Commercial Fungicide Formulations Induce In Vitro Oospore Formation and Phenotypic Change in Mating Type in *Phytophthora infestans*

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ABSTRACT

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A wide range of commercially formulated fungicides cause in vitro effects on mating behavior in specific isolates of *Phytophthora infestans*, the causal agent of late blight of potato and tomato. Four isolates of *P. infestans* representing each of the four common US genotypes, US-1, US-6, US-7, and US-8 and varying in their sensitivity to metalaxyl, were exposed to a variety of fungicides used to control late blight in petri dish assays at concentrations ranging from 1 to 100 µg a.i./ml. Exposure of each of these normally heterothallic single mating type isolates of *P. infestans* to 9 of the 11 commercial fungicide formulations tested resulted in the formation of oospores after 2 to 4 weeks. The highest numbers of oospores were formed on media amended with Ridomil 2E (metalaxyl) and Ridomil Gold EC (mefenoxam) at 0.1 to 10 µg a.i./ml, averaging as many as 471 and 450 oospores per petri dish, respectively. Several other fungicides including Maneb, Manzate (Mancozeb), Curzate (cymoxanil + mancozeb), and Acrobat MZ (dimethomorph + mancozeb) also induced oospore formation, producing from 0 to 200 oospores per plate at fungicide concentrations from 0.1 to 10 µg a.i./ml. The metalaxyl resistant isolates formed oospores in response to the fungicides more often than the metalaxyl sensitive isolates. No oospores were formed on media amended with Bravo (chlorothalonil) or Tattoo C (chlorothalonil + propamocarb HCl) and these compounds completely suppressed growth of the isolates at 0.1 and 1 µg a.i./ml. Three metalaxyl resistant A2 isolates mated with both A1 and A2 isolates after exposure to the fungicides Ridomil 2E and Ridomil Gold EC. Alterations in mating type expression were also observed in a metalaxyl sensitive A1 isolate after exposure to Benlate (benomyl). Copious amounts of chemicals are applied annually to potato and tomato production areas to control late blight. Our results indicate that a wide range of chemically diverse fungicides can induce normally heterothallic metalaxyl resistant isolates of *P. infestans* to form oospores in vitro after short exposures to the fungicides.

Additional keywords: fungicide resistance, Irish potato famine, oomycetes, potato late blight, *Solanum tuberosum*.

Late blight caused by the oomycete pathogen *Phytophthora infestans* (Mont.) de Bary, is a devastating disease of potato and tomato worldwide. The cost of chemical control of late blight worldwide may well exceed 1 billion annually, although actual losses in the field and storage are likely several fold higher. The cost of controlling late blight in the Columbia Basin area of Washington and Oregon alone in 1995 was estimated to have approached 30 million (23). The total amount of agricultural chemicals applied annually on potato is greater than for any other food crop (32). About 97% of potato hectarage are treated with fungicides (34).

Metalaxyl, introduced in the 1970s, has historically been the most effective therapeutic fungicide available for late blight control. In 1981, resistance to metalaxyl was first reported in field populations of *P. infestans* in the Netherlands and has been reported subsequently throughout Europe and other parts of the world (8,10,13,46). Resistance to metalaxyl was first reported in populations of *P. infestans* in the United States in 1990 and since then reports of resistance have been widespread (12,16,18). Concurrent with the increased incidence of fungicide-resistant strains was the appearance of exotic pathogen strains and the A2 mating type in Europe (10,18,22). These factors combined have led to a resurgence of late blight in the last two decades (18). The severity of recent epidemics has led to the Environmental Protection Agency granting a Section 18 Emergency Exemption for certain fungicides to be used in the United States (18). Several of these compounds have since been registered for use in potato production in the United States.

Phytophthora infestans is a normally heterothallic species and reproduces predominately by asexual means. Two specific mating types (compatibility types), A1 and A2, are necessary for sexual reproduction. Each mating type is bisexual and capable of producing male or female gametangia that fuse and result in the formation of thick-walled survival structures called oospores. Both mating types of the pathogen were first reported in Mexico, whereas only the A1 mating type was found elsewhere in the world until the 1980s when the A2 mating type was reported in Europe and subsequently in Asia, South America, and North America (5,11,17,18,22,30,41,45). A shift in the predominance of mating type from the A1 to the A2 has appeared in North American populations of *P. infestans* in recent years concomitant with the use of the fungicide metalaxyl and the widespread occurrence of metalaxyl resistant isolates in field populations.

Oospore formation has been reported in heterothallic species of *Phytophthora* in response to intraspecific or interspecific stimulation, culture aging, compounds produced by antagonistic fungi such as *Trichoderma* spp., root exudates, and treatment with fungicides such as chloroneb, ethazol, and metalaxyl (1,3,26,33,36,

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39,40,42,43,47,48). A single isolate can act as male or female during heterothallic hybridizations (outcrossing) or simultaneously act as male and female during homothallic hybridization (selfing). The propensity to produce oospores in vitro by either outcrossing or selfing appears to be isolate dependent in *P. infestans*, i.e., most isolates are prone to outcrossing; however, some isolates are capable of selfing (25,28,43). Formation of oospores and complete mating type conversions of A1 isolates of *P. parasitica* have been reported in aged single mating type cultures and in vitro in the presence of metalaxyl and chloroneb (1,3,26). The production of oospores and mating type conversion of both A1 and A2 isolates of *P. infestans* in the presence of metalaxyl have also been reported previously (3,28).

Although some fungicides may induce single isolates of *P. infestans* to form oospores, only a limited number of fungicides have been evaluated in previous work (3). Quantification of the effect of diverse classes of fungicides on numbers of oospores produced in vitro by single isolates has never been evaluated in previous in vitro studies. In addition, the fungicides most commonly used on potatoes have not been evaluated for their in vitro effects on oospore formation in single isolates of *P. infestans*. Isolates of *P. infestans* differ in sensitivity to metalaxyl (12,13). However, the differential impact of diverse classes of fungicides on oospore formation in single isolates that are either sensitive or resistant to metalaxyl has never been examined previously.

The resurgence of late blight in the past two decades and the presence of new highly virulent genotypes of the pathogen emphasize the need to intensify research on the biology of *P. infestans*. Growers are increasingly relying on diverse classes of fungicides for late blight control in fields, and we know little of their effects on pathogen biology. The objectives of this study were to determine the in vitro effects of commercially formulated fungicides used on potato and tomato on formation of oospores and the phenotypic expression of mating type in single isolates of *P. infestans* that differ in sensitivity to metalaxyl.

MATERIALS AND METHODS

Source of isolates. Cultures of *P. infestans* were either isolated from diseased potato and tomato plants in North Carolina or were donated (Table 1). All isolates used in this study were derived from single propagules of either sporangia or zoospores. Isolates of *P. infestans* tested included two A1 isolates and two A2 isolates (Table 1). Isolates for this study were chosen after preliminary in vitro screenings of over 40 isolates of *P. infestans* (US-1 [three isolates], US-6 [two isolates], US-7 [17 isolates], US-8 [10 isolates], US-13 [two isolates], US-18 [two isolates], and unknown genotypes [seven isolates]) on rye B media amended with Ridomil 2E following the same experimental protocol as described below.

TABLE 1. Isolate number, fungicide sensitivity, mating type, allozyme genotype, host of origin, and source of isolates of *Phytophthora infestans* used in the fungicide assays

Isolate	Metalaxyl sensitivity[a]	Mating type[b]	Genotype[c]	Host	Source
188.1.1	S	A1	US-1	Potato	Z. Punja
94-55	R	A1	US-6	Tomato	W. Fry
94-54	R	A2	US-7	Tomato	W. Fry
94-8-4	R	A2	US-8	Potato	P. Shoemaker

[a] Metalaxyl sensitivity determined by plating isolates on rye B media amended with 0, 5, and 100 µg a.i./ml metalaxyl. Isolates were characterized as sensitive if radial growth at 5.0 µg a.i./ml and 100 µg a.i./ ml was less than 40% of the nonamended control. Resistant isolates exhibited growth greater than 40% of the nonamended control at 5.0 µg a.i./ml metalaxyl.

[b] Mating type determined by pairing isolate with known A1 and A2 mating type isolates.

[c] Genotype determined by allozymes at the *glucose-6-phosphate isomerase* and *peptidase* loci.

Approximately 90% of isolates examined in preliminary screenings produced no oospores in response to Ridomil 2E. Approximately 10% of the isolates screened produced oospores in single culture, and four isolates were used in subsequent experiments (Table 1). The isolates chosen for further testing represented the four common US genotypes, US-1, US-6, US-7, and US-8 as determined by allozymes at the *glucose-6-phosphate isomerase* and *peptidase* loci (Table 1; 16). Isolates of *P. infestans* were maintained on rye B agar (2) at 18°C and were preserved in cryogenic storage in 10% dimethyl sulfoxide.

Fungicide assays. Commercially formulated fungicides commonly used on potatoes or tomatoes were evaluated in this study (Table 2). We evaluated commercial formulations of the fungicides rather than the active ingredient alone to more realistically represent compounds that are used by growers in the field. The concentrations tested are commonly used in screening isolates for resistance to fungicides. Fungicide stocks were prepared in sterile deionized water and were added to molten rye B agar at 0, 0.1, 1, 10, and 100 µg a.i./ml. The media was continuously agitated while pouring to ensure even distribution of the fungicides. Rye B is routinely used in our laboratory for isolation and maintenance of cultures of *P. infestans*. We also tested clarified V8 agar but our isolates did not grow well on this medium and therefore rye B was chosen for further work. Agar disks (5-mm diameter) from actively growing rye B cultures of single isolates of *P. infestans* were plated on nonamended and fungicide-amended rye B media (100 × 15 mm petri dishes). Petri dishes were wrapped with Parafilm, inverted, and incubated at 18°C in the dark. Measurements of colony diameter (in millimeters) were made after 2 weeks incubation. The dishes were observed microscopically every 2 weeks for a 3-month-period for the presence of oospores. At the end of the 3-months, all oospores observed were removed from the petri dishes by separating the oospores from the agar medium with a sterile needle, and oospores were subsequently counted via microscopic enumeration. We quantified the number of oospores produced on a per petri dish basis rather than from individual agar disks removed from petri dishes because oospore distribution on the plate was not always uniform. Two replicate petri dishes at each fungicide concentration were used, and the experiment was repeated. The data reported were pooled from the two experiments. Oospore counts (number of oospores per petri dish) were transformed using a log transformation to normalize the data before analysis. A general linear models procedure (SAS version 6.12; SAS Institute, Cary, NC) was used to evaluate the interactions and main effects of fungicide, concentration, and isolate on oospore numbers. Data for the fungicides Tattoo C and Bravo were not included in the analysis, because neither of these compounds induced oospore formation.

Oospore viability assay. Viability of a subset of the oospores collected was determined by tetrazolium bromide assay (37). Five hundred microliters of 0.1% solution of 3(4,5-dimethyl thiazolyl

TABLE 2. Common name, trade name, and chemical class of the commercial fungicide formulations evaluated in the study

Trade name	Common name	Chemical class
Ridomil 2E	Metalaxyl	Phenylamide
Ridomil Gold EC	Mefenoxam	Phenylamide
Ridomil Gold/Bravo	Mefenoxam + chlorothalonil	Phenylamide/nitrile
Ridomil Gold/Copper	Mefenoxam + copper	Phenylamide/copper
Bravo	Chlorothalonil	Nitrile
Maneb	Maneb	Dithiocarbamate
Tattoo C	Propamocarb HCl + chlorothalonil	Dithiocarbamate/ nitrile
Curzate	Cymoxanil + mancozeb	Acetimide/ dithiocarbamate
Manzate	Mancozeb	Dithiocarbamate
Acrobat Mz	Dimethomorph + mancozeb	Morpholine/ dithiocarbamate
Benlate	Benomyl	Benzimidazole

1-2) 2,5-diphenyl tetrazolium bromide (MTT; Sigma Chemicals Co., St. Louis) in 0.1 M phosphate buffer (pH 5.8) was added to 500 µl of oospores suspended in sterile deionized water. Oospores were incubated in a water bath at 35°C for 2 to 2.5 h. Oospores were examined microscopically, and oospore viability was assessed after recording the number of blue- or red-staining oospores. At least 100 oospores for each isolate for each fungicide treatment (all concentrations combined) were counted when possible, otherwise as many oospores as were present in the sample were counted. Viability counts were done on oospores produced in each replication.

Mating type determination. Agar disks (5-mm diameter) containing mycelia were removed from cultures of the isolates grown on nonamended rye B media, from oospore-forming sectors containing mycelia, and oospores from the isolates grown on fungicide-amended rye B media. Agar disks were cut in half with a sterile scalpel and paired directly on fresh rye B media with known tester isolates of the A1 and A2 mating types. Isolates used for mating-type tests were 94-1 and 94-52 (A1 mating type) and 94-37 and 94-53 (A2 mating type). The tester isolates did not produce oospores in response to the range of fungicides tested in this study in preliminary experiments and have been used routinely in our laboratory for mating type evaluation. Petri dishes were incubated at 18°C in the dark, and cultures were observed for oospore formation after 10 to 14 days. Isolates grew to the center of the plates and oospores formed in the median between isolates. Isolates forming oospores with A1 tester isolates were rated as A2 mating type and isolates that formed oospores with A2 tester isolates were rated as A1 mating type.

RESULTS

Isolate growth and production of oospores. All isolates grew well on the nonamended rye B agar, and the range of colony diameters was 74.5 to 83 mm after 2 weeks (Table 3). Isolate 188.1.1 which is the US-1 genotype and sensitive to metalaxyl did not grow on rye B agar amended with Ridomil 2E, Ridomil Gold, Ridomil Gold + Copper, and Ridomil Gold + Bravo at fungicide concentrations greater than 0.1 µg a.i./ml (Table 3). Isolate 188.1.1 did not form oospores on media amended with any of the these fungicides (Table 4). However, isolate 188.1.1 did grow on media amended with Maneb at concentrations up to 1 µg a.i./ml and on media amended with Curzate, Manzate, and Acrobat MZ at concentrations up to 0.1 µg a.i./ml (Table 3). Isolate 188.1.1 also grew on Benlate-amended media at all concentrations tested (Table 3). Isolate 188.1.1 formed oospores after 2 to 4 weeks on both Maneb- and Benlate-amended media (Table 4) and by the end of the 3-month-period on media amended with 0.1 µg a.i./ml Benlate, Maneb, Curzate, Manzate, and Acrobat MZ (Table 4, Fig. 1E through I).

In contrast, isolates 94-55, 94-54, and 94-8-4, which are resistant to metalaxyl, grew on media amended with Ridomil 2E and Ridomil Gold at all concentrations tested (Table 3). Growth of these isolates was suppressed at fungicide concentrations greater than 1 µg a.i./ml on Ridomil Gold + Copper-amended media with the exception of isolate 94-8-4 which was able to grow on Ridomil Gold + Copper up to and on 10 µg a.i./ml (Table 3). Isolates 94-55, 94-54, and 94-8-4 formed oospores on media amended with Ridomil 2E and Ridomil Gold more often and in greater numbers than did isolate 188.1.1 which is metalaxyl sensitive (Table 4, Fig. 1A and B). The metalaxyl resistant isolates formed oospores within 2 weeks on media amended with Ridomil 2E, Ridomil Gold, or Ridomil Gold + Copper (Table 4) and higher numbers of oospores were observed after 3 months on media amended with these fungicides than the nonamended controls (Fig. 1A through C). Growth of the metalaxyl resistant isolates was suppressed on media amended with the Ridomil Gold + Bravo formulation (Tables 3), and lower numbers of oospores were formed on this medium (Fig. 1D) than on the other Ridomil formulations (Fig. 1A,B, C). Only isolate 94-54 was able to grow on and form oospores in the presence of Ridomil Gold + Bravo at 0.1 µg a.i./ml.

Isolate 94-55, the US-6 genotype, formed oospores on media amended with eight different fungicides after 2 to 4 weeks (Table 4, Fig. 1), but no oospores were formed on nonamended controls even after 3 months (Table 4, Fig. 1). Isolate 94-54, the US-7 genotype, also formed oospores on media amended with eight different fungicides after 2 to 4 weeks (Table 4) and on media amended with nine different fungicides by the end of the 3-month-period (Table 4, Fig. 1). Isolate 94-8-4, the US-8 genotype, formed oospores on media amended with eight different fungicides after 2 to 4 weeks and produced oospores on nonamended control plates after 3 months (Table 4, Fig. 1).

Oospores were not observed with any isolate on media amended with Bravo or Tattoo C (Table 4). Most of the isolates did not

TABLE 3. Colony diameter (mm) of isolates of *Phytophthora infestans* after 2-week growth on nonamended or different concentrations of fungicide-amended rye B agar at 18°C in the dark

Fungicide formulation	µg a.i./ml	188.1.1	94-55	94-54	94-8-4
Control	0	74.5	83	83	83
Ridomil 2E	0.1	15	83	83	76.5
	1	0	27	68.8	76.5
	10	0	17.5	41.3	64.8
	100	0	12	26.5	45
Ridomil Gold EC	0.1	11	47	83	81.5
	1	0	15.8	25.5	72
	10	0	16.3	25.3	55.3
	100	0	11	10	33.5
Ridomil Gold + Copper	0.1	19	83	83	71
	1	0	60	83	78.3
	10	0	0	0	22
	100	0	0	0	0
Benlate	0.1	83	83	83	72.5
	1	78.5	83	83	78.3
	10	69.3	83	83	72.5
	100	17.3	40.3	55.5	11.3
Maneb	0.1	83	83	83	70
	1	48	83	83	77.5
	10	0	0	0	18.5
	100	0	0	0	0
Curzate	0.1	19	83	83	83
	1	0	0	0	59
	10	0	0	0	0
	100	0	0	0	0
Manzate	0.1	51.5	83	83	75
	1	0	21.5	0	0
	10	0	0	0	0
	100	0	0	0	0
Acrobat MZ	0.1	24	83	83	80.5
	1	0	8	0	24
	10	0	0	0	0
	100	0	0	0	0
Ridomil Gold + Bravo	0.1	18	0	83	0
	1	0	0	0	0
	10	0	0	0	0
	100	0	0	0	0
Bravo	0.1	0	0	83	0
	1	0	0	0	0
	10	0	0	0	0
	100	0	0	0	0
Tattoo C	0.1	0	0	41.5	0
	1	0	0	0	0
	10	0	0	0	0
	100	0	0	0	0

grow on media amended with these two fungicide formulations even at concentrations as low as 0.1 µg a.i./ml (Table 3). Only isolate 94-54 grew on these two fungicides at 0.1 µg a.i./ml.

Quantification of oospores. All oospores were removed from the plates at the end of the 3-month incubation period and the average numbers of oospores formed on nonamended or fungicide-amended rye B media were quantified (Fig. 1). The three-way interaction between fungicide, concentration, and isolate was highly significant (Table 5) indicating that the individual isolates responded differently to different concentrations of the fungicides (Fig. 1).

The US-1 isolate 188.1.1, which is sensitive to metalaxyl, did not form oospores on media amended with any metalaxyl or mefenoxam containing compound (Table 4, Fig. 1A through D). However, isolates 94-55, 94-54, and 94-8-4, which are all resistant to metalaxyl, formed high numbers of oospores even at low concentrations of the fungicides Ridomil 2E and Ridomil Gold EC (Fig. 1A and B). Among all isolates, the fewest number of oospores were formed on media amended with Ridomil Gold + Bravo (Fig. 1D). Only isolate 94-54 formed oospores on media amended with Ridomil Gold + Bravo at concentrations of 0.1 µg a.i./ml (Table 4, Fig. 1D). The three metalaxyl resistant isolates grew on media amended with Ridomil Gold alone but did not grow on media amended with Ridomil Gold + Bravo and few oospores were detected (Tables 3 and 4).

Generally, the largest numbers of oospores were formed at the lower concentrations of each fungicide examined, and in most cases no oospores were formed at 100 µg a.i./ml, however there were exceptions (Table 4, Fig. 1). For example, two of the metalaxyl resistant isolates grew and produced large numbers of

TABLE 4. Oospore production by isolates of *Phytophthora infestans* grown on nonamended and fungicide-amended rye B agar[a]

		188.1.1		94-55		94-54		94-8-4	
Fungicide formulation	Concn. µg a.i./ml	2 weeks	3 months	2 weeks	3 months	2 weeks	3 months	2 weeks	3 months
Control	0	–	+	–	–	–	–	–	+
Ridomil 2E	0.1	–	–	+	+	+	+	+	+
	1	NG	NG	+	+	+	+	+	+
	10	NG	NG	+	+	+	+	+	+
	100	NG	NG	+	+	+	+	–	+
Ridomil Gold EC	0.1	–	–	+	+	+	+	+	+
	1	NG	NG	+	+	+	+	–	+
	10	NG	NG	+	+	+	+	–	+
	100	NG	NG	+	+	+	+	–	–
Ridomil Gold Copper	0.1	–	–	+	+	+	+	+	+
	1	NG	NG	+	+	+	+	+	+
	10	NG	NG	NG	NG	NG	NG	–	+
	100	NG	NG	NG	NG	NG	NG	NG	NG
Benlate	0.1	–	+	+	+	–	+	–	+
	1	+	+	+	+	–	+	–	+
	10	+	+	+	+	–	–	+	+
	100	+	+	–	–	–	–	+	+
Maneb	0.1	+	+	–	–	–	+	+	+
	1	+	+	+	+	+	+	+	+
	10	NG	NG	+/SG	+/SG	NG	NG	–	+
	100	NG	NG	NG	NG	NG	NG	NG	NG
Curzate	0.1	–	+	+	+	+	+	+	+
	1	NG	+/NG	NG	+/SG	NG	+/SG	+	+
	10	NG	NG	NG	+/SG	NG	NG	NG	NG
	100	NG	NG	NG	NG	NG	NG	NG	NG
Manzate	0.1	–	+	+	+	+	+	+	+
	1	NG	NG	NG	NG	NG	+/SG	–	+
	10	NG	NG	NG	+/SG	NG	NG	NG	NG
	100	NG	NG	NG	NG	NG	NG	NG	NG
Acrobat MZ	0.1	–	+	+	+	–	+	+	+
	1	NG	NG	+	+	+/SG	+/SG	+	+
	10	NG	NG	NG	NG	NG	NG	NG	NG
	100	NG	NG	NG	NG	NG	NG	NG	NG
Ridomil Gold + Bravo	0.1	–	–	NG	NG	+	+	NG	NG
	1	NG	NG	NG	NG	NG	NG	NG	NG
	10	NG	NG	NG	NG	NG	NG	NG	NG
	100	NG	NG	NG	NG	NG	NG	NG	NG
Bravo	0.1	NG	NG	NG	NG	–	–	NG	NG
	1	NG	NG	NG	NG	NG	NG	NG	NG
	10	NG	NG	NG	NG	NG	NG	NG	NG
	100	NG	NG	NG	NG	NG	NG	NG	NG
Tattoo C	0.1	NG	NG	NG	NG	–	–	NG	NG
	1	NG	NG	NG	NG	NG	NG	NG	NG
	10	NG	NG	NG	NG	NG	NG	NG	NG
	100	NG	NG	NG	NG	NG	NG	NG	NG

[a] Presence or absence of oospores was determined after 2 weeks or 3 months incubation on fungicide-amended media at 18°C in the dark. – = no oospores produced; + = oospores produced; NG = no growth; +/SG = slight growth with oospores formed around site of original plug.

oospores on Ridomil 2E and Ridomil Gold EC at concentrations of 100 µg a.i./ml (Fig. 1A and B, Table 3). Growth of most of the isolates was either greatly reduced or completely suppressed at concentrations of 10 and 100 µg a.i./ml of most fungicides (Table 3).

Fungicide induction of oospore formation was most evident with the metalaxyl resistant isolates 94-55, 94-54, and 94-8-4. These three isolates formed oospores more often and in greater numbers than did the metalaxyl sensitive isolate 188.1.1 (Table 4,

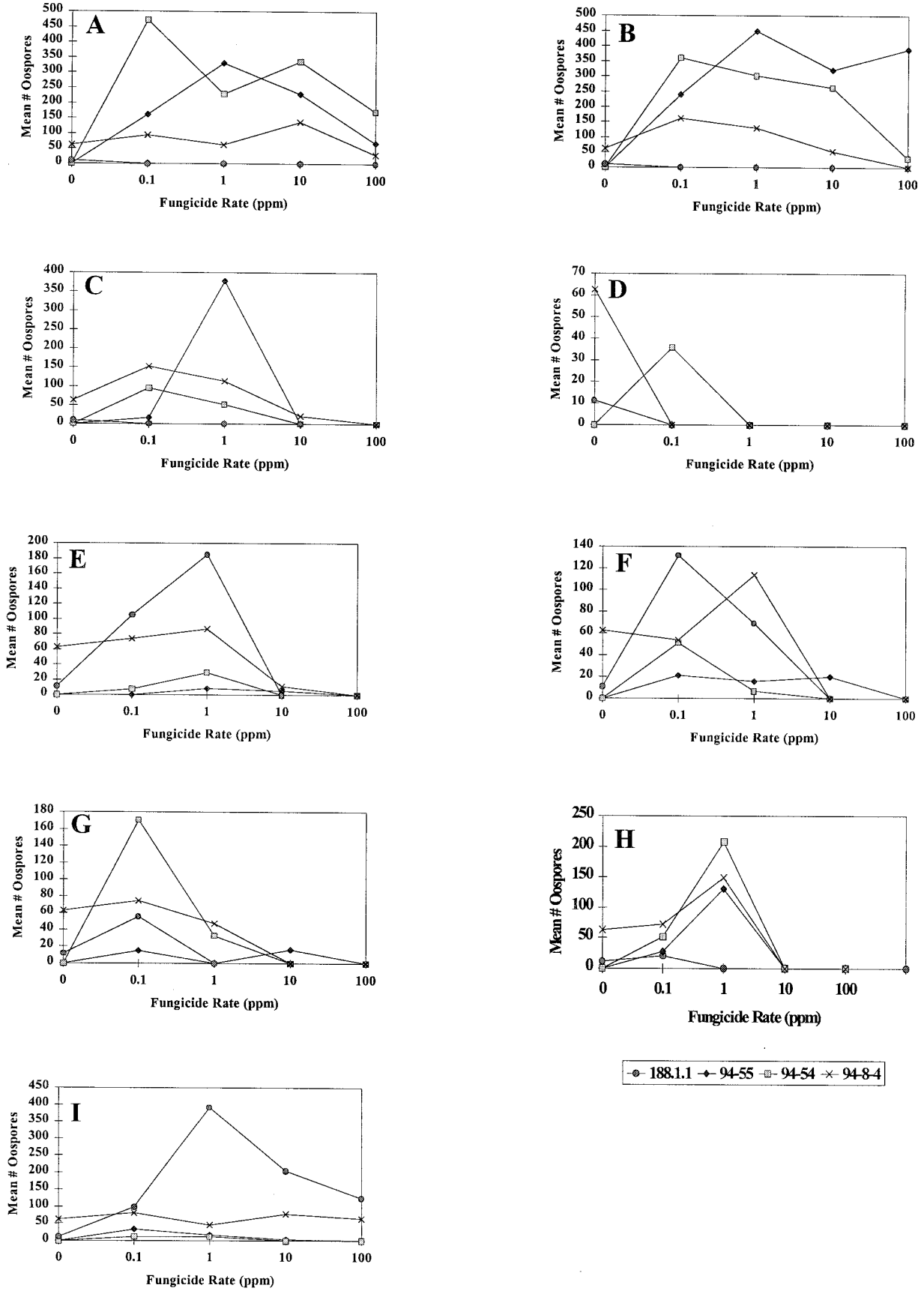

Fig. 1. Mean number of oospores formed after 3 months by isolates of *Phytophthora infestans* on 0, 0.1, 1, 10, and 100 µg a.i./ml of **A,** Ridomil 2E; **B,** Ridomil Gold EC; **C,** Ridomil Gold + Copper; **D,** Ridomil Gold + Bravo; **E,** Maneb; **F,** Curzate; **G,** Manzate; **H,** Acrobat MZ; and **I,** Benlate.

Fig. 1). Isolate 188.1.1 formed oospores in the presence of five of the fungicides at concentrations of 1 µg a.i./ml or less (Table 4, Fig. 1). However, isolate 188.1.1 formed oospores in the presence of Benlate at all concentrations tested (Table 4, Fig. 1I).

Oospore viability. Variable numbers of oospores formed on the fungicide-amended media so sample size was also variable for testing oospore viability (Table 6). Oospore viability ranged from 4.9 to 52.3% across all isolates and fungicides (Table 6). Oospore viability from control plates that were not amended with fungicides ranged from 20 to 33%. Viability of oospores from metalaxyl resistant isolates formed on Ridomil 2E and Ridomil Gold EC ranged from 20.4 to 31.5%. A subset of the oospores were germinated according to the method of Chang and Ko (4), Germination rates were very low, generally less than 5%, and not all germinating oospores resulted in viable progeny.

Phenotypic expression of mating type. Isolates grown on rye B media and not exposed to fungicides in vitro or on fungicide-amended rye B media were tested to determine mating type in paired crosses with known tester isolates on nonamended rye B agar. Isolates 94-55 and 188.1.1 are the A1 mating type and isolates 94-54 and 94-8-4 are the A2 mating type. Isolates that had not been exposed to fungicides in vitro formed oospores with the opposite mating type in repeated crosses on nonamended rye B agar as expected (Table 7). In contrast, isolates 94-55, 94-8-4, and 94-54, obtained from Ridomil Gold-amended media at 1 µg a.i./ml, formed oospores with tester isolates of both the A1 and A2 mating types in paired crosses in repeated experiments (Table 7). Similarly, isolates 94-55 and 94-8-4, obtained from media amended with 10 µg a.i./ml Ridomil Gold, formed oospores with both the A1 and A2 tester isolates (Table 7). Isolate 94-55, obtained from media amended with 10 µg a.i./ml Ridomil 2E, formed oospores with both tester isolates (Table 7). Similarly, isolate 94-8-4, obtained from media amended with 1 µg a.i./ml of Benlate, formed oospores with both the A1 and A2 tester isolates (Table 7).

TABLE 5. General linear models analysis of variance of numbers of oospores produced in vitro in response to fungicide, concentration, and isolate of *Phytophthora infestans*

Source of variation[a]	df	Mean square	*Pr* > *F*
Fungicide	8	65.45	0.0001
Concentration	3	147.99	0.0001
Fungicide × concentration	24	7.99	0.0001
Isolate	3	44.34	0.0001
Fungicide × isolate	24	25.54	0.0001
Concentration × isolate	9	7.49	0.0001
Fungicide × concentration Isolate	72	3.34	0.0001

[a] Tattoo C and Bravo were not included in the analysis because the isolates did not form oospores in response to these fungicides. Fungicides were tested in petri dish assays at concentrations of 0.1, 1.0, 10, and 100 µg a.i./ml.

DISCUSSION

We have demonstrated that a wide range of chemically diverse fungicides induce normally heterothallic isolates of *P. infestans* to form oospores in vitro after short periods of exposure to the chemicals. We have also demonstrated that the metalaxyl resistant isolates we tested are more likely to form oospores in response to the fungicides we evaluated than the single metalaxyl sensitive isolate we tested. Third, alterations in the phenotypic expression of mating type were observed in some of these isolates after exposure to Ridomil Gold, Ridomil 2E, and Benlate.

Fungicides such as chloroneb, ethazol, and metalaxyl induce oospore formation in single isolates of heterothallic species of *Phytophthora* (1,3,26,33), however, only a very limited number of fungicides were evaluated in previous work. We examined the differential impact of diverse classes of fungicides on oospore induction in isolates of *P. infestans* that differed in sensitivity to metalaxyl. We expanded the range of fungicides studied to include fungicides most commonly used on potatoes for late blight control, and quantified the impact of the fungicides on numbers of oospores of *P. infestans*.

The numbers of oospores produced in single culture via fungicide induction in our work were not insignificant although the numbers were less than those reported from sexual crosses (35,43). More importantly, the oospores obtained via fungicide induction were viable. Oospore viability ranged from 4.9 to 52.3% in our study. Others have used the staining assay with tetrazolium bromide to determine the viability of oospores derived from sexual crosses and reported viability ranges from 38.8 to 88.2% (35). The maximum percentages of viable oospores derived from fungicide induction in our study were lower than those derived from sexual crosses by others but are within the range reported previously (35). Although the tetrazolium bromide assay may overestimate viability, other methods such as plasmolysis may actually underestimate viability (35). Germination rates of oospores derived from both selfing and outcrossing has been reported to be very low (35, 40). We attempted to germinate some of the oospores observed in these experiments, but germination rates were less than 5% and not all germinating oospores resulted in a viable culture. Thus, germination was not a good indicator of viability so we chose to use a viability staining assay instead. Further work is needed to understand the conditions affecting dormancy and germination of both hybrid oospores and oospores induced by fungicides.

The actual rate of commercial fungicide that *P. infestans* may encounter in the field is variable and depends on the compound applied and environmental factors. We used a range of fungicide concentrations in our work based on the active ingredients for each commercial fungicide. The range of concentrations tested is commonly used for fungicide resistance screening studies. Our results demonstrate that the interaction between fungicide, concentration, and isolate was significant and that the isolates responded differently to different concentrations of the fungicides.

TABLE 6. Viability of oospores obtained from fungicide-amended and nonamended rye B agar media after 3 months

	% Viable				Numbers of oospores			
Fungicide formulation[a]	188.1.1	94-55	94-54	94-8-4	188.1.1	94-55	94-54	94-8-4
Control	20	–	–	33.3	15	–	–	66
Ridomil 2E	–	31.5	20.4	29.6	–	108	319	267
Ridomil Gold	–	29.5	28.6	24.7	–	268	21	239
Maneb	22.8	18.8	47.8	31.7	193	16	23	126
Ridomil Gold + Copper	–	ND	52.9	20.2	–	ND	17	173
Curzate	34.3	52.3	16.7	18.3	178	44	30	164
Acrobat MZ	30.6	44.1	15	24.1	49	34	133	145
Manzate	4.9	30	29.4	23.8	162	10	109	172
Benlate	22.9	ND	15.4	27.2	894	ND	13	335

[a] Fungicide treatment from which oospores were removed. Percent viability calculated based on all concentrations (0.1, 1, 10, and 100 µg a.i./ml) of each fungicide combined for each isolate. Number of tetrazolium bromide-treated oospores counted for all concentrations of each fungicide, including oospores from all replications. – indicates no oospores produced on this treatment. ND = not determined.

Generally, the largest numbers of oospores formed at the lower concentrations of each fungicide. This was probably due to the fact that growth of most isolates was greatly reduced or completely suppressed at high concentrations of the fungicides. However, two of three metalaxyl resistant isolates studied grew and produced relatively high numbers of oospores on media amended with high concentrations of Ridomil Gold and Ridomil 2E.

The presence of fungicide-induced oospores in the field may represent a further means for creating genetic variability in the pathogen population. In recent years there has been concern that the coincidence of the A1 and A2 mating types of *P. infestans* in the same field could lead to outcrossing, generating strains with increased pathogenicity, or higher levels of resistance to fungicides. There have already been several reports of the possible occurrence of sexual reproduction in field populations (15,21,44,49). However, these reports nearly always assume that the presence of the A2 mating type arose from migration from Mexico (18,20). Judelson (25) indicated that selfing should be considered in the evolution of *P. infestans* because in vitro studies from sexual crosses reveal that certain isolates of *P. infestans* appear more prone to selfing than to outcrossing. Our results demonstrate that many commercially formulated fungicides induce oospore formation in certain isolates of *P. infestans*.

Our data indicate that some of the fungicides we tested cause alterations in the phenotypic expression of mating type in certain isolates of *P. infestans*. Isolates exposed to Ridomil 2E or Ridomil Gold formed oospores with the same and opposite mating type in controlled crosses in our experiments. Further work is needed to determine whether the other commercial fungicides cause a similar response. It is unclear whether the phenotypic change in mating type expression that we observed is a result of ameiotic production of oospores via apomixis or meiotic formation or true selfing. We can not rule out the possibility of somatic mutations due to fungicide exposure. Further experiments are needed using molecular analysis before a genetic mechanism can be assigned to the biological phenomenon we have observed.

Sexual compatibility in *P. infestans* is thought to be determined by the interaction between diffusible mating hormones and their receptors (27). Where a conversion of mating type was noted previously, both hormone production and reception were changed in all sexual variants resulting from aging or fungicide treatment (3,26). The presence of a receptor that represses the expression of the A1 mating type with one molecular configuration and A2 mating type with another configuration was postulated (26). It was further postulated that aging and fungicide treatment changed mating type through alteration of the molecular configuration of the repressor (3,26). Presumably, the presence of both mating types in a single culture would lead to oospore formation due to mating type conversion.

The commercially formulated fungicides we tested may be acting as hormone mimics by binding to the receptors for the mating type hormones in *P. infestans*. The chemicals we examined that have a demonstrated effect on the induction of oospore formation come from several different classes of fungicides with different molecular structures (Table 2). Many hormone mimicking or endocrine disrupting chemicals have been reported from diverse classes of fungicides (7,9,29). It has been reported that synthetic compounds do not have to resemble the chemical structure of the natural hormones or growth factors that they mimic or disrupt (29). The mating type hormones in *P. infestans* have not been purified and their receptor sites have not yet been identified so we can not support or refute the hypotheses that the commercially formulated fungicides we examined act as hormone mimics. However, the isolates we used may provide a valuable in vitro model system for testing the effects of a range of agricultural chemicals on hormone systems in a culturable organism in the future.

Recently, a model was proposed to explain the basis of mating type determination and unusual segregation of the chromosomal region containing the mating type locus of *P. infestans* (24). In developing this model a group of isolates of *P. infestans* were identified that yielded unusually large numbers of self-fertile progeny from in vitro sexual crosses. Molecular analyses demonstrated that markers linked to the mating type locus in these isolates were more variable than were control loci not linked to the mating type locus. The loci linked to the mating type locus in these isolates were commonly duplicated, translocated, absent, or present in a homozygous condition (24). It was suggested that these self-fertile isolates are trisomic for the mating type alleles and that perhaps the expression of the mating type genes is not closely regulated or balanced in these isolates. Isolates described in our study may be representative of the isolates described by Judelson (24). It is unclear how commonly these types of isolates occur in nature. Potentially, commercially formulated fungicides may induce oospore formation due to the instability of the mating type locus in these isolates. On the other hand, the commercially formulated fungicides themselves may be causing the instability of the mating type locus. Further genetic analysis is now needed using the molecular markers linked to the mating type locus to test these hypotheses.

The role of oospores in the population biology of late blight epidemics is poorly understood. Oospores have been reported in naturally occurring tissue infected with *P. infestans* (6,14, 19,31,38,39,45). If oospores occur in nature either through selfing or outcrossing they represent a mechanism for increased genetic variation in pathogen populations as well as enhanced survivability of the pathogen (14,18,43,45). The coincidence of both A1 and A2 mating types in commercial potato and tomato growing regions throughout most of the world has resulted in concern that outcrossing may generate strains with increased aggressiveness and virulence or higher levels of fungicide resistance. Our work demonstrates another potential avenue by which oospores could be produced in this pathosystem and warrants further studies in plants.

Migrations of virulent and fungicide-resistant strains in the past two decades have led to a worldwide resurgence of potato and tomato late blight (18). Copious amounts of chemicals are applied annually to potato and tomato production areas in an effort to control late blight. These results demonstrate that the fungicides applied to manage this disease have major nontarget effects on the reproductive biology of the pathogen. Studies are now in progress to examine the effects of some of these fungicides on oospore formation in vivo. If the in planta behavior of *P. infestans* is similar to that observed in vitro, fungicides used for control of this devastating disease may have considerable impact on the reproduction, survival, and epidemiology of this important plant pathogen.

TABLE 7. Mating type determination of isolates recovered from nonamended or fungicide-amended rye B media

Isolate	Treatment[a]	Type	Oospore production[b]	
			A1 tester	A2 tester
188.1.1	Control	A1	0/1	1/1
94-55	Control	A1	0/3	3/3
94-8-4	Control	A2	3/3	0/3
94-54	Control	A2	4/4	0/4
94-55	Ridomil Gold, 1 µg a.i./ml	A1	4/10	9/10
94-8-4	Ridomil Gold, 1 µg a.i./ml	A2	5/5	4/5
94-54	Ridomil Gold, 1 µg a.i./ml	A2	5/5	5/5
94-55	Ridomil Gold, 10 µg a.i./ml	A1	3/5	5/5
94-8-4	Ridomil Gold, 10 µg a.i./ml	A2	4/5	3/5
94-54	Ridomil Gold, 10 µg a.i./ml	A2	0/1	0/1
94-55	Ridomil 2E, 10 µg a.i./ml	A1	2/2	1/2
94-8-4	Benlate, 1 µg a.i./ml	A2	2/2	2/2

[a] Fungicide treatment and concentration from which agar plug was removed from oospore forming sector for pairing with known tester isolates. Control cultures came from isolates grown on rye B media not amended with fungicides.

[b] Number of plates with oospores formed in pairings with known tester isolates. All crosses were repeated at least twice.

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