either MS suspensions at ratios (MR:MS) of 1:1, 1:5, 1:10, 1:20, and 1:40 or with H_2O at ratios of 1:1, 1:5, 1:10, 1:20, and 1:40 by volume. Each inoculum suspension was inoculated into about 40 intact cucumber plants (80 cotyledons) drenched with metalaxyl (5 mg per pot) one day before inoculation. Inoculation was done by placing one 10-μl inoculum droplet on the upper surface of each leaf. Inoculated plants were kept in a dew chamber 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 ($\sim$150 μEinsteins$\cdot m^{-2} \cdot s^{-1}$). Percentage of infected cotyledons was visually determined at 7 days after inoculation. To induce fungal sporulation, infected plants were placed in a dew chamber (17 C, in darkness) for 20 hr. Plants having one true leaf were inoculated by spraying inoculum suspension on upper leaf surfaces and were treated further as described above.

RESULTS

The metalaxyl treatment prevented the infection of cucumbers inoculated with the metalaxyl-sensitive strains MS1 and MS2, but did not control infection in plants inoculated with the resistant strains MR1 and MR2 of *P. cubensis*. Percentage infection with MR strains decreased as the proportion of water increased (Table 1). At a dilution of 1:40, only 9% (maximum) of the inoculated cotyledons became infected. The addition of metalaxyl-sensitive sporangia to inoculum containing metalaxyl-resistant sporangia substantially increased the percent infection of metalaxyl-treated plants (Table 1). Thus, inoculation with a mixture consisting of 9% MR sporangia and 91% MS sporangia (mixing ratio 1:10, MR:MS) resulted in about 70% infection compared to about 30% infection with inoculum made of MR sporangia alone at the same concentration. The increased infection capacity of MR + MS sporangia relative to that of MR sporangia alone (synergy factor) ranged between 1.0 and 3.9 depending on the mixing ratio. The synergy factor was minimal (1.0) at a 1:1 ratio and maximal (3.9) at a 1:20 (MR:MS) mixing ratio.

Similar results (but with a maximal synergy factor of 6.2 at a 1:40 mixing ratio) were obtained when disease readings were taken in plants inoculated at the first true leaf stage (Table 2). The rate of decline in number of lesions with dilution of inoculum was much greater in plants inoculated with MR2 sporangia alone than in plants inoculated with MR2 mixed with MS2 sporangia.

Sequential inoculation experiments showed that a primary inoculation of metalaxyl-treated plants with a sensitive strain increased the infectivity (or zoospore release on leaf surface) of a resistant strain subsequently inoculated at 1–24 hr. In the experiment presented in Table 3, treated plants were first

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TABLE 1. Infection of metalaxyl-treated cucumber plants (at the cotyledonary leaf stage) by inoculation with metalaxyl-resistant (MR) sporangia of *Pseudoperonospora cubensis* alone or mixed with metalaxyl-sensitive (MS) sporangia[a]

Mixing ratio by volume (MR:H_2O or MR:MS)	Cotyledons infected (% ± S.D.) and synergy factor[b]								
	Experiment 1			Experiment 2			Experiment 3		
	MR1 + H_2O	MR1 + MS1	Synergy factor	MR2 + H_2O	MR2 + MS2	Synergy factor	MR2 + H_2O	MR2 + MS1	Synergy factor
1:1	100	100	1.0	100	100	1.0	100	100	1.0
1:5	43.7 ± 9	100	2.3	45.0 ± 8	96.5 ± 7	2.1	43.3 ± 8	95.4 ± 2	2.2
1:10	28.7 ± 11	71.4 ± 6	2.5	32.0 ± 7	69.3 ± 11	2.2	29.3 ± 2	67.6 ± 9	2.3
1:20	14.4 ± 5	56.2 ± 3[c]	3.9	22.0 ± 3	76.7 ± 5[c]	3.5	15.9 ± 4	52.7 ± 4[c]	3.3
1:40	7.5 ± 9	14.9 ± 7	2.0	9.0 ± 9	13.2 ± 6	1.4	8.5 ± 9	13.6 ± 4	1.6

[a] Soil drench of 5 mg a.i. of metalaxyl per 10-cm-diameter pot, 10 plants per pot, four pots per treatment.
[b] Synergy factor was calculated by using the formula: ([cotyledons (%) infected with MR + MS]/[cotyledons (%) infected with MR + H_2O]). MS sporangia alone (five per cotyledon) failed to produce infection on metalaxyl-treated plants.
[c] Sporangia produced on these plants in a moist atmosphere were found to be metalaxyl-resistant.

TABLE 2. Infection of metalaxyl-treated cucumber plants[a] at the first true leaf stage by inoculation with metalaxyl-resistant (MR) sporangia of *Pseudoperonospora cubensis* alone or mixed with metalaxyl-sensitive (MS) sporangia

Mixing ratio by volume (MR:H_2O or MR:MS)[b]	Number of lesions on the first true leaf ± S.D.		
	MR2 + H_2O	MR2 + MS2	Synergy factor[c]
1:1	26.4 ± 2	28.3 ± 3	1.1
1:5	16.0 ± 2	23.2 ± 1	1.4
1:10	9.8 ± 2	20.1 ± 4	2.0
1:20	5.0 ± 2	18.0 ± 3	3.6
1:40	2.4 ± 1	14.9 ± 2[d]	6.2

[a] Two one-true-leaf plants per 10-cm-diameter pot treated with a soil drench of 5 mg a.i. metalaxyl per pot. Ten pots per treatment.
[b] MR2 sporangial suspension (500 sporangia per milliliter) was either mixed with H_2O at ratios indicated or with MS2 sporangial suspension (500 sporangia per milliliter) at similar ratios. MS2 sporangia alone failed to produce any infection in plants treated with metalaxyl.
[c] See formula in Table 1.
[d] Sporangia collected upon sporulation were metalaxyl-resistant.

TABLE 3. The effect of prior inoculation with a metalaxyl-sensitive strain on the infectivity of a metalaxyl-resistant strain of *Pseudoperonospora cubensis* in metalaxyl-treated cucumber plants[a]

Interval between inoculations (hr)	Cotyledons infected with MR2 strain[b] (% ± S.D.)		
	Primary inoculation with water	Primary inoculation with MS2 strain	Synergy factor[c]
0	13.4 ± 2.4	48.5 ± 4.3	3.6
1	14.3 ± 5.2	53.4 ± 1.9	3.7
2	14.7 ± 3.9	56.7 ± 3.9	3.9
4	15.9 ± 2.5	66.8 ± 4.7	4.2
6	14.9 ± 3.2	60.5 ± 5.1	4.1
12	13.1 ± 6.9	51.0 ± 3.9	3.9
24	12.9 ± 3.5	41.3 ± 7.2	3.2

[a] See footnote *a* in Table 1.
[b] Primary inoculation with MS2 strain was done with 5 ± 1 sporangia per droplet per cotyledon, and the second inoculation with the MR2 strain was done with 0.25 ± 0.2 sporangia per droplet per cotyledon. MS2 sporangia alone produced no infection in treated plants.
[c] See footnote *b* in Table 1.

inoculated with the MS2 strain (5 ± 1 sporangia per cotyledon) or water and thereafter, at various intervals after the first inoculation, were inoculated with the MR2 strain (0.25 ± 0.2 sporangia per cotyledon). Percentage of cotyledons infected was 3.2–4.2 times higher in plants first inoculated with the MS2 strain than in those first inoculated with water.

When fungicide-treated plants were first inoculated with a resistant strain (0.25 ± 0.2 sporangia per cotyledon) and thereafter with a sensitive strain (five sporangia per cotyledon), the synergy factor reached a level of 4.3, 2.7, and 1.6 for plants inoculated with MS2 at 0, 1, and 2 hr after inoculation with MR2, respectively, as compared to plants first inoculated with water. Lengthening the interval period between inoculations resulted in equal infectivity (synergy factor 1.0) of MR2 + MS2 and MR2 + H_2O.

DISCUSSION

Results presented in this paper indicate that sporangia of *P. cubensis* resistant to metalaxyl gain a higher infection capacity in metalaxyl-treated cucumbers when mixed with sporangia sensitive to metalaxyl, compared to when mixed with water alone. This implies that sensitive sporangia, by themselves noninfective to fungicide-treated plants, sensitize the leaf tissue to resistant sporangia. The magnitude of this synergy usually reached a level of 3–4 and sometimes a level of 6. Sensitive sporangia were found to sensitize the host not only when mixed in inoculum suspension with resistant sporangia, but also when inoculated singly at 1–24 hr prior to inoculation with the resistant sporangia. Synergy was best expressed in mixed strain inoculation at a ratio of 1:20 (resistant:sensitive) in the inoculum suspension for cotyledons, and 1:40 for true leaves. Several synergistic interactions in pathogenesis by fungi have been described (7, and the literature cited therein). Wild and Eckert (7) stated that synergy is most apparent when one of the interacting microorganisms is inhibited by some environmental factor, yet retains the ability to stimulate the pathogenic activity of the associated microorganism. In our system, the MS sporangia are inhibited by metalaxyl and yet stimulate, in an unknown manner, the infectivity of resistant sporangia to fungicide-treated plants.

Metalaxyl inhibits nucleic acid synthesis of metalaxyl-sensitive strains of oomycetes (3) and strongly inhibits mycelial growth (6) in vitro, but has a slight effect on zoospore release and cyst germination (1,6). We assume, therefore, that in the mixed strain inoculation of metalaxyl-treated cucumbers with *P. cubensis*, initial establishment of both strains occurs equally well (4), but further fungal growth occurs in the resistant strain only. With no experimental evidence we can only speculate on how MS strains stimulate the infectivity of an MR strain. One possibility is that MS sporangia stimulate release of zoospores from MR sporangia on the leaf surface. Another possibility is that enzymes secreted by the MS germlings make penetration of MR germlings into mesophyll cells more efficient. Such a case was demonstrated by Wild and Eckert (7) in mixed strain inoculations of benomyl-treated oranges with benomyl-sensitive and -resistant strains in which pectinases secreted by the sensitive germlings increased infectivity of the resistant propagules. This possibility could explain the enhanced infectivity we obtained when MR inoculation followed that of MS inoculation and the lack of such enhancement when inoculations were conducted in the reverse sequence.

The phenomenon of synergy we report here may have enhanced the spread of metalaxyl-resistant strains of *P. cubensis* in commercial plastic greenhouses in Israel in the period from 1978 to 1980 (5).

LITERATURE CITED

1. Cohen, Y., Reuveni, M., and Eyal, H. 1979. The systemic antifungal activity of Ridomil against *Phytophthora infestans* on tomato plants. Phytopathology 69:645-649.
2. Cohen, Y., Reuveni, M., and Samoucha, Y. 1983. Competition between metalaxyl-resistant and -sensitive strains of *Pseudoperonospora cubensis* on cucumber plants. Phytopathology 73:1516-1520.
3. Fisher, D. J., and Hayes, A. L. 1982. Mode of action of the systemic fungicides furalaxyl, metalaxyl and ofurace. Pestic. Sci. 3:330-339.
4. Hickey, E. L., and Coffey, M. D. 1980. The effects of Ridomil on *Peronospora pisi* parasitizing *Pisum sativum*: An ultrastructural investigation. Physiol. Plant Pathol. 17:199-204.
5. Reuveni, M., Eyal, H., and Cohen, Y. 1980. Development of resistance to metalaxyl in *Pseudoperonospora cubensis*. Plant Dis. 64:1108-1109.
6. Staub, T., Dahmen, H., and Schwinn, F. J. 1980. Effects of Ridomil on the development of *Plasmopara viticola* and *Phytophthora infestans* on their host plants. Z. Pflanzenkr. Pflanzenschutz 87:83-91.
7. Wild, B. L., and Eckert, J. W. 1982. Synergy between a benzimidazole-sensitive isolate and benzimidazole-resistant isolates of *Penicillium digitatum*. Phytopathology 72:1329-1332.