Techniques

Estimation of Metalaxyl Resistance in *Phytophthora infestans*

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ABSTRACT

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A method for detecting low frequencies of resistance to metalaxyl was developed for *Phytophthora infestans*. Potato tuber disks were used because they were more susceptible to the blight fungus than leaf disks or intact plants. When resistance of 5–95% was anticipated in a mixed sporangial population, tuber disks (10 × 3 mm) were placed in petri dishes on either water or 100 mg/L of metalaxyl and inoculated with 3 to 6 sporangia per disk. When 0.01–1% resistance was anticipated, disks were placed on 100 mg/L of metalaxyl and inoculated with 50–800 sporangia per disk. The number of disks on metalaxyl supporting fungal sporulation at 1 wk after inoculation was used to compute resistant frequency. The bioassay is simple, accurate, and may be used as a tool to assess aspects of selection pressure imposed on the pathogen by phenylamide fungicides in the field.

Additional keywords: control strategies, late blight, oomycetes.

Shortly after metalaxyl, a phenylamide fungicide, was introduced for the control of late blight, fungicide resistance appeared on a widespread scale (1). Resistance was usually confirmed when breakdown of control had occurred. The lack of quantitative methods for estimating fungal resistance to phenylamide fungicides caused difficulties not only in clarifying suspected cases of resistance but also in designing and validating antiresistance strategies (11,12). Sozzi and Staub (11) recently have reported a semiquantitative method for monitoring sensitivity of *Phytophthora infestans* (Mont.) de Bary to metalaxyl. However, they concluded that "detection of 0.1% resistant sporangia in a mixed population of *P. infestans*, as it occurs in an early phase of resistance development or in crops treated with mixtures of phenylamides with residual compound, was not possible" (11).

In this paper we report on a method to monitor sensitivity of *P. infestans* to metalaxyl with which the occurrence of 0.01–100% resistant sporangia in a sporangial population can be accurately confirmed.

METHODS AND MATERIALS

Plant material. Tests were conducted with potato (*Solanum tuberosum* L. 'Alpha') tuber disks from tubers of about 100 g that

had been stored at 10 C in the dark for as long as 9 mo. Disks (10 mm diameter, 3 mm thick) were cut from tubers with the aid of an electric slicer and a cork borer. Disks were immediately washed with running tap water, blotted dry, and used for inoculation at 1 hr after being cut from old tubers or 3 hr after being cut from young tubers. Some tests were done with potato leaf disks (10 mm diameter) or with 8-wk-old potted plants.

Fungal isolates. Three metalaxyl-sensitive (MS) and three metalaxyl-resistant (MR) field isolates of *P. infestans* were used. MS1, MS2, and MS3 were collected from potato in blighted fields at Sufa (1986), Bet-Kama (1983), and Nir-Eliyahu (1984), Israel, respectively. MR1, MR2, and MR3 were collected at Gevouloth (1984), Bror-Hayil (1985), and Mishmereth (1986), Israel, respectively. Isolates were kept on detached potato leaflets or tuber slices (3 cm diameter, 5 mm thick) on water-saturated filter paper in petri dishes at 16 C. Isolates were periodically tested for sensitivity to metalaxyl by inoculating potato tuber disks lying on filter paper saturated with concentrations of metalaxyl (Ridomil 25 WP, Ciba-Geigy, Basel, Switzerland) ranging from 0.1 to 1000 mg active ingredient per liter. Details on the sensitivity to metalaxyl of the six isolates are given in the Results section (Table 1).

Inoculum preparation. Tuber disks (3 cm) were inoculated with a sporangial suspension of *P. infestans* and incubated in petri dishes at 16 C in the dark. After 7 days, freshly formed sporangia were collected from the tuber disk surface by gently shaking the disk in cold (4 C) tap water. The sporangial concentration was adjusted to 2×10^4 (5–95% resistant sporangia) or to 8×10^4 sporangia per milliliter (0.01–1% resistant sporangia) based on the mean of 10 counts in a hemacytometer. Mixed-isolate inocula were prepared by thoroughly mixing isolate MS1 with MR1, MS2 with MR2, and MS3 with MR3 by volume to produce suspensions containing 0.01, 0.1, 1, or 5–95% (5% stepwise) resistant sporangia. Sporangial suspensions were kept in an ice bath until used.

Inoculation. Inoculum droplets (10 μl each) were produced with the aid of a Nichiryo 8100 syringe dispenser (Nichiryo Co. Ltd., Chiyoda-Ku, Tokyo, Japan). Inoculum suspensions that were adjusted with the aid of a hemacytometer to 20,000 and then diluted to 10,000, 5,000, 2,500, 1,250, 625, 312, and 156 sporangia per milliliter were found to contain (two experiments with 10 droplets counted in each) 105 ± 10, 48 ± 6, 26 ± 4, 12 ± 2, 7 ± 1, 3.2 ± 0.97, and 1.95 ± 0.87 sporangia per 10-μl droplet, respectively. Unless stated otherwise, 20 tuber disks or 20 leaf disks were placed in a 9-cm-diameter glass petri dish on a 7-cm-diameter filter paper (Whatman No. 1) with 3 or 1.5 ml of water, respectively. A 10-μl inoculum droplet was placed on the surface of each disk. Dishes were incubated at 20 C in the dark. One week after inoculation, the number of tuber disks supporting fungal sporulation was determined with the aid of a stereomicroscope. Intact plants were inoculated by placing inoculum droplets (one per leaflet) on 30 tagged leaflets per plant (three plants per treatment). Plants were incubated in a moisture-saturated atmosphere at 18 C in the dark for 20 hr and then transferred to a growth chamber at 20 C (12 hr light per day, 180 μE $m^{-2} \cdot s^{-1}$). A week later the number of leaflets with lesions was counted.

Resistance frequency in mixed sporangial populations. For assaying proportion of sporangial droplets in the range of 5–95% resistant sporangia, 20 tuber disks were placed on water and 20 disks on 100 mg of metalaxyl per liter. Disks were inoculated with a mean of 3.12–6.25 sporangia per disk depending on the pair of isolates mixed. The number of disks supporting fungal sporulation was counted 1 wk later.

The percentage of resistant sporangia in the population was computed by dividing the number of tuber disks supporting fungal sporulation on metalaxyl by the number of tuber disks supporting sporulation on water and multiplying the result by 100.

For assaying proportion of sporangial droplets in the range of 0.01–1% resistant sporangia, 40 tuber disks (20 per dish) were placed on 100 mg of metalaxyl per liter and inoculated with 50, 100, 200, 400, or 800 sporangia per disk. The percentage of disks supporting fungal sporulation (P) was determined at 1 wk after inoculation and was used to compute the percentage resistant according to the following formula: $\%R = P\ (IE/S)$ where P = percentage disks supporting fungal sporulation, IE = number of sporangia of the respective resistant isolate required to produce infection (with sporulation) in 90% of the disks inoculated (see below), and S = number of sporangia inoculated per disk. The fraction IE/S is a correction factor that compensates for the increased number of sporangia used in the assay when the suspected range of resistant sporangia is 0.01–1%.

Data analysis. Each experiment was repeated three or four times. Data from infection efficacy experiments (Fig. 1) were analyzed for their fitness to the probit model using the PROBIT procedure (8). This procedure provided IE90 values (infection

TABLE 1. Dosage-response data for the pathogenicity of three metalaxyl-sensitive (MS) and three metalaxyl resistant (MR) field isolates of *Phytophthora infestans* to potato tuber disks in the presence of metalaxyl

Metalaxyl (mg a.i./L)	Tuber disks with fungal sporulation (% ± S.D.)[a]					
	MS1	MS2	MS3	MR1	MR2	MR3
0	100	100	100	100	100	100
0.01	58±8	68±8	70±5	100	100	100
0.1	13±3	5±0	3±3	100	100	100
1	0	0	0	100	100	100
10	0	0	0	100	100	100
100	0	0	0	98±1	100	100
1000	0	0	0	10±0	15±0	15±0

[a] One-centimeter tuber disks (3-mm thick) were placed in petri dishes (20 per dish, three dishes per isolate per concentration) on metalaxyl-saturated (3 ml) filter paper (No. 1, 7-cm diameter) and inoculated with 25 sporangia each. Dishes were incubated at 20 C in the dark. At 7 days after inoculation, the proportion of the 60 disks per isolate on which fungal sporulation was visible was recorded.

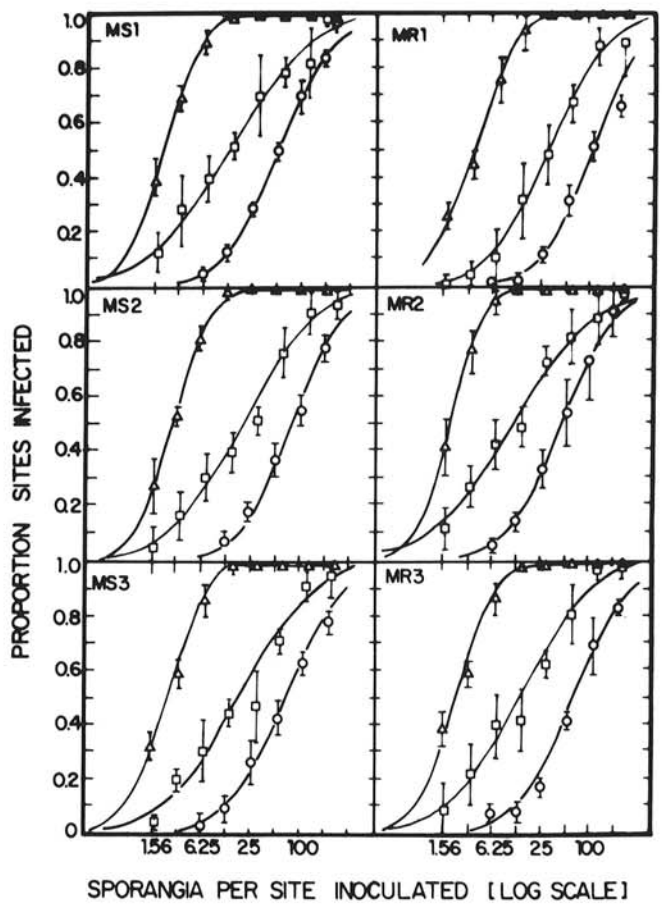

Fig. 1. Density-dependent infectivity of sporangia of six isolates of *Phytophthora infestans* to tuber disks (Δ), leaf disks (□), and whole plants (o) of potato (cv. Alpha) in growth chambers at 20 C. Each value represents an average of three experiments ± standard deviation.

efficiency = 0.90) that represent the number of sporangia required to support sporulation in 90% of the sites inoculated. The parameters of the probit line (intercept and slope) were used to compute the probability curves presented in Figure 1. The relationship between resistance frequency observed (y) and resistance frequency expected (x) was analyzed by the NOINT linear regression option (8) for the equation $y = x$ (Fig. 2). Chi-square tests were done to determine the probability of agreement between the observed frequency of resistance in a test and the frequency of resistance expected in that test (see Results).

RESULTS

Sensitivity of isolates to metalaxyl. MS isolates were completely controlled in inoculated tuber disks lying on filter paper containing 1 mg/L of metalaxyl, whereas MR isolates were still sporulating at 1,000 mg/L of the fungicide (Table 1). After 50 passages on fungicide-free tuber disks at 20 C, no change in sensitivity to metalaxyl was noticed in either MS or MR isolates.

Infection efficiency. The frequency of successful infections for each isolate depended on inoculum concentration and tissue inoculated (Fig. 1). Pathogenicity of the fungus was lowest to intact plants and highest to tuber disks. In intact plants, at least a mean of 6.25–25 sporangia per droplet was required to produce a lesion as compared to a mean of 1.56 sporangia (the lowest concentration tested) required to produce a lesion in tuber disks (Fig. 1). The number of sporangia required to produce sporulating lesions in 90% of the inoculated sites (IE90) is given in Table 2. Mean IE90 for all isolates was significantly lower ($P < 0.0001$) in tuber disks compared with leaf disks and in leaf disks compared with intact plants. Mean IE90 for MS isolates was not significantly different ($P = 0.05$) from that of MR isolates in any of the tissues inoculated.

Additional tests (data not presented) revealed that infection efficiency of the MR isolates on tuber disks in the presence of 100 mg/L of metalaxyl was not significantly different from that obtained on water (Fig. 1 and Table 2).

Isolates showed similar pathogenicity to tubers stored 0–9 mo after harvest prior to inoculation.

Detecting resistance in mixed populations. Tuber disks were used for detecting resistance in mixed sporangial populations because they were the most susceptible tissue to our isolates of *P. infestans*. The relationships between the percentage of metalaxyl-resistant sporangia in the inoculum mixture (expected frequency) and the percentage of resistance observed in the tuber disk bioassay are presented in Figure 2. In all three mixed-isolate tests, a very high correlation to the $y = x$ model was found between these two percentages. Inoculation tests conducted with half or two times the sporangial concentrations mentioned above resulted in a poor fit to the above model.

A much more concentrated inoculum mixture was required for detecting resistance frequencies of 0.01–1%. Resistance of 0.01% in a sporangial population was detected when at least 400–800 sporangia (depending on isolates mixed) were applied to each tuber disk (Table 3). Higher concentrations were not used due to bacterial contamination. Resistance of 0.1% was detected with at least 100–200 sporangia per disk, and resistance of 1% was detected with at least 50–100 sporangia per disk (Table 3). A chi-square test showed that the assay could accurately predict the frequency of resistance in the range of 0.01–1% (Table 4). The probability level associated with the chi-square statistic used to test agreement between computed and expected percentage of metalaxyl resistance was above 0.9 in 13 out of 27 cases. In only two cases, this probability of agreement was 0.5–0.7 (Table 4).

DISCUSSION

In epidemiological studies of the dynamics of fungal populations, it is crucial that the early stages of the epidemic be monitored. A variety of methods have been used to monitor

TABLE 2. Infection efficiency (IE90) of six isolates of *Phytophthora infestans* to tuber disks, leaf disks, and intact plants of potato at 20

Isolate[b]	Number of sporangia required to support sporulation in 90% of the sites inoculated (IE90)[a]		
	Tuber disks	Leaf disks	Whole plants
MS1	6	116	262
MS2	8	128	355
MS3	8	142	374
MR1	10	146	444
MR2	5	106	214
MR3	7	102	315

[a] Tissue was inoculated with a single 10-μl inoculum droplet containing a mean of 1.56, 3.12, 12.5, 25, 50, 100, or 200 sporangia. Proportions of sites supporting sporulation were recorded at 1 wk after inoculation (Fig. 1). IE90 values were calculated after probit transformation of the data in Figure 1 with the aid of PROBIT (8). Goodness-of-fit chi-square statistics ranged between 0.82 and 0.99.

[b] MS = metalaxyl-sensitive isolate; MR = metalaxyl-resistant isolate.

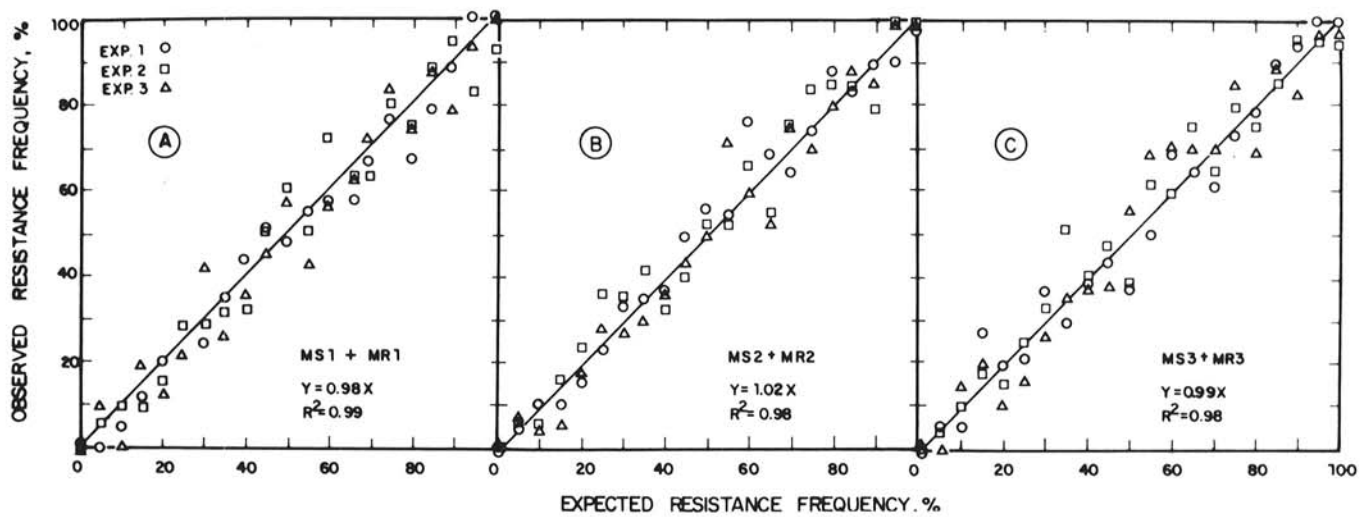

Fig. 2. The relationships between the percentage of metalaxyl-resistant sporangia of *Phytophthora infestans* in three inoculum mixtures (expected, x-axis) and the percentage of potato tuber disks supporting sporulation on 100 mg of metalaxyl per liter (observed, y-axis). Inoculum mixtures of metalaxyl-sensitive (MS) isolates and metalaxyl-resistant (MR) isolates were used. Experiments in **A**, with isolates MS1 and MR1, **B**, with isolates MS2 and MR2, and **C**, with isolates MS3 and MR3, were conducted three times each with a of mean 6.25, 3.125, and 6.25 sporangia per disk, respectively. These concentrations provided the best adjusted R^2 values to the $y = x$ model. The F statistic for the regressions and their statistical significance were: **A**, $F = 3,593$, $P = 0.0001$; **B**, $F = 4,827$, $P = 0.0001$; and **C**, $F = 4,963$, $P = 0.0001$, respectively.

TABLE 3. Percentage of potato (cv. Alpha) tuber disks supporting sporulation of *Phytophthora infestans* on 100 mg of metalaxyl/ L 1 wk after inoculation with a sporangial suspension containing 0.01–1% metalaxyl-resistant (MR) sporangia

Isolates mixed	MR (%)	Tuber disks infected (% ± S.D.)[a] sporangia per tuber disk inoculated				
		50	100	200	400	800
MS1 + MR1	0.01	0	0	0	0	1.3 ± 2.5
	0.1	0	0	1.9 ± 1.2	5.6 ± 1.3	6.9 ± 1.3
	1	0	8.1 ± 2.4	20.6 ± 3.8	48.1 ± 5.2	56.3 ± 8.5
MS2 + MR2	0.01	0	0	0	2.5 ± 2.0	3.1 ± 3.8
	0.1	0	1.9 ± 1.3	6.3 ± 3.2	7.5 ± 2.0	13.1 ± 2.4
	1	4.4 ± 3.1	14.4 ± 2.4	28.1 ± 4.3	65.6 ± 8.8	78.8 ± 2.5
MS3 + MR3	0.01	0	0	0	0	1.3 ± 1.4
	0.1	0	0	2.5 ± 2.0	6.3 ± 3.2	8.8 ± 3.2
	1	0	12.5 ± 2.0	26.9 ± 2.4	66.3 ± 3.2	77.5 ± 7.0

[a]Sporangia of the respective metalaxyl-sensitive (MS) and metalaxyl-resistant (MR) isolates were mixed to contain 0.01, 0.1, or 1% MR. Sporangia concentration was calibrated with the aid of a hemacytometer (10 counts) to 8×10^4 sporangia/ml and then diluted 2-, 4-, 8-, and 16-fold with cold tap water. Forty potato tuber disks were placed on metalaxyl in two petri dishes (20 per dish) and inoculated with each sporangial suspension. Each value represents an average ± standard deviation from four separate experiments.

TABLE 4. Computed resistance percentages according to *P, IE,* and *S* in a tuber disk assay with metalaxyl-sensitive (MS) and metalaxyl-resistant (MR) isolates of *Phytophthora infestans*[a]

Isolates mixed	%*R* expected	*IE*	%*R* computed from *P*, with *S* of				
			50	100	200	400	800
MS1 + MR1	0.01	10	0	0	0	0	0.016****
	0.1		0	0	0.095****	0.14****	0.086****
	1		0	0.81***	1.030****	1.202***	0.703**
MS2 + MR2	0.01	5	0	0	0	0.031**	0.019****
	0.1		0	0.095****	0.157***	0.093****	0.082***
	1		0.440*	0.720**	0.703**	0.820**	0.493*
MS3 + MR3	0.01	7	0	0	0	0	0.011****
	0.1		0	0	0.088****	0.110****	0.077***
	1		0	0.875****	0.942****	1.160***	0.678**

[a] *P* is percentage of tuber disks infected (with sporulation) (data taken from Table 3); *S* is number of sporangia (MS + MR) applied to each tuber disk; *IE* is infection efficiency as determined by number of MR sporangia required to produce infection (with sporulation) in 90% of the tuber disks inoculated. Values taken from Figure 1 (see also Table 1). %*R* computed with the formula: $\%R = P(IE/S)$. Asterisks indicate the probability level associated with the chi-square statistic used to test agreement between %*R* computed and %*R* expected (chi-square test): *0.5–0.7; **0.7–0.8; ***0.8–0.9; ****0.9–0.99.

populations of fungicide-resistance foliar pathogens; most of them involve excising sporulating lesions from leaf tissue and streaking the lesions across fungicide-amended and unamended agar to dislodge spores. Fungicide resistance is determined according to spore germination or growth of germ tubes (7).

Phenylamide fungicides have little effect on spore germination (direct or indirect) of oomycetes (1) including *P. infestans* (5), thus making the agar-amended methods unsuitable for monitoring purposes. Techniques that involved inoculation of host tissue were therefore developed. Reuveni et al (9) and Cohen et al (6) exposed metalaxyl-treated and untreated potted cucumber plants to naturally dislodging sporangia of *Pseudoperonospora cubensis* in experimental or commercial plastic houses and estimated percentage resistant sporangia according to the ratio between disease severities (9) or number of lesions (6) developed. Samoucha and Gisi (10) harvested sporangia of *P. infestans* and *Plasmopara viticola* and inoculated them onto oxadixyl-treated and untreated potato or grape plants, respectively, and estimated percentage resistance as 100 times the ratio between disease severity developed on treated and untreated plants. These methods are not accurate enough, nor can they detect resistance of low frequencies.

An improved method was recently reported by Sozzi and Staub (11) to monitor sensitivity of *P. infestans* to metalaxyl. With their method, metalaxyl-treated and untreated potato leaf disks (15 mm) were each inoculated with a single inoculum droplet containing 250 sporangia, and percentage sporulating disks a week later served as percentage resistant sporangia in the original inoculum. With that method (reference 11, Fig. 3), percentage disks sporulating on 100 ppm active ingredient metalaxyl were 0, 0, 40, 90, and 100 for inoculum mixtures containing 0, 0.1, 1, 10, and 100% resistant sporangia, thus indicating a low correlation between expected and observed percentages of resistant sporangia.

This paper reports on a bioassay for the accurate detection of metalaxyl resistance in mixed sporangial populations of Israeli field isolates of *P. infestans*. With the aid of this bioassay, a frequency of resistant sporangia of as low as 0.01% could be detected. The bioassay is simple, inexpensive, requires no green leaf tissue of potato, and young as well as old tubers can be used. To save labor we have chosen to use only 40 potato tuber disks in each test. Increasing the number of disks per test will probably further increase the reliability of the results. The bioassay can easily be adapted to detect lower levels of metalaxyl resistance by simply reducing the concentration of metalaxyl in the plates. The accuracy of the present method may be attributed to the higher infection efficiency of *P. infestans* to potato tuber disks relative to potato leaf disks or whole plants. The infection efficiency of the MR isolates was therefore a parameter required for obtaining accurate results on resistance frequency in fungal populations.

The bioassay developed in this study was used by Cohen and Samoucha (2,3) to monitor phenylamide sensitivity in potato crops treated with various oxadixyl-containing fungicides. The bioassay has proven to be a be useful tool to assess aspects of the development of resistance in the field (2–4).

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