Environmental and Genetic Factors Influencing Self-Fertility in *Phytophthora infestans*

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ABSTRACT

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Phytophthora infestans is generally regarded as heterothallic—requiring physical proximity of two individuals of different mating type (A1 and A2) for oosporogenesis. Recent reports of limited selfing in young cultures of this oomycete stimulated us to investigate factors contributing to the phenomenon. The ability to produce oospores rapidly (within 2 weeks) in pure, single individual cultures (self-fertility) was tested in 116 individual isolates. The 116 isolates were from geographically diverse locations (16 countries) and were genetically diverse. Mating type and growth medium were the most prominent factors in determining if an isolate would be self-fertile. The majority of A2 isolates (45 of 47 tested) produced oospores when grown on a 50:50 mixture of V8 and rye B me-

dium. In contrast, the majority of A1 isolates (65 of 69 tested) did not produce oospores on this medium. None of the 116 isolates produced oospores when grown on rye B medium (with no V8 juice). Further tests on representative A1 and A2 isolates revealed that oatmeal agar, tomato juice agar, and V8-juice agar all induced the A2 mating type isolate to produce oospores but did not induce the A1 mating type isolate to produce oospores. Calcium carbonate and pH did not alter the self-fertile oospore production in either A1 or A2 mating type isolates. For in vivo tests, the application of fungicide to potato or tomato leaf tissue either before or after inoculation did not stimulate any individual isolate (one A2 and one A1 isolate) to produce oospores in infected tissue. However, in all of the controls for all experiments (in vivo and in vitro), many oospores were produced rapidly if both strains grew in physical proximity.

Additional keywords: growth media, late blight.

The role of sexual reproduction in the biology and epidemiology of *Phytophthora infestans* (Mont.) De Bary, causal organism of late blight of potato and tomato, has become increasingly important in the last decade (10,38). Recent migrations of A1 and A2 mating type isolates (14) and the occurrence of resistance to certain fungicides used to control these isolates have created the risk of sexual reproduction in the field (21,25). Presumably sexual reproduction, and thus, the formation of oospores will enable a nonparasitic survival mechanism for the pathogen in addition to providing an opportunity for production of new genotypes in areas of the world where neither of these events was known previously (9).

In heterothallic species of *Phytophthora*, an interaction between two mating types (A1 and A2) is generally necessary for oospore formation (13,37). However, there are reports of oospores generated in pure culture of a heterothallic species (16,33,36). Several studies have shown that older cultures of *Phytophthora* spp. form oospores in pure culture, however younger cultures do not (2,4,42). While oospore production in older cultures of *Phytophthora* spp. does not appear to be mating-type specific, there are certain events that evoke oospore production in younger isolates of A2 mating type but not A1 mating type. One such event is wounding a culture by cutting with a cork borer or scalpel (32). Exposure to avocado root extract induces oospore formation within 7 days in pure cultures of A2 but not A1 mating type isolates of *P. cinnamomi* (43). Two *Trichoderma* spp. produce a volatile compound that cause the production of oospores in A2

cultures of many *Phytophthora* spp. (3,30,31). Several fungicides evoke oospore production in isolates of *P. capsici*, *P. cinnamomi*, *P. infestans*, and *P. parasitica* in culture (1,6,29,40,41).

A recent study in our laboratory has shown that isolates of the US-7 and US-8 clonal lineages of *P. infestans* (both are A2 mating type) were able to rapidly produce viable oospores in pure culture (36). This self-fertility was not due to the age of the culture, contamination, mixture of isolates, or to heterokaryosis, because single-zoospore (uninucleate) isolates derived from self-fertile isolates were phenotypically and genotypically indistinguishable from the parental isolate. In addition, by analyzing the *glucose-6-phosphate isomerase* (*Gpi*) allozyme patterns of eight oospore progeny, at least some of the progeny produced in pure culture were recombinant (products of true selfing; 36).

In this report, we continue our study of rapid oospore production in *P. infestans*. By examining this phenomenon in laboratory culture we were able to test the influence of genetic factors such as mating type and genotype, as well as cultural factors, such as medium composition and pH on self-fertility. Additionally, we report on attempts to induce self-fertility in plant tissue.

MATERIALS AND METHODS

Isolates. This study utilized 116 isolates of *P. infestans* from 16 countries collected between 1981 and 1997 and included isolates that were diverse in mating type (69 A1 isolates and 47 A2 isolates), *Gpi* genotype (10 genotypes), and RG57 genomic fingerprint (more than 25 fingerprint patterns; Table 1). All *P. infestans* isolates were obtained from the culture collection at Cornell University. Additionally, two isolates (one A1 and one A2 mating type) each of *P. cinnamomi*, *P. cambivora*, *P. cryptogea*, *P. drechsleri*, and *P. nicotianae* were obtained from A. Drenth (University of Queensland, Australia). All isolates were main-

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tained on rye B medium (5) and were subcultured using a cork borer to cut a 5-mm diameter disk of agar containing mycelium and transferring this disk to other media.

Oospore production. The ability of the 116 isolates to produce oospores in pure culture was tested by culturing each on a medium composed of 50% rye B (5) and 50% V8 agar (26,36) and on rye B medium alone. Each isolate was tested at least twice with different preparations of V8/rye B agar and new subcultures of each isolate. After 2 weeks each culture was examined microscopically for the presence of oospores and scored as positive or negative.

Production of single-zoospore isolates. Sporangia from 2-week-old cultures grown on rye B medium were washed with distilled H₂O and placed at 4°C for 4 h to induce zoospore release (24). Released zoospores were separated from sporangia and mycelial fragments by filtering through 10- μ m nylon mesh fabric (HD3-10 super nylon mesh; Tetko Inc., Kansas City, MO). The filtrate containing zoospores was plated onto 0.75% (wt/vol) water agar and incubated at 18°C with 18 h of light per day for 5 days. Germinating zoospores were identified with a dissecting microscope, picked from the water agar with a scalpel, and placed on rye B agar (5,36). For some single-zoospore progeny, mating type was determined on agar plates with known A1 and A2 tester isolates (14); *Gpi* genotype was determined by cellulose acetate electrophoresis (18).

Medium composition. The ability of liquid media, CaCO₃, tomato juice agar, and oatmeal agar to induce oospore production was also tested. For this aspect of the study only two isolates of *P. infestans* were used, US940507 (A1 mating type, US-1 clonal lineage) and US940480 (A2 mating type, US-8 clonal lineage). A member of the US-1 clonal lineage was used, because this lineage is widely used in *P. infestans* research. A member of the US-8 clonal lineage was used because it is a very common and destructive lineage throughout the United States and Canada (14). Specific isolates in each lineage were chosen because they are commonly used in our laboratory. The additional media tested were standard V8 agar at pH 6.0 (26), V8 broth, V8 agar without CaCO₃ adjusted to pH 6.0 with 5 N NaOH, rye B agar, rye B agar with 2 g/liter CaCO₃, pea broth (17), pea broth with 2 g/liter CaCO₃, oatmeal agar (33), and 20% (vol/vol) tomato juice agar (identical to V8 agar except V8 juice was replaced with tomato juice). No media were clarified, however rye B agar and pea broth are both naturally quite clear. Each medium was tested in two separate experiments with different preparations of media and new subcultures of each isolate. In addition to placing each isolate on each medium individually, the two isolates (A1 and A2 mating type) were paired on each medium as a positive control to ensure that oospore formation could occur. The presence or absence of oospores was determined microscopically and scored as positive or negative.

The effect of pH on oospore formation was tested with rye B agar and V8/rye B agar which were prepared and autoclaved and the pH was adjusted to 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, or 7.5 using either 1 N HCl or 5 N NaOH. For each of these experiments a single 5-mm agar disk containing isolate US940507 (A1) or US940480 (A2) was placed in the center of a petri dish containing the medium. As a positive control, both were paired on each medium as described above. A preliminary experiment was done with rye B agar with a pH range of 5.0 to 7.5. Subsequently, experiments were done with V8/rye B agar with a pH range of 5.0 to 7.5 in one and a pH range of 4.5 to 6.5 in the other. Each treatment was replicated three times (isolate-pH-medium combination). Oospore production was determined by removing two agar plugs (5 mm in diameter) from each plate (one from near the center of the culture and one from the edge). Each culture plug was squashed between a microscope slide and a cover slip and all oospores in each plug were counted microscopically. A correlation analysis between pH and number of oospores was performed using SAS Systems (version 6.11, SAS Institute Inc., Cary, NC).

Diffusion through polycarbonate. To determine if compounds in V8 agar necessary to induce oospore formation were water soluble, sterile 0.2 μ m polycarbonate membranes were used (45 mm in diameter; Millipore Corp., Boston), which are nonpenetrable by *P. infestans* hyphae but allow water and water-soluble compounds to pass through (34,35). Two-week-old cultures of *P. infestans* isolates grown on rye B agar were used in this study. In addition to isolates US940480 and US940507, isolate US970001, a member of the US-17 clonal lineage, was added to the study, because of recent epidemics of late blight on tomato in eastern United States caused by the US-17 clonal lineage (19). Mycelial disks (20 mm in diameter) were cut from each culture and placed individually in petri dishes (55 mm in diameter). Two replicates (20-mm disks of agar with mycelium) from each isolate were used for each of the following experimental treatments: (i) no treatment; each mycelial disk was placed mycelial side up in the empty dish; (ii) polycarbonate alone; each mycelial disk was placed in the dish and covered with a sheet of polycarbonate membrane; (iii) polycarbonate + rye B; each mycelial disk was placed in the dish and covered with polycarbonate membrane and a 20-mm disk (nonmycelial) of rye B agar was placed on top; and (iv) polycarbonate + V8; each mycelial disk was placed in the dish and covered with polycarbonate membrane and a 20-mm disk (nonmycelial) of V8 agar was placed on top. Mycelia of *P. infestans* could not grow on, around, or through the polycarbonate membrane, and no mycelium was detected in any nonmycelial agar disks at the end of the experiment. All petri dishes were wrapped and incubated at 15°C in the dark for 2 weeks. This experiment was done twice, for a total of four replicates per treatment. The total number of oospores in each 20-mm mycelial disk was counted microscopically by squashing the agar between slide and cover slip.

Production of oospores in leaflets. Detached leaflets of *Solanum tuberosum* L. cv. Superior, and *Lycopersicon esculentum* Mill. cv. Vendor were used in this experiment. Leaflets were detached from the middle third of 6-week-old plants and inoculated with two droplets (25 μ l) of sporangia (20,000 per ml) from isolate US940480 (A2 mating type, US-8 clonal lineage), US940507 (A1 mating type, US-1 clonal lineage), or US970001 (A1 mating type, US-17 clonal lineage). As a control, leaflets were inoculated with a mixture of either US940507 (A1) and US940480 (A2) or US970001 (A1) and US940480 (A2) using sporangial suspensions containing a total of 20,000 sporangia per ml with equal numbers of the two isolates. Inoculations were made in small moist chambers (inverted petri dishes containing solidified water agar [1.5%, wt/vol] in the base) with two leaflets per chamber. Leaflets were incubated in the dark at 20°C. Two chambers (four leaflets with two inoculation sites per leaflet) were sampled weekly for 6 weeks to determine if oospores were present. This was done by removing 1.5-cm disks containing the initial inoculation sites and some surrounding leaf tissue from the leaflet and boiling the disks in 95% ethanol for 1 min to clear the tissue (36). Presence or absence of oospores was determined by microscopically examining each 1.5-cm disk in its entirety.

Effect of fungicides on production of oospores in leaflets. The influence of diverse fungicides on oospore formation in infected leaflets was also investigated. *P. infestans* isolates US940507 (US-1, A1) and US940480 (US-8, A2) were used in this experiment. Leaflets were inoculated with two droplets (25 μ l) of sporangia (10,000 per ml) from US940507, US940480, or with a mixture of US940507 and US940480 sporangia. Leaflets were either floated on water in a 150-mm petri dish (the first time the experiment was performed) or placed in small moist chambers as described above (the next two times the experiment was performed), with three leaflets per dish. All leaflets were maintained in a 20°C growth chamber in the dark except when sprayed with fungicides.

Fungicides were applied at the labeled-field rate with 100 ml of each fungicide mixed per application. Treatments and rates were as follows: Acrobat (dimethomorph + mancozeb), 1.25 g/100 ml

(2.25 lb in 21.5 gal/A); Bravo Weather Stik (chlorothalonil), 0.87 ml/100 ml (1.5 pt in 21.5 gal/A); Curzate 60 WDG (cymoxanil + mancozeb), 0.7 g/100 ml (1.25 lb in 21.5 gal/A); Ridomil 4EC (metalaxyl), 0.12 ml/100 ml (3.2 oz in 21.5 gal/A); Tattoo C (propamocarb + chlorothalonil) 1.4 ml/100 ml (2.3 pt in 21.5 gal/A). A water spray was included as a control. All treatments were applied with hand-held spray bottles. The bottles were calibrated by spraying water on microscope slides of known area. The microscope slides were weighed after application of water, and distance and spray time were adjusted for an application of $\approx 2 \mu\text{l}$ of water per cm^2 . New fungicide suspensions were made for each application. Fungicides were applied at 12 h before inoculation and 24, 48, and 72 h after inoculation.

A randomized complete block design was used for the treatment combination of *P. infestans* isolate–fungicide–time for a total of 72 treatments. There were three replicates per treatment and a replicate consisted of three inoculated leaflets (one moist chamber) with two inoculation sites per leaflet. Thus, there were 18 leaf disks sampled and counted per treatment. The experiment was performed three times.

To evaluate the fungicide effect on oospore production, the numbers of oospores in treated leaflets were quantified. Leaf disks (8-mm diameter) were cut from the inoculation sites 14 days after inoculation, macerated in 3 ml of sterile distilled water, and mixed thoroughly. The total number of oospores in a 50- μl aliquot was counted microscopically by mounting the droplet on a slide and scanning each field of view for oospores using a 10 $\times$ objective lens. The mean number of oospores per square centimeter was calculated.

RESULTS

Oospore production on V8/rye B agar. Most A2 isolates produced oospores on V8/rye B agar, however most A1 isolates did not (Table 1). All cultures grew normal on the V8/rye B medium with aerial mycelium and none had appressed-mycelium which would be characteristic of a true cross (16,36). The V8/rye B mixture was used, because some isolates would not grow on V8 agar. For A2 mating type isolates, 37 of 47 produced oospores on V8/rye B agar in each trial of the experiment and 45 of 47 produced oospores in at least one trial of the experiment (Table 1). Among A2 isolates tested, only two isolates from Japan (JP880003 and JP880012) were negative for oospore formation on V8/rye B agar every time they were tested. Eight additional isolates (five from Mexico, two from Japan, and one from Poland) produced oospores in one of two or one of three trials of the experiment (Table 1).

Four of sixty-nine A1 isolates were unusual in that they produced oospores on V8/rye B agar, and of these, two were from Mexico and two were from the United Kingdom (UK). Of the two unusual A1 isolates tested from Mexico, one (MX830086) produced oospores each time it was grown on V8/rye B agar with many oospores per plate and the other (MX885597) produced very few oospores in only one of three trials. Neither isolate MX830086 nor ME885597 produced oospores on rye B agar. Of the two unusual A1 isolates from the UK (UKXX0011 and UK820012), both made many oospores each time they were placed on V8/rye B, but no oospores were observed when isolates were grown on rye B.

Because isolates MX830086, UKXX0011, and UKXX0012 were the only A1 mating type isolates of *P. infestans* tested that consistently produced oospores, we attempted to obtain cultures derived from single (uninucleate) zoospores from each of these isolates. Unfortunately, neither of the isolates from the UK produced many sporangia (although both produced large amounts of aerial hyphae), and no zoospores were obtained from these isolates. Eight single-zoospore isolates were obtained from MX830086. All produced oospores on V8/rye B agar and were identical to the parental isolate in mating type (A1) and *Gpi* genotype (100:100).

Upon testing other species of *Phytophthora* for self-fertility on V8/rye B agar, oospores were observed in only one of three tests for one isolate (*P. cambivora* isolate UQ2633 [A1 mating type]). In this replicate, oospores were dispersed throughout the plate and the culture was fluffy with no appressed-mycelium characteristic of a true cross (16,36). No oospores were observed in the A2 mating type isolate of *P. cambivora* or in A1 or A2 mating type isolates from *P. cinnamomi*, *P. cryptogea*, *P. drechsleri*, or *P. nicotianae*.

Medium composition. All of the isolates were tested for oospore production on V8/rye B as well as rye B agar, and two isolates were tested on additional media. Isolates were tested on rye B agar to be certain that the presence of V8 was inducing oospore formation. None of the 116 isolates tested produced oospores on rye B agar.

In a further study of the effect of medium composition on oospore formation, nine media were tested using two *P. infestans* isolates US940507 (A1 mating type, US-1 clonal lineage) and US940480 (A2 mating type, US-8 clonal lineage). Isolate US940507 did not produce oospores on any medium tested (Table 2). However, isolate US940480 produced oospores on 20% V8 agar, 20% V8 agar without CaCO_3 , oatmeal agar, and 20% tomato juice agar (Table 2). Interestingly, US940480 alone did not produce oospores in any of the liquid media tested. Matings between US940507 and US940480 produced oospores on each medium (Table 2).

pH of V8/rye B agar. Oospores were not produced on rye B agar by either isolate US940507 or US940480 at any pH level tested (5.0 to 7.5). On V8/rye B agar at pH levels ranging from 4.5 to 7.5, no oospores were produced by isolate US940507. In contrast, isolate US940480 produced oospores at all pH levels tested. The number of oospores per plug was similar within the same plate (comparing the plug taken from the center of the plate and the edge of the plate), however oospores were highly variable between plates at all pH levels and ranged from 5 to 155 oospores. While there appeared to be an increase in oospore production at pH 6, the number of oospores in an agar plug was highly variable among replicates (ranging from 5 to 133 oospores per plug). There was no significant correlation ($r = -0.05$; $P = 0.707$) between the number of oospores produced by isolate US940480 and pH.

Diffusion through polycarbonate. Three *P. infestans* isolates were included in this experiment (US940480, US940507, and US970001). Exposure of cultures growing on rye B agar to V8 agar medium induced oospore formation in the A2 isolate (US940480) despite the partition imposed by the polycarbonate membrane (Table 3). This treatment resulted in sporadic oospore production in the two A1 isolates. Isolate US940507 produced one oospore in one disk in one of the four repetitions. Isolate US970001 produced 10 to 15 oospores in three of the four repetitions. Exposure to polycarbonate alone or polycarbonate and rye B resulted in sporadic oospore production (3 to 20 oospores per disk) in the A2 isolate but not in either of the A1 isolates tested. Exposure of isolate US940480 to V8 agar across a polycarbonate membrane consistently resulted in production of more oospores (50 to 100 oospores per disk) than exposure to polycarbonate alone or to polycarbonate and rye B medium (Table 3).

Oospore production in leaflets. The ability of three isolates to produce oospores in host tissue was investigated. In tomato or potato leaflets infected by a mixture of US940507 (US-1, A1) plus US940480 (US-8, A2) or US970001 (US-17, A1) plus US940480, oospores were observed in all leaflets within 1 week after inoculation. No oospores were observed in diseased tissue infected individually with US940507, US940480, or US970001 in any weekly observation through 5 weeks after inoculation. However, at 6 weeks postinoculation, several weeks after the leaf tissues had rotted, a few oospores (fewer than 10 per 1.5-cm disk) were observed in only three of the nine potato leaflets inoculated with US940480. No oospores were observed in tomato leaflets inoculated with US940480 at 6 weeks. Additionally, a few oospores

(fewer than five/lesion) were observed in only two of the nine potato leaflets inoculated with US970001 at 6 weeks postinoculation but no oospores were observed in tomato leaflets at 6 weeks after inoculation. No oospores were observed in either potato or tomato leaves infected by US940507.

Effect of fungicides on production of oospores in leaflets.

The presence of fungicides did not stimulate oospore production by either US940507 (US-1, A1) or US940480 (US-8, A2) within 14 days after inoculation, whether fungicides were applied to leaflets before or after inoculation. No oospores were observed with either isolate in any of the leaflets sprayed with any of the fungicides at any time (12 h before inoculation to 72 h after inoculation). Additionally, no oospores were observed at 14 days

after inoculation when leaflets were inoculated with individual *P. infestans* isolates and sprayed with water (Table 4).

However, when leaflets were coinoculated with both isolates (US940507-A1 and US940480-A2), oospores typically formed if a lesion formed. Frequently, fungicides suppressed the establishment of a lesion and therefore the occurrence of oospores. Some fungicides also suppressed oospore production when lesions were formed (Table 4). When leaflets were coinoculated with both the A1 and A2 isolates and sprayed with water, large numbers of oospores were produced within 14 days after inoculation. When fungicides, to which isolates were sensitive, were applied prior to inoculation, infection and consequently oospore production were prevented. Only isolate US940507 was sensitive to metalaxyl, and

TABLE 1. *Phytophthora infestans* isolates used in this study and oospore production on V8/rye B agar.

Isolate	Country	Date ^a	Clonal lineage ^b	MT ^c	Gpi ^d	Oospore ^e	Source/Citation ^f
AUX0002	Australia	1990		A1	86/100	—,—	(15)
SU900005	Belorussia	1990	PO-4	A1	90/100	—,—	(15)
SU900008	Belorussia	1990	PO-4	A1	90/100	—,—	(15)
SU900010	Belorussia	1990	PO-4	A1	90/100	—,—	(15)
SU900011	Belorussia	1990	PO-4	A1	90/100	—,—	(15)
SU900012	Russia	1990	PO-57	A2	100/100	+,+	(16)
SU900016	Russia	1990	PO-57	A2	100/100	+,+	(16)
CA910057	Canada	1991	US-6	A1	100/100	—,—	(14)
CA910058	Canada	1991	US-6	A1	100/100	—,—	(14)
CA940056	Canada	1992		A2	100/111	+,+	(21)
EC950017	Ecuador	1995		A1	100/100	—,—	(11)
EC950073	Ecuador	1995		A2	100/100	+,+	(11)
EC96088	Ecuador	1996		A1	86/100	—,—	(11)
EC96132	Ecuador	1996		A1	86/100	—,—	(11)
IS930001	Israel	1993		A1	100/100	—,—	CUPCC
IS940001	Israel	1994		A1	100/100	—,—	CUPCC
IS960001	Israel	1996		A1	100/100	—,—	CUPCC
IS970001	Israel	1997		A1	100/100	—,—	CUPCC
IS970002	Israel	1997		A1	100/100	—,—	CUPCC
JP880001	Japan	1988	JP-1	A2	100/100	+,+	(23)
JP880002	Japan	1988	JP-1	A2	100/100	—,—	(23)
JP880003	Japan	1988	JP-1	A2	100/100	—,—	(23)
JP880004	Japan	1988	JP-1	A2	100/100	+,+	(23)
JP880005	Japan	1988	JP-1	A2	100/100	+,+	(23)
JP880010	Japan	1988	US-1	A1	86/100	—,—	(23)
JP880012	Japan	1988	JP-1	A2	100/100	—,—	(23)
JP880015	Japan	1988	US-1	A1	86/100	—,—	(23)
JP880017	Japan	1988	US-1	A1	86/100	—,—	(23)
JP880023	Japan	1988	US-1	A1	86/100	—,—	(23)
JP880026	Japan	1988	JP-1	A2	100/100	+,+	(23)
MX830086	Mexico	1983		A1	100/100	+,+	(20)
MX830096	Mexico	1983		A1	100/100	—,—	(20)
MX830053	Mexico	1983		A2	100/100	+,+	(20)
MX830055	Mexico	1983		A2	100/100	+,+	(20)
MX830058	Mexico	1983		A1	100/100	—,—	(20)
MX830061	Mexico	1983		A2	100/100	+,+	(20)
MX830068	Mexico	1983		A1	100/100	—,—	(20)
MX830071	Mexico	1983		A2	100/100	—,—	(20)
MX830072	Mexico	1983		A1	100/100	—,—	(20)
MX830075	Mexico	1983		A2	100/100	+,+	(20)
MX860122	Mexico	1986		A2	100/100	+,+	(20)
MX880152	Mexico	1988		A2	100/111/122	+,+	(20)
MX885078	Mexico	1988		A2	86/86	+,+	CUPCC
MX885352	Mexico	1988		A1	86/100	—,—	CUPCC
MX885597	Mexico	1988		A1	86/100	—,—	CUPCC
MX885616	Mexico	1988		A2	86/86	—,—	CUPCC
MX885794	Mexico	1988		A1	100/122	—,—	CUPCC
MX885795	Mexico	1988		A1	100/100	—,—	CUPCC
MX885828	Mexico	1988		A1	86/122	—,—	CUPCC
MX885830	Mexico	1988		A1	86/122	—,—	CUPCC
MX890202	Mexico	1989	US-8	A2	100/111/122	+,+	(20)
MX890206	Mexico	1989	US-8	A2	100/111/122	—,—	(20)

(continued on next page)

^a Year isolate was collected.

^b Clonal lineage determined by RG57 genomic fingerprint.

^c Mating type.

^d Glucose-6-phosphate isomerase isozyme pattern.

^e Oospore production on rye B/V8 agar: + = oospores present; — = oospores absent. Comma separates results from different replicates.

^f CUPCC indicates isolate obtained from Cornell University *Phytophthora* culture collection.

metalaxyl was less effective at preventing lesion and oospore formation. All of the fungicides had some efficacy in suppressing lesions and oospore formation when applied within 24 h of inoculation; dimethomorph and cymoxanil were the most effective in reducing oospore formation (<100 oospores/cm²) when applied up to 3 days after inoculation of detached leaflets (Table 4).

DISCUSSION

While *P. infestans* is appropriately regarded as a heterothallic species, the term heterothallic is not absolute when applied to

this species. There have been many reports of oospores in single-isolate cultures of *P. infestans* (7,12,16,28), but the number of oospores in these situations is usually only a small fraction of the oospores produced in response to an individual of the alternate mating type (36). Additionally, in some cases the oospores are produced much more slowly than in true crosses (8,22). We have described the effect of some of the factors that influence oospore production in single-individual isolates.

Mating type was an important factor in predicting whether a single-isolate individual of *P. infestans* might be capable of rapidly producing oospores (Table 1). Most single-isolate individuals

TABLE 1. (continued from preceding page)

Isolate	Country	Date ^a	Clonal lineage ^b	MT ^c	Gpi ^d	Oospore ^e	Source/Citation ^f
MX890208	Mexico	1989	US-8	A2	100/111/122	++,	(20)
MX890222	Mexico	1989	US-8	A2	100/111/122	++,	(20)
MX910014	Mexico	1991		A2	100/111	++,	CUPCC
MX920101	Mexico	1992		A2	111/122	++,	CUPCC
MX930003	Mexico	1993		A2	100/111	++,	CUPCC
PE850015	Peru	1985	US-1	A1	86/100	-,	(15)
PE950006	Peru	1995		A1	100/100	-,	CUPCC
PE950007	Peru	1995		A1	100/100	-,	CUPCC
PE950008	Peru	1995		A1	100/100	-,	CUPCC
PE960005	Peru	1996		A1	100/100	-,	CUPCC
PE96015	Peru	1996		A1	86/100	-,	CUPCC
PE960027	Peru	1996		A1	100/100	-,	CUPCC
PE960032	Peru	1996		A1	100/100	-,	CUPCC
PE96040	Peru	1996		A1	100/100	-,	CUPCC
PE960043	Peru	1996		A1	100/100	-,	CUPCC
PH900006	Philippines	1989	US-1	A1	86/100	-,	(23)
PH910021	Philippines	1991	US-1	A1	86/100	-,	(23)
PO870016	Poland	1987	US-1(PO-1)	A1	86/100	-,	(38)
PO880034	Poland	1988		A2	100/100	++,	(38)
PO880035	Poland	1988	PO-9	A1	100/100	-,	(38)
PO880057	Poland	1988	PO-11	A1	90/100	-,	(38)
PO880058	Poland	1988	PO-12	A1	90/100	-,	(38)
PO900111	Poland	1990	PO-32	A2	100/100	-,+,+	(38)
PO900139	Poland	1990	PO-28	A1	100/100	-,	(38)
PO900149	Poland	1990	PO-50	A1	100/100	-,	(38)
SK910011	Korea	1991	JP-1	A2	100/100	++,	(16)
SK910021	Korea	1991	JP-1	A2	100/100	++,	(16)
UG95007	Uganda	1995	US-1	A1	86/100	-,	CUPCC
UKXX0011	United Kingdom	pre 1984	US-1	A1	86/100	++,	(15)
UK820012	United Kingdom	1982		A1	100/100	++,	(15)
UK890018	United Kingdom	1989		A1	100/100	-,	(39)
UK890020	United Kingdom	1989		A1	100/100	-,	(39)
US810003	United States	1981	US-1	A1	86/100	-,	(14)
US920159	United States	1992	US-6	A1	100/100	-,	(21)
US920199	United States	1992	US-8	A2	100/111/122	++,	(21)
US920219	United States	1992	US-7	A2	100/111	++,	(21)
US920210	United States	1992	US-7	A2	100/111	++,	(21)
US940298	United States	1994	US-16	A1	100/111	-,	(19)
US940349	United States	1994	US-7	A2	100/111	++,	(36)
US940477	United States	1994	US-8	A2	100/111/122	++,	(27)
US940478	United States	1994	US-11	A1	100/100/111	-,	(19)
US940480	United States	1994	US-8	A2	100/111/122	++,	(27)
US940486	United States	1994	US-7	A2	100/111	++,	(27)
US940494	United States	1994	US-12	A1	100/111	-,	(19)
US940499	United States	1994	US-1	A1	86/100	-,	(27)
US940501	United States	1994	US-1	A1	86/100	-,	(27)
US940502	United States	1994	US-14	A2	100/122	++,	(19)
US940504	United States	1994	US-13	A2	100/100	++,	(19)
US940507	United States	1994	US-1	A1	86/100	-,	(27)
US950301	United States	1995	US-8	A2	100/111/122	++,	(36)
US950304	United States	1995	US-11	A1	100/100/111	-,	(19)
US950305	United States	1995	US-11	A1	100/100/111	-,	(19)
US960012	United States	1996	US-8	A2	100/111/122	++,	(19)
US960014	United States	1996	US-17	A1	100/122	-,	(19)
US970001	United States	1996	US-17	A1	100/122	-,	(19)
US970007	United States	1997	US-8	A2	100/111/122	++,	(36)
US970008	United States	1997	US-17	A1	100/122	-,	CUPCC
US970011	United States	1997	US-8	A2	100/111/122	++,	(36)
US970016	United States	1997	US-8	A2	100/111/122	++,	(36)
US970051	United States	1997	US-11	A1	100/100/111	-,	CUPCC
US970064	United States	1997	US-8	A2	100/111/122	++,	CUPCC

of A2 mating type could be stimulated to produce some oogonia and antheridia within 2 weeks. In contrast, in the absence of the alternate mating type, we were unsuccessful in stimulating most individuals of A1 mating type to produce gametangia in the same time span. Importantly, both A1 and A2 mating type isolates appeared to grow at the same rate on V8/rye B medium, and oospore formation was not correlated to mycelial mass. It is possible that A1 isolates would have produced oospores if the cultures were left for an extended period of time, as shown in previous studies (2,4,42). However, this study was specifically aimed at rapid oospore formation, and A2 isolates appear to be more receptive to this rapid induction.

While our sample size with other heterothallic species of *Phytophthora* was much smaller, there appeared to be no generalities applicable to the genus regarding mating type and propensity for rapid oospore production in single-isolate cultures. Of the other species, there was only one isolate (A1 of *P. cambivora*) that we could stimulate rapid production of oospores in a single-isolate culture. We were unsuccessful in stimulating rapid oospore production in other single-isolate cultures of *P. cambivora*, *P. cinnamomi*, *P. cryptogea*, *P. drechsleri*, and *P. nicotianae*.

For the isolates of *P. infestans* capable of producing oospores as single-isolate individuals at least some of the time, some aspects of the culture medium had a noticeable impact. Agar medium containing V8 juice, tomato juice, or oatmeal stimulated oospore production in A2 isolates within 2 weeks. On other media, single-individual cultures did not produce oospores. This was true for any liquid medium we tested and for rye B medium. It is unknown why liquid medium, in particular V8 broth, would not support the formation of oospores in single-individual cultures (Table 2). Tomato juice or V8 juice when added to agar had a positive effect on oosporogenesis even in the presence of rye medium, because when V8 juice was added to rye medium, oospores could be stimulated in capable isolates. CaCO₃ and pH levels between 4.5 and 7.5 had no detectable effect.

TABLE 2. Production of oospores on culture medias using two isolates of *Phytophthora infestans* individually and in combination^a

Medium	US940507 (US-1; A1)	US940480 (US-8; A2)	US940507 × US940480
20% V8 agar (26)	–	+	+
20% V8 broth	–	–	+
20% V8 agar – CaCO ₃	–	+	+
Rye B agar (5)	–	–	+
Rye B agar + 2 g/liter CaCO ₃	–	–	+
Pea broth (17)	–	–	+
Pea broth + 2 g/liter CaCO ₃	–	–	+
Oatmeal agar (33)	–	+	+
20% Tomato juice agar	–	+	+

^a 20% V8 agar contains 2 g/liter CaCO₃ at pH 6.0. 20% V8 agar – CaCO₃ increased to pH 6.0 with 5N NaOH. + and – indicate presence and absence of oospores, respectively (results of two repetitions). Numbers in parenthesis indicate references.

TABLE 3. Induction of oospore production by mycelia grown on rye B agar in response to polycarbonate membrane and media treatments

Medium	US940507 (US-1; A1)	US940480 (US-8; A2)	US970001 (US-17; A1)
Polycarbonate/V8 agar	+	+++	++
Polycarbonate/rye B agar	–	++	–
Polycarbonate alone	–	+	–
Control	–	–	–

^a Number of oospores produced (average of four repetitions) by mycelium grown on a 20-mm disk of rye B agar. Rating scale: – = no oospores; + = 1 to 10 oospores; ++ = 11 to 25 oospores; +++ = 25 to 100 oospores present in each disk.

Experiments involving polycarbonate membranes produced two intriguing results. First, there appears to be a substance in V8 agar that is water-soluble and can diffuse through the polycarbonate membrane and induce oospore formation (Table 3). This result was expected when the experiment was performed with isolate US940480, which produced oospores when grown on V8/rye B medium. However, oospores were also detected in both A1 mating type isolates tested. This was unexpected because neither isolate US940507 nor US970001 had produced oospores in pure culture when grown on V8/rye B medium. The second interesting result was that polycarbonate disks alone stimulated the production of oospores in isolate US940480, which was the isolate most prone to oospore production. The reason for this is unknown, however it seems possible that the effect of the diffusible substance from V8-juice agar and the effect of the polycarbonate membrane are additive and together stimulate oospore production in two A1 isolates.

In contrast to the situation in culture, rapid oospore production did not occur when isolates were grown in potato or tomato leaf tissue. However, at 6 weeks after infection in severely decomposed tissues, single-isolate oospores were detected. At this time, many other microbes also colonized the tissues. Leaf tissue did not specifically suppress oospore production, because when A1 and A2 isolates were coinoculated (with sporangia of an A1 and sporangia of an A2 isolate) on a leaflet of either tomato or potato, oospores were detectable within 1 week after inoculation.

Because others have reported that fungicides can cause oospore production in single isolates of several *Phytophthora* spp. in culture (1,6,29,40,41), we wanted to determine the effect of fungi-

TABLE 4. Effect of fungicides on oospore formation in detached potato leaflets using two isolates of *Phytophthora infestans* individually or in combination

Fungicide ^a	Time ^b	Mean number oospores/cm ²		
		US940507	US940480	US940507 × US940480 ^c
Acrobat	–12	0	0	0
	24	0	0	10.0 ± 17.3
	48	0	0	11.1 ± 16.5
	72	0	0	23.3 ± 40.4
Bravo WS	–12	0	0	0
	24	0	0	31.1 ± 25.3
	48	0	0	2897.8 ± 2408.3
	72	0	0	2712.2 ± 1123.1
Curzate 60 WDG	–12	0	0	0
	24	0	0	0
	48	0	0	24.4 ± 31.0
	72	0	0	87.8 ± 73.8
Ridomil 4EC	–12	0	0	25.6 ± 38.6
	24	0	0	45.5 ± 13.9
	48	0	0	1392.2 ± 1179.7
	72	0	0	1175.6 ± 879.4
Tattoo C	–12	0	0	0
	24	0	0	45.5 ± 50.6
	48	0	0	894.4 ± 788.2
	72	0	0	1596.7 ± 1738.3
Water	–12	0	0	2250.0 ± 117.9
	24	0	0	1624.5 ± 294.9
	48	0	0	744.3 ± 5.7
	72	0	0	1125.0 ± 294.7

^a Active ingredient of trade name fungicides (Acrobat = dimethomorph + mancozeb; Bravo WS = chlorothalonil; Curzate 60 WDG = cymoxanil + mancozeb; Ridomil 4EC = metalaxyl; Tattoo C = propamocarb + chlorothalonil) sprayed on detached potato leaflets. All fungicides were applied at the label-recommended rate.

^b Time of fungicide application. –12 = 12 h before inoculation; 24 = 24 h after inoculation; 48 = 48 h after inoculation; 72 = 72 h after inoculation.

^c ± standard deviation of the mean.

cides on self-fertility of *P. infestans* in plant tissue. Fungicides applied to detached leaflets in laboratory tests had a predictable effect on the production of oospores: if fungicides prevented infection, there were no oospores, whether inoculation was done with single isolates or mixtures of an A1 and A2 isolate. In the absence of fungicides, inoculations with mixtures of an A1 and A2 isolate resulted in lesions and oospores appeared within 1 week after coinoculation.

Several fungicides suppressed the appearance of oospores when a leaflet became infected after coinoculation. Under the conditions of our test, dimethomorph + mancozeb (Acrobat) and cymoxanil + mancozeb (Curzate) were most effective at suppressing the occurrence of oospores in infected leaflets. In the 14 days of observation following inoculation, no oospores were detected in leaflets inoculated with single-isolate individuals whether or not fungicide had been applied. Because this work was done in detached leaflet it is not known if similar results would be obtained in whole plants in the field. However, based on these studies, we hypothesize that oospores would not be produced by a single isolate on plants in the field.

Looking at the combined results presented here, as well as results from previous studies, it appears that the heterothallic nature of *Phytophthora* is leaky. There are many cultural practices that can over-ride heterothallism including age of culture (2,4,42), other organisms (30,31), wounding (32), and addition of fungicide to culture medium (1,6,29,40,41). In these cases, it is possible that the organism is responding to stress. Additionally, A2 mating type isolates are more prone to rapid oospore production in pure culture than A1 mating type isolates when grown on medium containing V8 juice. However, *P. infestans* grows well on the V8/rye B medium and do not appear to be stressed. There appear to be many stimuli that can induce oospore production in *P. infestans*.

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