Phytophthora megasperma var. sojae: Development of Wild-Type Strains for Genetic Research

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ABSTRACT

Single oospore and zoospore progeny of four field isolates representing three races of the soybean pathogen *Phytophthora megasperma* var. *sojae* exhibited great variability in pathogenicity, growth rates, and colony morphology. To obtain lines suitable for genetic research, the field isolates were repeatedly selfed (self-fertilized) and single oospore progeny were selected for high aggressivity, good zoospore and oospore production, and germinability, high growth rates, and typical colony morphology. Fifth generation (S_5) inbred lines were obtained that bred true for the above characters when either single zoospores or single oospores were cultured. Techniques were developed for the production of zoospores in sufficient numbers for mutagenesis, and a water-soaking method was used to obtain 50% germination of oospores. The inbred S_5 progeny are considered to be wild-type lines and they offer the prospect of obtaining reliable auxotrophic strains for genetic studies with *P. megasperma* var. *sojae*.

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The genetics of *Phytophthora* spp. is controversial. The vegetative mycelial stage in various species has been interpreted to be haploid (8, 20, 26, 27), diploid (3, 7, 22, 25) or polyploid (22). Evidence has been presented for the occurrence of cytoplasmic inheritance (5, 28), somatic recombination (6, 15, 16), sexual recombination (8), and heterokaryosis (6, 15). However, much of the published work has been plagued by technical problems, including the lack of sufficient independent nuclear markers, poor oospore germinability, and uncontrolled variability likely caused by the use of native field isolates.

Phytophthora megasperma (Drechs.) var. *sojae* A. A. Hildb. is a pathogen of soybean [*Glycine max* (L.) Merr.] roots and stems (2) and offers many characteristics that are desirable for genetic work, particularly the inheritance of pathogenicity. The fungus can be cultured on natural and synthetic media and can be manipulated to form sexual oospores or asexual zoospores uniquely without mixtures of the two. The organism is homothallic and oospore germination is higher than with heterothallic species, approximating 50% with our current techniques. At least two dominant resistance genes have been found in soybeans and three physiologic races of *P. megasperma* var. *sojae* are known (2, 9, 17, 23). A race 4 has recently been discovered (24), but it was not included in this work. In this paper, "*field isolate*" refers to an isolate obtained from nature and not manipulated in the laboratory for more than a single zoospore isolation; "*wild-type*" strains will refer to selfed, inbred progeny.

Early experiments with field isolates of *P. megasperma* var. *sojae* races 1 and 2 disclosed considerable variability in growth, morphology, and aggressivity among the single oospore and single zoospore progeny, consistent with the previous observations of Hilty and Schmitthenner (10). The virulence of the progeny, however, was consistently the same as the field isolates in our tests. In this paper *virulence* is used synonomously with vertical pathogenicity (19) and is defined as a qualitative pathogenesis factor determined by interaction between the appropriate differential soybean cultivars and fungus isolates; interaction between susceptible host genotypes and virulent races, therefore, give susceptible host reactions, and those between resistant host genotypes and avirulent races give resistant reactions. *Aggressivity* is considered synonomous with horizontal pathogenicity (19) and is defined here as a quantitative factor for severity of infection by a genetically virulent fungus race.

Although we obtained drug-resistant mutants (race 1, cycloheximide resistant, and race 2, *p*-fluorophenylalanine resistant) of *P. megasperma* var. *sojae* by treatment of cysts of the field isolates with chemical mutagens, no stable auxotrophic mutant was obtained. Variability impeded further progress when attempts were made to obtain quantitative genetic data from progeny (single oospores and zoospores) of the drug-resistant mutants. However, crosses of these drug-resistant strains led to the recovery of dual-drug-resistant recombinant oospore progeny. When the virulence of ten dual-drug-resistant oospore progeny from separate crosses was determined on the D60-9647 differential soybean variety (17), seven were race 1 and three were race 2. Although the data suggested segregation for virulence in the cross, the ratio was not considered reliable because race 2 recombinants of low aggressivity would mimic the race 1 progeny.

These observations of variation in aggressivity, colony morphology, and linear growth rates of single-spore progeny indicated that the starting field isolates were so heterotic that they were unsuitable for critical genetic research. It was clear, therefore, that meaningful genetic experimentation with the fungus depended upon the

development of wild-type strains of the various races which would breed true for normal morphology, vigorous growth rates, the proper aggressivity, the proper virulence, and suitable sporulation and germination of propagules, when single zoospore or oospore isolations were made. This paper describes the development of true-breeding inbred lines of the three *P. megasperma* var. *sojae* races, and includes techniques developed for production and germination of zoospores and oospores at levels sufficient for genetic work. The oospore progeny of successive selflings will be referred to in this paper as S_1, S_2, etc. The zoospore progeny of these successive generations will be designated S_1Z, S_2Z, etc. We anticipate that the wild-type strains will enhance chances for success with mutagenesis procedures, and will allow determination of the inheritance mechanisms in this fungus.

MATERIALS AND METHODS.—Fungus isolates and their single-oospore and single-zoospore progeny were maintained on cleared V-8 juice agar (V8-JA) (27) or sucrose-KNO_3 minimal synthetic agar (sucrose, 15 g/liter; KNO_3, 2.0 g/liter; $MgSO_4 \cdot 7H_2O$, 0.2 g/liter; $FeSO_4 \cdot 7H_2O$, 1 mg/liter; $CaCl_2 \cdot 2H_2O$, 10 mg/liter; thiamin-HCl, 1 mg/liter; K_2HPO_4, 1.04 g/liter; KH_2PO_4, 1.90 g/liter; β-sitosterol, 20 mg/liter; $ZnSO_4 \cdot 7H_2O$, 1 mg/liter; $CuSO_4 \cdot 5H_2O$, $NaMoO_4 \cdot 2H_2O$, and $MnCl_2 \cdot 2H_2O$, each 0.02 mg/liter; Bacto-agar (Difco), 15 g/liter. The race 1 isolates used were: P174, originally obtained from A. A. Hildebrand, Canada Dept. of Agriculture, Harrow, Ontario, Canada, has been repeatedly subcultured for at least 15 years; P900, obtained from A. F. Schmitthenner (23) (his isolate #507); P406, a race 2 isolate obtained from F. L. Morgan, U.S. Department of Agriculture, Southern Soybean Laboratory, Stoneville, Mississippi (17); and P892, a race 3 isolate supplied by Schmitthenner (23) (his isolate #573). Race designations were determined by inoculation of standard differential soybean cultivars (2, 9).

Oospores were harvested aseptically from 28 ± 7-day-old petri dish cultures on V8-JA by homogenizing the outer 1-cm of the mycelial mat with approximately 200 ml of sterile deionized water in a Waring Blendor for 5 minutes (1-minute intervals) at full speed. The oospores were then separated from the mycelium by washing and centrifugation (approximately 1,000 *g*) three to eight times. The washed oospores were resuspended in sterile, deionized water to give the desired dilutions and, unless otherwise treated, were immediately spread on 1.5% water agar (WA) plates. Nongerminated oospores were selected immediately by viewing the overlayed plates with a dissecting microscope and circling individual oospores with a 0.5-mm diameter loop. Each oospore on its WA plug was then transferred to a WA plate that had been divided into 25 squares (25 oospores per plate). Transferred oospores were incubated on a laboratory bench at ambient temperature (approximately 24 C) and light conditions (fluorescent light for approximately 9 hours), and checked for the appearance of germinated oospores at 2- to 3-day intervals for at least 21 days. Germinated oospores were then transferred to V8-JA. At least two 5-mm diameter plugs from each established single-oospore colony were transferred to fresh V8-JA and observed for (i) morphological colony appearance, (ii) linear growth rate, (iii) aggressivity and virulence on soybean plants, and (iv) tested for oospore and zoospore production and germination.

Oospores were soaked in 5 ml water in capped test tubes for various times at various temperatures. In some experiments, oospores were treated with Helicase (Industrie Biologique Française, Gennevilliers, France) or β-glucuronidase (Calbiochem, La Jolla, California) at various concentrations in 0.05 M potassium acetate, pH 4.5, or water. Treated oospores were washed by centrifugation, diluted, overlayed onto WA plates, and picked in the manner described above for nontreated spores.

For zoospore production, 7-day-old mycelial mats from cultures on liquid synthetic medium were washed three times with sterile water and blended for 20 seconds with approximately 100 ml of sterile distilled water in a Waring Blendor. Four milliliters of minced mycelium were added to petri plates containing 4 ml of pea-broth (14). After incubation for 3-6 days in darkness at 25 C, the pea-broth was removed, the fungus washed three times with water, and finally 9 ml of water was added to each plate. Sporangia formed and a few zoospores were liberated within 5-7 hours. Zoospores were harvested at 10-12 hours, when they were most abundant, by passing them through fluted filter paper (Eaton-Dikeman Co., Mt. Holly Springs, Pennsylvania, coarse porosity) to remove traces of hyphae. The yield of zoospores varied considerably, but most experiments resulted in the production of 10^4 to 10^5 zoospores per petri plate culture. The zoospore suspensions were diluted and spread onto WA plates where encystment occurred almost immediately, and germ tubes appeared within 1-2 hours.

TABLE 1. Oospore germination and colony establishment for successive single-oospore generations of four field isolates of *Phytophthora megasperma* var. *sojae*

Single-oospore[a] generation	Oospore germination (%)[b]				Colony establishment (%)[c]			
	P174	P900	P406	P892	P174	P900	P406	P892
S_1	20	16	33	24	74	98	92	93
S_2	18	19	49	21	75	95	92	94
S_3	23	7	9	20	...	100	93	93
S_4	39	24	14	8	89	100	93	95
S_5	26	8	23	11	93	98	90	100

[a] In each isolate generation, 200-500 progeny were screened.

[b] Oospores were directly isolated and observed for germination every two to three days up to 21 days when values reported were taken.

[c] Germinated oospores were transferred to V8-JA as soon as they were detected and resultant colony establishment determined.

Individual germinated cysts were transferred to V8-JA for monosporous colony formation.

To determine if zoospores were uninucleate, nuclei were stained immediately after harvesting, as follows (1): the zoospores were fixed for 1.0-1.5 hours in 4% glutaraldehyde, washed 5 minutes in 0.02 M potassium phosphate buffer (pH 7.0), and stained for 4 minutes with Giemsa in phosphate buffer. Lugol's iodine was used for destaining for 20 seconds or less. The spores then were washed 1 minute with xylene and mounted in clove oil for microscopic observation.

Soybean seedlings were grown 5-7 days in 10-cm diameter pots in growth chambers and inoculated in hypocotyl wounds (14). Disease reactions were evaluated after 48 hours.

TABLE 2. Effect of water-soaking on oospore germination of *Phytophthora megasperma* var. *sojae*

Isolate generations[a]	Germination (%)[b]		
	Not soaked	Soaked in water[c]	
		25 C	36 C
P174 S_3, S_4	25.0	19.0	58.0
P900 S_3, S_4, S_5	14.4	19.0	53.1
P406 S_3, S_4, S_5	17.2	16.0	55.0
P892 S_3, S_4, S_5	15.3	18.0	51.5

[a]For each isolate 600 to 1,000 progeny were screened.
[b]Percentage germination was determined 21 days after harvest. Data for isolate P174 are an average of two experiments; P900, seven experiments; P406 and P892, each three experiments. A minimum of 400 oospores were analyzed per experiment.
[c]Temperature and time: 36 C for 48 hours; 25 C for 48 or 72 hours.

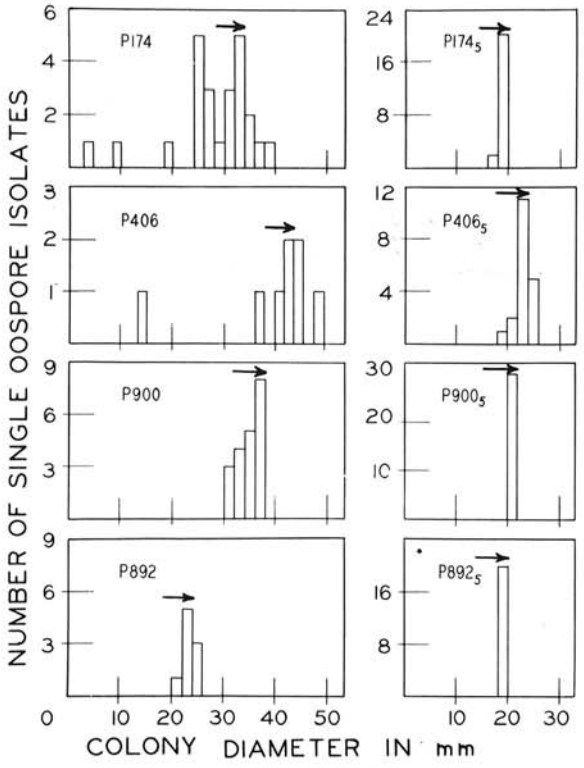

Fig. 1. Growth rates for S_1 progeny (left) and corresponding S_5 progeny (right) of four isolates of *Phytophthora megasperma* var. *sojae*. Data were taken after three days on V8-JA except for P174 (4 days), P406 (5 days), and P900 (4 days). Arrows denote mean growth rates of parent cultures (field isolates for S_1 progeny and S_4 lines for S_5 progeny).

RESULTS.—*Oospore germination.*—Untreated or water-soaked oospores (S_1, S_3, and S_5) transferred directly to V8-JA did not germinate appreciably (a maximum of 1% was observed with approximately 3,500 single oospores). Germination on water agar was higher, but nonsynchronous, and continued for a period of 35 days; 95% of the spores that germinated did so within the first 21 days and established colonies when transferred to V8-JA (Table 1). Only the successive selfed generations of isolate P174 showed increased colony establishment. Soaking oospores (S_3, S_4, and S_5) 48 hours in water at 25 or 36 C before plating eliminated much of the variability in germination (Table 2) and water-soaking at 36 C resulted in much higher ($50 \pm 10\%$) germination than with oospores soaked at 25 C or directly plated on WA. Water-soaked oospores typically germinated via a sporangium, while directly plated spores germinated by a germ tube.

Pretreatment of the oospores with Helicase at various concentrations from 0.025% to 5.0% for 1-72 hours at 25, 30, and 36 C did not stimulate oospore germination; to the contrary, several treatments resulted in decreased germination relative to control spores incubated only in water or buffer. Purified β-glucuronidase similarly did not improve oospore germination.

Heterotic nature of field isolates and the development of inbred lines.—Colony morphology and linear growth rates were variable in single-oospore colonies in the first and second selfed generations of isolates P174 and P406, but were fairly uniform in the first generation of isolates P900 and P892 (Fig. 1). With succeeding generations, morphology was uniform among progeny and the variation in linear growth decreased. There was a tendency toward colonies of slightly smaller diameters when the S_5 generation was reached (Fig. 1, Table 3). Oospore progeny of all the isolates exhibited the same virulence on the soybean differential cultivars as did the field isolates. All S_4 and S_5 progeny of isolates P406 and P900 exhibited the same aggressivity as the parent isolates. Several S_1 progeny of isolate P892 were less aggressive than the field isolate (Table 3), but further selfing of highly pathogenic S_1 colonies resulted in S_2 to S_5 progeny with aggressivity uniformly indistinguishable. Isolate P174 produced 40% nonaggressive or weakly-aggressive oospore progeny in the S_1 generation; further selfing of selected highly-aggressive S_1 progeny also resulted in weakly-aggressive progeny in the S_2 to S_4 generations (10-20%). Pathogenic variability was not noted in the S_5 generation of P174. Continuous selection for high aggressivity through the selfing series resulted in S_5 progeny of P174 that were still not as aggressive as P900, but were more aggressive than the field isolate of P174.

The field isolates and successive inbred generations of isolates P406, P892, and P900 showed little variation in oospore morphology, but there was a trend toward more

TABLE 3. Colony establishment, growth-rate, and virulence of single-zoospore progeny from field isolates and fifth-generation inbred cultures of *Phytophthora megasperma* var. *sojae*

Fungus isolate[a]	Zoospore colonies established (%)[b]	Growth of mono-zoospore colonies (mm)[c]	Virulence on soybean genotypes[d]			
			Typical reactions[e]			Atypical reactions[e]
			rps	*Rps*	*rps*^{2}	
P900	85	36 ± 4	15	0	0	1
$P900_5$	82	32 ± 1	16	0	0	0
P406	50	26 ± 1	13	0	13	2
$P406_5$	95	22 ± 1	15	0	15	0
P892	65	21 ± 1	16	16	0	0
$P892_5$	85	21 ± 1	16	16	0	0
P174	52	...	...	...	...	...
$P174_5$	67	...	...	...	...	...

[a]Field isolates are identified by isolate number and the fifth generation mono-oospore culture by the isolate number with the subscript 5.

[b]At least 50 germinated zoospores were tested from each culture.

[c]Mean and standard deviation measurements of 20 or more single-zoospore colonies from each culture with three replicates, and 3 days growth except, 4 days growth for P900 and $P900_5$.

[d]The *rps* soybean genotype is susceptible to all races of *P. megasperma* var. *sojae; RPS* (2) and *rps*^{2} (9) are single genes for resistance.

[e]Number of typical (susceptible or resistant) reactions on soybean genotypes; atypical (avirulent) reactions on all soybean genotypes.

abundant production of oospores in later generations. There was a great deal of variation in oospore size in field isolate P174 and this persisted throughout the five inbred generations.

Zoospore isolations and selection of wild-type strains.—There was little difference between S_5 inbred generations of the various isolates with respect to sporangia production and number of zoospores released. We experienced considerable difficulty in early work obtaining sufficient numbers (10^6 to 10^8) of *P. megasperma* var. *sojae* zoospores for mutagenesis. After trying several methods, the pea-broth technique described here was the only one that approached the high yield of zoospores we desired. We have successfully stored encysted zoospores in water at 9 C and found them to germinate (at 1 week, 70%; and at 3 weeks, 40%). This capability is useful since harvested zoospores may be accumulated for mutagenesis experiments.

Colony establishment of single zoospores from the S_5 lines was the same or better than the respective field isolates (Table 3), and variation in colony morphology and growth rate was less in the S_5 generations. Only one binucleate zoospore (from field isolate P174), and none with more than two nuclei, were observed in over 3,500 stained zoospores from the various field- and S_5 isolates. Three single-zoospore cultures from the field isolates were avirulent (Table 3), but all S_5 lines were virulent and of indistinguishable aggressivity on the appropriate soybean differentials.

Selected S_5 progeny of the four *P. megasperma* var. *sojae* isolates were designated $P174_5$, $P900_5$, $P406_5$, and $P892_5$, and their single-zoospore progeny were then screened for desirable characters. The selected zoospore isolates will be defined henceforth as wild-type strains of *P. megasperma* var. *sojae*. Their full designations are $P900_5$ Z-1, $P406_5$ Z-8, and $P892_5$ Z-25. Because of its depressed aggressivity, isolate P174 is not presently being used for genetic work.

DISCUSSION.—Several workers have noted extreme variability in the single-oospore and single-zoospore progeny of *Phytophthora* spp. and this variation often persisted with subsequent single-propagule isolations (3, 5, 8, 10, 28). It is therefore surprising that suitable inbred wild-type lines of *Phytophthora* spp. have not been developed heretofore as a prelude to mutagenesis and genetic work. Our preliminary mutagenesis and crossing experiments with *P. megasperma* var. *sojae* clearly indicated that the field isolates were unsuitable for such efforts. We therefore undertook the development of inbred wild-type lines of three *P. megasperma* var. *sojae* races. Several factors support the conclusion that the inbreeding program resulted in true-breeding lines of the fungus: (i) the aggressivity of oospore and zoospore progeny to soybean seedlings was less variable after successive oospore generations, (ii) variation in linear growth and morphology of successive oospore generations decreased, and (iii) the variation in linear growth and morphology of single zoospore isolates from S_5 progeny was notably less than that of zoospores from the field isolates.

Since the S_5 single-zoospore selections bred true with respect to all these characters and still exhibited growth rates nearly the same as the starting field isolates, they are now considered as wild-type strains, and are currently being used in mutagenesis and genetic work. We anticipate that these wild-type strains will be useful for the development of suitable auxotrophic mutants for forced auxotrophic crosses to determine the inheritance of virulence genes and other markers in the fungus. Already, our mutagenesis experiments (Long et al., *unpublished*) have resulted in the isolation of four different auxotrophic mutants that are not leaky and that have remained stable after repeated mass transfers and zoospore or oospore subcultures.

Germination of directly plated *P. megasperma* var. *sojae* oospores varied greatly between different generations, but colony establishment was consistently high (Table 1). Germination of water-soaked oospores (Table 2) is adequate for genetic studies. It is not clear whether water-soaking enhances germination through a

leaching effect or by a physical alteration of an unknown dormancy mechanism. It has been reported that β-glucuronidase enhanced germination of oospores of the closely related fungus *P. megasperma* from alfalfa (21). In our experiments, however, neither β-glucuronidase nor Helicase showed stimulatory effects with *P. megasperma* var. *sojae* but, instead, these were deleterious under some conditions.

The microscopic evidence of Ho et al. (13) suggested that zoospores of *P. megasperma* var. *sojae* are uninucleate but they did not report counts on large numbers of zoospores. Since zoospores must be very nearly 100% uninucleate to facilitate genetic work, we examined the nuclear number in zoospores. Our conclusion that the zoospores of *P. megasperma* var. *sojae* are almost exclusively uninucleate seems warranted because we observed only one binucleate zoospore in approximately 3,500 examined.

Although we experienced variation in spore yields, the pea-broth method for zoospore production was the only one that resulted in the large numbers of *P. megasperma* var. *sojae* zoospores required for mutagenesis experiments. The techniques of Ho and Hickman (12) and Ho (11) resulted in substantial zoospore production, but did not readily yield the large numbers of zoospores that we desired.

Hilty and Schmitthenner (10) showed variation in aggressivity and cultural characters among single-zoospore isolates from field isolates of *P. megasperma* var. *sojae*. They found one single-zoospore isolate that was avirulent and one that exhibited altered virulence, since it attacked normally resistant cultivars. We could not confirm the occurrence of altered virulence in any single zoospore or oospore progeny of the field isolates, but variation in the aggressiveness of single-zoospore and -oospore progeny was common, especially in the S_1 and S_2 progeny of some races.

Isolate P174 lost aggressivity after an extended time in culture. In addition, this isolate was clearly more variable than the other three isolates for all characters studied. It is noteworthy that, although the selfing program resulted in an S_5 selection of P174 that was more aggressive and much less variable than the field isolate, aggressivity was not restored to the level of the P900 race 1 isolate. The presumed lost aggressiveness of isolate P174 therefore appears irrecoverable by single-sporing and selection.

Our data not only demonstrate the feasibility of obtaining strains of *Phytophthora* spp. suitable for meaningful genetic studies, but also underscores the dangers inherent in using virulence (4, 8, 18), and morphological characters (3, 8) for genetic analyses. The development of several reliable genetic markers is imperative, if the question of ploidy in *Phytophthora* spp. is to be adequately resolved and inheritance mechanisms elucidated.

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