

## Genetics of *Phytophthora infestans*: Characterization of Single-Oospore Cultures from A1 Isolates Induced to Self by Intraspecific Stimulation

R. C. Shattock, P. W. Tooley, and W. E. Fry

Visiting fellow, research associate, and professor, respectively, Department of Plant Pathology, Cornell University, Ithaca, NY 14853. R. C. Shattock acknowledges the support of the NATO Science Programme and Ciba-Geigy Corporation. P. W. Tooley and W. E. Fry acknowledge support from the USDA (CRGO Grant 0034157) and from Ciba-Geigy Corporation. Permanent address of senior author, School of Plant Biology, University College of North Wales, Bangor, Gwynedd, LL57 2UW, U.K. Accepted for publication 25 September 1985.

### ABSTRACT

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Selfed oospores were produced by A1 compatibility type isolates of *Phytophthora infestans* by pairing with A2 compatibility types of either *P. infestans* or *P. drechsleri* positioned on the opposite sides of a polycarbonate membrane. Nine single-oospore cultures were derived from one A1 isolate, and one single-oospore culture was derived from another A1 isolate. Segregation for some (but not all) observed characteristics was

detected in the progeny. Two of nine progeny from one isolate were segregants for electrophoretic variants of the enzyme peptidase. Hyphae of all nine progeny grew more compactly than those of the parents. There was no segregation for compatibility type. Virulence of two progeny cultures was identical with that of the parent. The other progeny were either nonpathogenic or failed to sporulate in vitro.

Heterothallic species of *Phytophthora* are bisexual and are capable of self-mating and producing oospores if stimulated chemically by the presence of a strain of the opposite compatibility type (7,8), other fungi (2), mechanical damage (11), or organic substances (10). Oospore formation in A1 compatibility type cultures of *P. infestans* in the presence of either A2 isolates of *P. infestans* or other *Phytophthora* species has been reported recently (20,21). In both cases, in vitro stimulation of self-mating in *P. infestans* occurred although hyphal contact between the paired cultures was prevented by a polycarbonate membrane.

These previous studies did not show whether selfed oospores could produce vegetative thalli which would be identical to, or different from, the parent thallus. If oospores resulted from apomixis, then selfed oospores contribute nothing to variability in the pathogen population. If, on the other hand, selfed oospores result from meiosis and recombination, they would be a potential source of variation in this species. The role of selfed oospores in nature, however, is unknown. If selfed oospores do occur and if they produce vegetative thalli, they represent a means of survival for *P. infestans* (21) as well as a mechanism for creating genetic variability.

This research was done to determine if selfed oospores of *P. infestans* could germinate and produce vegetative thalli and to determine if selfed oospores result from automixis or apomixis.

### MATERIALS AND METHODS

**Cultures.** The A1 isolates of *P. infestans* had diverse origins. Two isolates (118 and 127) were collected from the field in New York State. Two isolates (B130 and 40/34) were collected from the field in North Wales. One isolate (BT), derived from a field isolate in Wales, was resistant to chloramphenicol and streptomycin (16). The three isolates from Wales were derived from single zoospores.

Two isolates of A2 compatibility type were used: one was *P. infestans* (isolate 126) (=WV445) (12,20), and the other was *P. drechsleri* (isolate PC3) obtained from Wales (21).

Isolates were cultured on rye A agar (4) or clarified V-8 juice

(C-V8) agar at 18 C in darkness (20). For long-term storage, isolates were placed under mineral oil and held at 18 C in the dark.

**Oospore induction, extraction, and germination.** Oospores were induced by pairing isolates of opposite compatibility type across polycarbonate membranes (pore size, 0.2  $\mu$ m) (Nuclepore Corp., Pleasanton, CA) according to a modification of the method of Shen et al (20). The modifications were that  $\beta$ -sitosterol (40  $\mu$ g/ml) was added to C-V8 agar and that nine paired hyphal plugs (14 mm in diameter) were positioned equidistant from one another in 9-cm-diameter petri dishes. The polycarbonate membrane separated members of a pair. For control treatments, hyphal plugs were paired with fresh sterile C-V8 agar plugs. Petri dishes contained 1.5% distilled water agar (DWA) to maintain high humidity and were incubated in an inverted position. Oospores in hyphae were clearly visible when observed with a dissecting microscope. The number of oospores in each hyphal plug were counted. Oospores were extracted from isolates that produced more than 100 oospores per plug.

Extraction of 1- to 4-mo-old oospores from hyphal mats was accomplished with the aid of water snails (*Ampullaria* spp. and *Planorbis* spp.). Oospores and hyphae scraped from the surface of agar plugs were fed to snails that had been starved for 48 hr (18). Feeding occurred in glass dishes containing several centimeters of distilled water. Fecal pellets containing oospores were collected after 24 and 48 hr. The pellets were concentrated by sedimentation and were then homogenized manually with a glass tissue grinder. The homogenate was treated with HgCl<sub>2</sub> (30  $\mu$ g/ml) for 4-5 min to surface-sterilize the oospores.

Oospores were collected on 47-mm-diameter polycarbonate membrane (pore size, 12  $\mu$ m) by using a vacuum source and were rinsed immediately with sterile distilled water to remove HgCl<sub>2</sub>. Oospores were then resuspended in sterile water (5 ml) and spread over 0.6% DWA in a 9-cm-diameter petri plate. The plates were incubated at 18 C under continuous blue light (430-490 nm) with background white light. Some oospore suspensions were not immediately placed on DWA, but were stored at 4 C in darkness for up to 4 wk until used.

Single-oospore cultures were derived from germinated oospores. The germinated oospores were transferred from DWA to either rye B agar (4) containing vancomycin (200  $\mu$ g/ml) and rifampicin (50  $\mu$ g/ml) or to droplets of particle-free rye A broth containing the above antibiotics. The droplets were positioned on the lid of inverted petri dishes containing 1.5% DWA. Germinating oospores (germlings) were incubated at 20 C in darkness and monitored

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TABLE 1. Relative numbers of selfed oospores produced by different isolates of *Phytophthora infestans* and *P. drechsleri* in pairings with opposite compatibility types across polycarbonate membranes

A1 isolates	Oospores (no.) <sup>a</sup> produced			
	by A1 isolates		by A2 isolates	
	<i>P.i.</i> 126 (A2)	<i>P.d.</i> PC3 (A2)	<i>P.i.</i> 126 (A2)	<i>P.d.</i> PC3 (A2)
118 <sup>b</sup>	+++	++	0	++
127 <sup>b</sup>	++	++	0	+
B130 <sup>b</sup>	+	... <sup>c</sup>	0	...
BT	++	+	0	++
40/34	++	++	0	+

<sup>a</sup> Plus signs are used to represent the approximate number of selfed oospores produced in each pairing by the A1 or A2 isolate. + = between 10 and 100 oospores per plug; ++ = between 100 and 1,000 oospores per plug; +++ = greater than 1,000 oospores per plug. Counts represent an average of nine plugs.

<sup>b</sup> Selfing occurred in control treatments of these isolates.

<sup>c</sup> Not tested.

closely for further hyphal development. Developing colonies were transferred to antibiotic-free rye A agar or C-V8 agar.

**Characterization of progeny.** Parental isolates and their progeny were compared in several respects. Morphology and sporulation were examined on C-V8 agar and rye A agar, respectively. Rates of hyphal extension were determined on rye A agar, and increases in dry weight were determined in 10% C-V8 broth. Compatibility type was determined with tester isolates 515 (A1) and 503 (A2) of *P. infestans* (22).

Virulence of parents and progeny was assessed by inoculating detached leaflets obtained from several different *R*-gene differential potato genotypes with a mixture of sporangia and zoospores by following the method of Shattock et al (15). Inoculated leaflets were incubated in inverted petri dishes containing 1.5% DWA. The differential series included potato genotypes R1; R2; R3; R4; R5; R7; R10; R1,2; R1,4; and R2,3. The cultivar Norchip, which lacks any known *R*-genes, and which has a very low level of race-nonspecific resistance was also included. The isozyme banding patterns at two enzyme loci, glucosephosphate isomerase (*GPI-I*) and peptidase (*PEP*), were determined for parent and progeny cultures. The methods for electrophoresis were those described by Tooley and Fry (22).

## RESULTS

All A1 isolates of *Phytophthora infestans* produced oospores when paired across polycarbonate membranes with A2 isolates of *P. infestans* (Table 1). The A2 isolate of *Phytophthora drechsleri* was only slightly less effective than the A2 isolate of *P. infestans* (isolate 126) in stimulating oospore production in A1 isolates of *P. infestans* (Table 1). The A1 isolates differed in the numbers of oospores produced. Isolate 118 produced more than 1,000 oospores per plug, while B130 produced fewer than 100. The A1 isolates stimulated oospore production in the A2 isolate of *P. drechsleri*, but not in the A2 isolate of *P. infestans* (Table 1).

The requirement for stimulation by A2 cultures for oospore production in the A1 compatibility isolates 118, 127, and B130 was not absolute. A few scattered oospores appeared even when paired with sterile agar across the polycarbonate membrane.

Germination of oospores was observed in only two of the five A1 isolates. Only a very small proportion of oospores germinated, of which only a few developed into mycelial cultures. Germination of oospores of isolate 127 were first observed after 8 days of incubation on 0.6% DWA. Of 15 germinated oospores observed and transferred over a period of 25 days, three developed into small colonies, but only one of these survived. Oospores of isolate 127 were 35 days old when extracted via snails, and were stored at 4 C in darkness for an additional 35 days after surface sterilization.

Nine single-oospore cultures were derived from a 14-wk-old culture of isolate 40/34. Approximately 3,500 oospores were recovered from fecal pellets and incubated on DWA. Of these, 161 germinated oospores were observed over a period of 40 days, and transferred to rye B agar or drops of rye A broth. Fifty-two of the 161 germings produced small colonies, but only nine of these continued to grow into mycelial cultures.

The mode of germination was consistent for all oospores; each produced a germ sporangium (Fig. 1A). Subsequent germ tubes (Fig. 1B) formed a branched hyphal colony on agar or in broth. Germings not transferred immediately often produced secondary sporangia.

The single-oospore cultures differed from their parental cultures in some, but not all, aspects. Hyphal extension rates on rye A agar of all single-oospore cultures were slower than those of the parents (Table 2). The growth rate of the single-oospore progeny of isolate 127 (127/1) on agar was less than half that of the parental isolate. Also, progeny from isolate 40/34 (40/34/1-9) grew at 27-66% the rate of the parent on agar. In contrast, only one single-oospore culture, 40/34/2, exhibited a significantly reduced growth in 10% C-V8 broth culture (Table 2). This disparity of growth rate by isolates in broth and on agar medium was apparently due to morphological changes in hyphal branching pattern. Single-oospore isolates had highly branched, thickened hyphae relative to

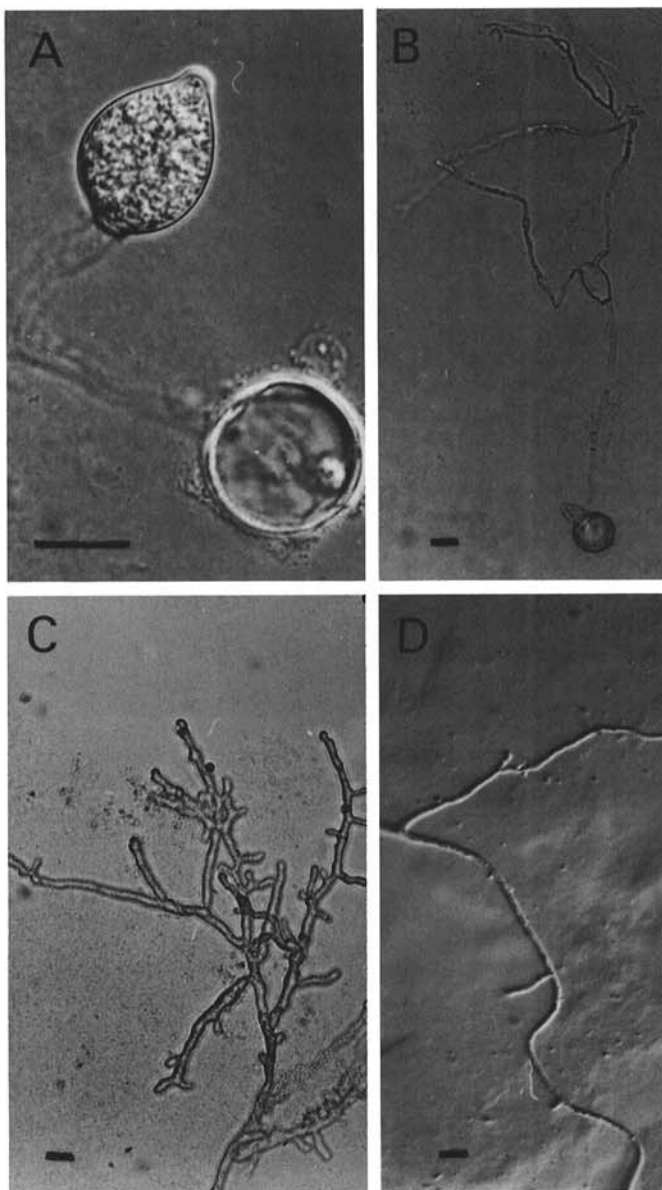


Fig. 1. *Phytophthora infestans*: A, germinated selfed oospore of isolate 40/34; B, hyphal development from the germ sporangium; C and D, branching pattern of hyphae of single-oospore culture of isolate 40/34/3 and of isolate 40/34, respectively, on rye A agar. Bar = 20  $\mu$ m.

the parental isolates (Fig. 1C and D). All progeny cultures were A1 compatibility type.

The virulence of only two progeny could be assessed, and they were both identical to the parent. Only isolates 40/34/4 and 40/34/5 sporulated in vitro and released zoospores when sporangia were chilled in distilled water for 3 hr at 10 C. When these were tested for virulence on detached leaflets, they produced sporulating lesions on the cultivar Norchip and on the same *R*-gene differentials as did the parental isolate, 40/34 (Table 2). However, the progeny produced lesions 24–36 hr later than did the parent. Some progeny (40/34/6) produced sporangia in vitro, but sporangia failed to release zoospores. Attempts to infect differentials with these sporangia failed. Other progeny (40/34/8) and (40/34/9) failed to sporulate at all.

Segregants were observed at one of two enzyme loci examined electrophoretically. The parental isolate 40/34 (heterozygous genotype 92/100 at the *PEP* locus) yielded two of nine single-oospore progeny of homozygous genotype 100/100 (Table 2). The other seven progeny were identical to the parent. At the other enzyme locus that was examined (*GPI-1*), the parental culture 40/34 was of homozygous genotype 100/100, so that if segregation occurred via selfing, it would not be detected among the progeny, and all progeny had the same genotype as the parent (Table 2). No changes in banding pattern were observed at either enzyme locus in the single-oospore culture derived from isolate 127.

## DISCUSSION

This study confirms previous reports (20,21) of intra- and interspecific stimulation of selfing in A1 isolates of *P. infestans*. The A2 isolate 126 (=WV445) was also used by Shen et al (20), but they did not report whether or not it was stimulated by A1 isolates to produce oospores. Its failure to be stimulated in our study puts it into sexuality group 5 as described by Ko (8). Members of this group produce  $\alpha_2$ , a postulated hormone which induces production of gametangia in A1 isolates but are unresponsive to the hormone  $\alpha_1$  produced by A1 isolates. We found that *P. drechsleri* (isolate PC3) belongs to sexuality group 4 (8), as do some recently collected A2 isolates of *P. infestans* of Mexican origin (22), all of which were stimulated to self by isolates 127 and BT (*unpublished*).

Our study differs from some previous ones because we used polycarbonate membranes to prevent physical contact between cells of opposite mating type. In other studies, selfing had been identified in one or both partners involved in direct matings between compatible strains of heterothallic species of *Phytophthora* by using fluorescent dyes (6) or cytological markers (14) to identify hyphae or by analyzing genetical data (1).

Direct observation of germination of selfed oospores of heterothallic species of *Phytophthora* has only been reported once before (3). Six and 25 single-oospore progeny from A2 isolates of *P. cinnamomi* and *P. drechsleri*, respectively, produced by using *Trichoderma* (2), were characterized. Although the progeny segregated for cultural characteristics, mating type remained unchanged. Since the determinants of the A2 compatibility type are thought to be heterozygous (9,13), selfing might be expected to produce segregants.

The identification of two homozygous segregants at the *PEP* locus indicates that selfing had occurred. By inference, the other single-oospore progeny are also the result of automixis involving reduction division and fertilization and not apomixis in which fertilization is absent. Unfortunately, we were able to analyze only two progeny for virulence, the other trait for which we might expect to see segregation. Virulence on potato genotypes with major gene resistance could increase via selfing if this trait is recessive as with many other fungal pathogens (5) and if the isolates 127 and 40/34 were heterozygous at virulence loci. The two progeny tested for virulence retained the same virulence pattern as the parental type.

Shaw (19) has stated that questions about the inheritance of virulence and other important traits can be addressed by analyzing cultures from selfed oospores. While this is undoubtedly true, it is essential to increase not only the level of germination of oospores from that witnessed here and elsewhere (2), but also to increase the

TABLE 2. Characteristics of parental and single-oospore progeny isolates of *Phytophthora infestans*

Isolate	Dry wt <sup>a</sup>	Growth rate <sup>b</sup>	Compati- bility type	Virulence	Genotype at enzyme locus:	
					<i>GPI-1</i> <sup>c</sup>	<i>PEP</i> <sup>d</sup>
127	100 a <sup>w</sup>	11.0 a <sup>w</sup>	A1	2,3,4,7,10	86/100 <sup>s</sup>	92/100 <sup>s</sup>
127/1	38 b	4.6 b	A1	N.T. <sup>y</sup>	86/100	92/100
40/34	100 a	12.6 a	A1	1,2,4,5,10	100/100	92/100
40/34/1	105 a	3.6 g	A1	N.P. <sup>z</sup>	100/100	100/100
40/34/2	55 b	3.8 g	A1	N.T.	100/100	92/100
40/34/3	102 a	6.5 d	A1	N.T.	100/100	100/100
40/34/4	98 a	8.2 b	A1	1,2,4,5,10	100/100	92/100
40/34/5	103 a	7.6 c	A1	1,2,4,5,10	100/100	92/100
40/34/6	115 a	5.6 e	A1	N.P.	100/100	92/100
40/34/7	101 a	4.6 f	A1	N.P.	100/100	92/100
40/34/8	118 a	3.4 g	A1	N.T.	100/100	92/100
40/34/9	90 a	5.6 e	A1	N.T.	100/100	92/100

<sup>a</sup> Dry weight when grown in 10% C-V8 broth and expressed as percentage of the growth of the parental isolate. Mean dry weight of isolate 127 and isolate 40/34 was 10.2 and 16.3 mg, respectively.

<sup>b</sup> Mean rate of increase (millimeters per day) of colony diameter on rye A agar at 18 C without illumination.

<sup>c</sup> *GPI-1* = glucosephosphate isomerase.

<sup>d</sup> *PEP* = peptidase.

<sup>e</sup> Data for each group of parental and selfed progeny cultures (i.e., 127 and 127/1 versus 40/34 and 40/34/1 through 40/34/9) were analyzed separately and comparisons among means were performed within each group. Means within a column and parent-progeny group followed by the same letter do not differ significantly according to Duncan's multiple range test ( $P < 0.05$ ).

<sup>f</sup> The allele coding for the most commonly occurring isozyme at a locus is designated 100. When an allele coded for an isozyme that migrated 92% as far as the most common (reference) one, it was designated 92.

<sup>g</sup> Not tested due to lack of sporulation.

<sup>h</sup> Tested for virulence, but found to be nonpathogenic.

success rate of colony establishment. Unfortunately, germination of selfed oospores and colony establishment may remain problematic. This may be due to inbreeding depression since Shattock et al (17) have shown that the percentage germination of oospores from conventional genetic crosses can be much higher than those observed here.

The data we presented show that selfed oospores of A1 isolates of *P. infestans* produced in vitro by intraspecific stimulation are viable and will germinate to produce mycelial colonies. If interspecific stimulation occurs in natural populations of *Phytophthora* (21), then selfing may generate variation in populations of *P. infestans* of a single compatibility type.

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