Infectivity to Potato, Sporangial Germination, and Respiration of Isolates of *Phytophthora infestans* from Metalaxyl-Sensitive and Metalaxyl-Resistant Populations

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ABSTRACT

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Twenty field isolates of *Phytophthora infestans* from different locations in Israel were compared for infectivity on potato plants (cv. Alpha) in growth chambers. Lesions developed on significantly larger proportions of inoculated leaflets on plants inoculated with metalaxyl-resistant (MR) isolates and maintained for 3, 6, or 9 hr in a moisture-saturated atmosphere than on plants similarly inoculated with metalaxyl-sensitive (MS) isolates. No significant difference in infection frequency was recorded between MR and MS populations in plants kept for 20 hr in a moisture-saturated atmosphere. Germination tests in distilled water at 16 C revealed a significantly faster rate of zoospore liberation from MR than from MS sporangia at 60–150 min, but not at 240 min. Sporangia of the MR isolates consumed more than three times as much oxygen during zoosporogenesis as sporangia of the MS isolates. We propose that the greater infectivity of MR sporangia compared with MS sporangia results from faster indirect germination.

Additional keywords: fitness, oomycetes, phenylamide fungicides.

Phenylamides (such as metalaxyl) are highly efficient systemic fungicides active against plant pathogens belonging to the Peronosporales (5). Field isolates resistant to these compounds have appeared in several oomycetous species as a result of selection pressure (5). In some species, such as *Pseudoperonospora cubensis* and *Phytophthora infestans*, the resistant isolates exhibited greater fitness on their hosts than the sensitive isolates (5,8,13,15). The greater fitness of the metalaxyl-resistant (MR) population of *P. infestans* in Israel compared with the metalaxyl-sensitive (MS) population was expressed in the significantly larger lesions induced in potato foliage (13).

In this study, we examined the infectivity to potato foliage of sporangia from the two populations of *P. infestans* that occur

in Israel in an attempt to clarify the reasons for the predominance of MR isolates in the country (4) despite the withdrawal of metalaxyl-containing fungicides for late blight control by potato growers.

MATERIALS AND METHODS

Plant material. All experiments were conducted with the potato (*Solanum tuberosum* L.) cultivar Alpha. Plants were grown in the greenhouse (19–29 C) from certified potato tubers in 1.5-L pots containing sandy loam, one tuber (about 50 g per tuber) per pot, and were fertilized twice a week with 1% N:P:K (20:20:20) solution. Plants were used for inoculation tests 6–7 wk after planting, when they had three or four shoots, each with nine or 10 compound leaves.

Fungal isolates. Ten MS and 10 MR isolates of *P. infestans* were collected from blighted potato fields. Details on the location, cultivar, and date of collection of isolates are given elsewhere (13,14).

Isolates were maintained on tuber slices (30 mm in diameter, 5 mm thick) kept on water-saturated filter paper in petri dishes at 16 C. The MR isolates were periodically tested for resistance to metalaxyl by inoculating them onto potato tuber disks lying on a filter paper saturated with a solution of metalaxyl (25 WP, 0.1–1,000 mg a.i./L). The MS isolates were controlled in tuber disks treated with 0.1 μg of metalaxyl per milliliter, whereas the MR isolates were still sporulating in disks treated with 1,000 μg/ml. Details on the sensitivity of the various isolates to metalaxyl are given elsewhere (13).

Inoculum preparation. Freshly produced sporangia were harvested into distilled water from potato tuber disks (30 mm in diameter, 5 mm thick) that had been inoculated with *P. infestans* 6 days before harvesting. Concentration was adjusted to $1–5 \times 10^4$ sporangia/ml, according to the experiment (see below), with the aid of a hemacytometer. Sporangia were used for inoculum increase on potato tuber slices, infection frequency tests with whole potato plants, germination tests, and respiration experiments.

Agar disks (5 mm in diameter) containing the fungus, which had been grown on rye seed agar (3) at 20 C in the dark, were used to initiate fungal colonies on rye seed broth medium (3). Fungal colonies were used for respiration experiments.

Biological variables. The following biological variables were measured in MS and MR isolates: infection frequency (proportion of inoculated leaflets that became infected), indirect germination (number of zoospores and proportion of empty sporangia produced in distilled water at 16 C), direct germination (proportion of sporangia producing germ tubes on water agar at 22–23 C), sporangial respiration (rate of oxygen consumption by sporangia maintained in water at 19 C), and mycelial respiration (rate of oxygen consumption by 7-day-old mycelial mats in water at 19 C).

Assessment of infection frequency. Three experiments were performed, with three plants inoculated per treatment (200 sporangia per site, one site per leaflet, 30 leaflets [mostly subterminal] per isolate). Experiments were conducted as described previously (13).

The inoculated plants were maintained in a dew chamber at 18–19 C in darkness for 3, 6, 9, or 20 hr to allow for infection by the pathogen and were then transferred to a growth chamber maintained at 20 C (50–60% RH, 12 hr light/day, 120 $\mu E \cdot m^{-2} \cdot sec^{-1}$) for symptom expression. Infection frequency was determined 6 days after inoculation.

Assessment of direct germination. Droplets (10 μl) of sporangial suspensions containing a mean of 150 sporangia each were placed on 1.5% water agar disks (1 cm in diameter, 5 mm thick) in closed petri dishes. Dishes were incubated at 22–23 C in darkness. The proportion of sporangia producing germ tube(s) was microscopically observed at 0, 2, 4, 8, and 12 hr in four disks (100 sporangia counted in each). Experiments were conducted twice, with 10 MS and 10 MR isolates in each experiment.

Assessment of indirect germination. Sporangial suspensions (2 ml each, 5×10^4 sporangia/ml) were placed in 30-ml beakers and incubated at 16 C in the dark. At 0, 30, 60, 90, 150, and 240 min, one beaker was withdrawn and 20 μl of 1% acid fuchsin in lactophenol was added. The proportion of empty sporangia and the number of zoospores in the suspensions were counted with the aid of a hemacytometer (four counts per beaker). The experiment was conducted three times, with the same 10 MS and 10 MR isolates in each experiment.

Oxygen consumption. Oxygen consumption was measured polarographically using a YSI oxygen monitor (model 53, Yellow Springs Instrument Co., Yellow Springs, OH) equipped with a Clark-type electrode (model YSO 5331). Sporangial suspensions (3 ml each, 2.5×10^4 sporangia/ml) were placed in a closed chamber at 19 C, and oxygen concentration was measured under continuous stirring. The oxygen concentration in the chamber decreased for at least 15 min. Oxygen consumption is given in micromoles of oxygen per sporangium per minute.

Similar tests were conducted with mycelial mats. The fungus was grown in liquid rye seed medium (3) in 35-mm petri dishes at 20 C in the dark. Seven days after the plates were inoculated, fungal colonies were withdrawn, washed twice with double-distilled water, blotted dry on a sterile filter paper, weighed (about 0.2 g), and placed in a chamber with 3 ml of double-distilled water, and oxygen consumption was measured for 15 min at 19 C. Both types of experiments were repeated three times with the same 10 MS and 10 MR isolates.

Sporangial weight. Sporangial suspensions (10 ml each) containing 3×10^5 sporangia were filtered through a Millipore membrane (22 mm in diameter, 8 μm pore size) whose weight had been previously determined. Sporangia were washed three times with 10 ml of distilled water. Membranes were dried at 50 C for 12 hr and weighed. Sporangial dry weight was calculated by dividing the net weight of the sporangia on a membrane by 3×10^5.

TABLE 1. Infection frequency[a] for *Phytophthora infestans* field isolates sensitive (MS) and resistant (MR) to metalaxyl

Isolate	Length of dew period (hr)			
	3	6	9	20
MS 1	0.05	0.30	0.60	0.78
MS 2	0.08	0.32	0.65	0.77
MS 3	0.02	0.27	0.53	0.84
MS 4	0.05	0.15	0.45	0.82
MS 5	0.06	0.31	0.67	0.95
MS 6	0.10	0.27	0.48	0.92
MS 7	0.10	0.26	0.62	0.90
MS 8	0.05	0.18	0.53	0.82
MS 9	0.00	0.15	0.40	0.82
MS 10	0.10	0.28	0.55	0.88
MR 1	0.17	0.55	0.66	0.67
MR 2	0.10	0.52	0.68	0.89
MR 3	0.10	0.55	0.70	0.82
MR 4	0.10	0.43	0.77	0.80
MR 5	0.07	0.37	0.63	0.90
MR 6	0.12	0.45	0.70	0.82
MR 7	0.17	0.48	0.73	0.73
MR 8	0.15	0.62	0.76	0.77
MR 9	0.10	0.18	0.75	0.80
MR 10	0.07	0.35	0.60	0.73
LSD[b]	0.11	0.14	0.16	0.06

[a]Proportion of inoculated potato leaflets (cv. Alpha) on which lesions developed. Results are means from three experiments.

[b]Least significant difference ($P = 0.05$) for comparing means of MS or MR isolates within a column.

RESULTS

The two populations exhibited a large variation in the variables measured (Tables 1–4). Isolates within each population differed significantly ($P < 0.05$) in infection frequency (Table 1), sporangial

germination (indirect, Table 2; direct, Table 3), and sporangial respiration (Table 4).

Contrast estimates computed for each variable (Table 5) revealed that the MR population had a significantly higher mean infection frequency, faster rate of zoospore liberation, faster sporangial direct germination, and higher rate of oxygen consumption than the MS population. The differences between the populations changed with time. The difference in infection frequency was greatest (about twofold) in plants incubated in dew conditions for 6 hr ($t = 8.32$, $P < 0.001$). No significant difference between populations was noted at 20 hr of dew (Tables 1 and 5). Similarly, the rate of zoospore liberation differed most between populations at 90 min of incubation in water ($t = 4.07$, $P < 0.001$); no significant difference was recorded at 240 min (Tables 2 and 5).

The proportion of empty sporangia (data not shown) was closely correlated with the number of liberated zoospores (at 90 min, $R^2 = 0.95$; at 150 min, $R^2 = 0.95$; at 150 min, $R^2 = 0.96$). Populations did not differ, however, in the mean number of zoospores liberated from a single sporangium (about 7.46 zoospores per empty sporangium in the MS isolates, compared to 7.31 zoospores per empty sporangium in the MR isolates). Direct germination of sporangia followed a similar pattern of behavior. After 4–8 hr of incubation on water agar at 22–23 C, the proportion of germinating sporangia was significantly higher in the MR than in the MS population (Tables 3 and 5). The difference dissipated at 12 hr of incubation.

Sporangia from the MR population consumed oxygen significantly faster than sporangia from the MS population during 15 min of incubation in water ($t = 5.17$, $P < 0.001$) (Table 5). Sporangia of the MS isolates consumed 1.33–3.84 $\times 10^{-8}$ μmol of oxygen per sporangium per minute, compared to 4.33–18.14 $\times 10^{-8}$ μmol per sporangium per minute consumed by sporangia of the MR isolates (Table 4). Populations did not differ in mean sporangial weight (5.9 $\pm$ 1.9 ng dry weight per sporangium for the MS isolates, compared to 5.3 $\pm$ 1.8 ng dry weight per sporangium for the MR isolates).

Oxygen consumption measurements taken from mycelial mats of the fungus (6–7% dry matter) in water (data not shown) were much lower than measurements from sporangia, with no significant difference between the MR and the MS populations (Table 5). Similar respiration rates have been reported by others (2). The two populations did not differ significantly in linear growth on rye seed agar medium (13).

Correlation coefficients among all variables measured were highly significant (Table 6). Resistance to metalaxyl was strongly correlated with infection frequency, sporangial direct germination, and sporangial respiration and to a lesser extent (though still

TABLE 2. Kinetics of indirect sporangial germination[a] of *Phytophthora infestans* field isolates sensitive (MS) and resistant (MR) to metalaxyl

Isolate	Incubation (min)[b]			
	60	90	150	240
MS 1	1.5	35.5	103.0	195.5
MS 2	3.0	109.5	151.0	193.5
MS 3	0	25.5	62.5	161.0
MS 4	0	18.0	53.5	171.0
MS 5	0	8.5	78.0	133.0
MS 6	0	13.0	51.5	138.5
MS 7	0	14.0	36.5	137.0
MS 8	0	22.5	71.5	180.5
MS 9	0	16.5	34.5	111.0
MS 10	0	30.0	99.5	204.5
MR 1	202.0	148.5	310.0	277.0
MR 2	40.0	61.5	124.0	209.5
MR 3	21.5	43.0	153.0	154.0
MR 4	3.5	51.5	119.0	209.5
MR 5	5.0	15.5	32.0	91.0
MR 6	0.5	25.0	72.0	159.0
MR 7	6.0	113.5	121.5	214.0
MR 8	2.0	76.0	34.5	171.5
MR 9	16.5	33.5	79.5	215.0
MR 10	7.0	91.0	202.5	254.0
LSD[c]	3.8	32.6	61.6	91.4

[a] Zoospores/ml, $\times 10^3$. Suspensions contained 5×10^4 sporangia/ml. Results are means from a single typical experiment.
[b] Minutes of incubation in double-distilled water at 16 C in the dark.
[c] Least significant difference ($P = 0.05$) for comparing means of MS or MR isolates within a column.

TABLE 3. Direct germination[a] of sporangia of *Phytophthora infestans* field isolates sensitive (MS) and resistant (MR) to metalaxyl

Isolate	Duration (hr)		
	4	8	12
MS 1	10.8	24.8	39.8
MS 2	18.0	31.0	50.3
MS 3	10.3	20.0	35.8
MS 4	9.3	19.3	33.5
MS 5	15.8	23.3	52.3
MS 6	8.3	19.8	50.3
MS 7	6.0	26.3	48.8
MS 8	14.0	13.8	45.0
MS 9	5.3	10.5	33.0
MS 10	13.3	27.5	52.8
MR 1	16.0	36.5	53.8
MR 2	15.0	28.5	44.0
MR 3	18.8	29.3	39.3
MR 4	16.5	35.0	58.5
MR 5	7.5	23.3	40.8
MR 6	12.0	24.8	35.3
MR 7	28.8	52.3	67.3
MR 8	21.5	37.8	55.5
MR 9	15.8	31.8	44.3
MR 10	30.5	46.8	55.3
LSD[b]	5.6	10.4	17.2

[a] Percentage germination on 1.5% water agar at 22–23 C in the dark. Results are means from a single typical experiment.
[b] Least significant difference ($P = 0.05$) for comparing means of MS or MR isolates within a column.

TABLE 4. Rate of oxygen consumption of sporangia of *Phytophthora infestans* field isolates sensitive (MS) and resistant (MR) to metalaxyl[a]

Isolate	Oxygen consumption (μmol/sporangium/min, $\times 10^{-8}$)
MS 1	2.75
MS 2	3.84
MS 3	2.83
MS 4	1.93
MS 5	3.09
MS 6	2.04
MS 7	2.02
MS 8	2.24
MS 9	1.33
MS 10	3.29
MR 1	12.24
MR 2	5.56
MR 3	5.56
MR 4	8.50
MR 5	4.33
MR 6	5.43
MR 7	18.14
MR 8	8.39
MR 9	6.54
MR 10	10.20
LSD[b]	7.80

[a] Freshly produced sporangia were suspended in double-distilled water (2.5×10^4 sporangia/ml). Oxygen comsumption was monitored polarographically for 15 min at 19 C. Oxygen concentration decreased linearly with time during the assay. Results are means from three experiments.
[b] Least significant difference ($P = 0.05$) for comparing means of isolates within a column.

TABLE 5. Sporangial respiration, mycelial respiration, zoospore liberation, direct germination, and infection frequency of metalaxyl-sensitive (MS) and metalaxyl-resistant (MR) field populations of *Phytophthora infestans*

Feature	Mean ± SD		Resistant vs. sensitive population		
	MR	MS	Contrast	t	$P > t$
Respiration					
Sporangia[a]	8.51 ± 6.1	2.62 ± 1.1	5.89	5.17	0.001
Mycelial mats[b]	4.58 ± 3.4	4.71 ± 2.5	−0.13	−0.12	0.905
Zoospore liberation[c]					
60 min	34.2 ± 60.7	0.6 ± 1.2	33.6	3.49	0.012
90 min	65.9 ± 48.4	29.3 ± 29.9	36.6	4.07	0.001
150 min	124.8 ± 89.5	74.2 ± 50.2	50.6	3.12	0.003
240 min	195.5 ± 80.4	162.6 ± 59.3	32.9	1.35	0.040
Empty sporangia[d]					
60 min	12.2 ± 18.6	0.9 ± 4.2	11.3	3.15	0.001
90 min	20.8 ± 16.9	8.0 ± 9.0	12.8	4.23	0.001
150 min	33.5 ± 21.8	17.7 ± 10.5	15.8	4.14	0.001
240 min	53.5 ± 20.6	47.6 ± 13.9	5.9	1.53	0.125
Direct germination[e]					
4 hr	18.2 ± 8.3	11.1 ± 4.6	7.1	4.78	0.001
8 hr	34.6 ± 11.4	21.6 ± 8.6	13.0	5.72	0.001
12 hr	49.4 ± 13.3	44.1 ± 14.2	5.3	1.70	0.092
Infection frequency[f]					
3 hr	0.11 ± 0.07	0.06 ± 0.05	0.05	3.12	0.003
6 hr	0.47 ± 0.12	0.25 ± 0.09	0.22	8.32	0.001
9 hr	0.70 ± 0.10	0.55 ± 0.12	0.15	5.44	0.001
20 hr	0.79 ± 0.07	0.85 ± 0.06	−0.06	1.88	0.076

[a] Micromoles $\times 10^{-8}$ of oxygen per sporangium per minute in water at 19 C.
[b] Micromoles of oxygen per 0.1 g (fresh weight) of mycelial mats in water at 19 C.
[c] Zoospores $\times 10^3$ liberated per milliliter of sporangial suspension (5×10^4 sporangia/ml) at 16 C.
[d] Proportion of empty sporangia that have liberated their zoospores.
[e] Proportion of sporangia producing germ tube(s) on water agar at 22–23 C.
[f] Proportion of inoculated leaflets on which lesions developed.

TABLE 6. Correlations among metalaxyl resistance, infection frequency, zoospore liberation, direct germination, and sporangial respiration for 20 field isolates of *Phytophthora infestans*

Variable	Infection frequency[a]	Zoospore liberation[b]	Direct germination[c]	Sporangial respiration[d]
Metalaxyl resistance[e]	0.73 (0.0003)[f]	0.47 (0.0364)	0.65 (0.0018)	0.72 (0.0003)
Infection frequency		0.59 (0.0067)	0.61 (0.0043)	0.63 (0.0031)
Zoospore liberation			0.76 (0.0001)	0.78 (0.0001)
Direct germination				0.90 (0.0001)

[a] For plants maintained in dew conditions for 6 hr after inoculation.
[b] At 90 min of incubation in water.
[c] Proportion of sporangia producing germ tube(s) on 1.5% water agar after 8 hr of incubation at 22–23 C in the dark.
[d] Rate of oxygen consumption for sporangial suspension in water.
[e] Fungal growth or absence of growth on potato tuber tissue in the presence of 100 μg of metalaxyl per milliliter.
[f] Significance value.

significant) with zoospore liberation. Sporangial respiration was strongly correlated with metalaxyl resistance, infection frequency, zoospore liberation, and direct germination.

DISCUSSION

The results presented here show that in the absence of selection pressure, isolates of *P. infestans* from the MR population in Israel are more infective to potato foliage than isolates from the MS population during relatively short periods of leaf wetness (dew). This greater infectivity was found to be significantly correlated with the rate and abundance of indirect sporangial germination in water.

According to Duniway (11), "indirect germination [of *P. infestans*] is a rate-limiting step in the infection process. Therefore, the steps in the infection process that follow indirect germination appear to have little influence on the speed with which *P. infestans* infects potato foliage." In fact, the temperature readings and duration of leaf wetness periods that are most strongly correlated with late blight increases in the field (10,16) and in growth chambers (18) are those that are most suitable for indirect germination (9). The speed and profusion of indirect sporangial germination suggest that indirect germination by sporangia of *P. infestans* contributes more to infection than does direct germination (11).

The capacity of the Israeli MR isolates of *P. infestans* to produce zoospores more rapidly and in larger quantities than the MS isolates is probably one of the major reasons for their wide predominance in the country (4) since they first appeared in 1982 (7). Another reason is the capacity of the MR isolates to produce significantly larger lesions in potato leaflets than the MS isolates (13). The increased infection frequency and lesion size explain our earlier observation that MR-induced late blight epidemics in potato progress significantly faster than MS-induced epidemics (13).

Sporangia of the MR isolates were also found (Tables 4 and 5) to consume significantly more oxygen (0.16 μmol of O_2 per gram of dry weight) during zoosporogenesis than sporangia of the MS isolates (0.04 μmol of O_2 per gram of dry weight). More isolates should be assayed to establish whether oxygen consumption may serve as a marker for metalaxyl resistance. The biochemical and genetic reasons for the enhanced oxygen consumption of the MR isolates are not known, but the fact that respiration is strongly correlated with the speed and abundance of zoospore liberation (Table 6) suggests that aerobic energy production is a rate-limiting factor in zoosporogenesis.

Zoosporogenesis consists of two phases: protoplasm cleavage and zoospore discharge. Because the latter process depends mainly on physical factors affecting sporangial turgor (12), cleavage seems to be the energy-demanding phase. Phosphorylation of soluble cytoplasmic β-1,3 glucans in *Phytophthora* species during zoosporogenesis (1) is probably one of these respiration-dependent, energy-demanding processes. Indeed, respiration inhibitors and fungicides that inhibit energy production, such as mancozeb and folpet, are strong inhibitors of zoosporogenesis in *P. infestans* (17,19; Y. Cohen, *unpublished data*). Sporangia of *P. infestans* exposed to water stress lose germinability and

infectivity to potato foliage (6), mainly because of the destruction of cellular and mitochondrial membranes, which is responsible for the lack of respiration and the inhibition of protein and nucleic acids synthesis (6).

The MR and the MS populations did not differ significantly in mycelial respiration (Table 5) (0.65×10^{-5} μmol of O_2 per gram of dry weight for the MR isolates, compared to 0.67×10^{-5} μmol of O_2 per gram of dry weight for the MS isolates). Similar rates ($0.5–0.6 \times 10^{-6}$ μmol of O_2 per gram of dry weight) have been reported previously for *P. infestans* (2). Nor did the two populations differ in linear growth in vitro (13). MR isolates, however, produced significantly larger lesions in potato leaflets (13), suggesting a higher metabolic activity in vivo.

This study and our earlier reports (13,15) support the conclusion that the greater fitness (competitive and noncompetitive) of Israeli MR isolates of *P. infestans* on potato foliage compared with MS isolates is a consequence of their faster and more abundant germination and their extensive colonization of the foliage. Both features indicate a higher metabolic activity of MR compared to MS isolates whose nature is yet to be defined.

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