TECHNIQUES FOR ISOLATING PHYTOPHTHORA MEGASPERMA VAR. SOJAE FROM SOIL AND PLANT TISSUE

by

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B.A., DePauw University, 1977

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A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Plant Pathology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1980

Approved by:

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INTRODUCTION

In 1955, Suhovecky (36) first reported a *Phytophthora* species as the incitant of a destructive root and stem rot of soybean [*Glycine max* (L.) Merr.], a disease that had appeared in northwestern Ohio as early as 1951 (37). Later in 1955, Skotland (35) described a similar disease in North Carolina caused by a fungus resembling *Phytophthora cactorum* (Leb. and Cohn) Schroet. In 1957, Herr (17) also identified the Ohio pathogen as *P. cactorum*. Kaufmann and Gerdemann (19) wrote in 1957 that they felt the pathogen was closely related to *Phytophthora megasperma* Drechs., but after further study described the fungus as a new species, *Phytophthora sojae* (20).

However, in 1959, Hildebrand (18) provided evidence that the fungus was morphologically indistinguishable from *P. megasperma*, yet differed physiologically and pathologically. He proposed a new taxonomic designation, *Phytophthora megasperma* Drechs. var. *sojae* Hildeb. This trinomial has received the widest acceptance (22) and will be used in this paper. The common name, Phytophthora rot, will be used to describe the disease caused by this fungus (4).

P. megasperma var. *sojae* (*Pms*) may infect soybeans during any stage of development of the plant. Seeds may be attacked, resulting in seed rot and pre-emergence damping-off. Post-emergence root and stem rot is noted by a watersoaked, brown discoloration and constriction of the stem (36). Older plants may display reduced vigor throughout the growing season. The lower leaves turn yellow, followed by chlorosis of the upper leaves, and death of the entire plant. Wilted leaves remain attached to the dead plant for several weeks.

The fungus destroys lateral and branch roots, and the taproot turns dark brown. A light brown lesion may progress up the stem 10-20 cm or more (18). Oospores are formed in the roots and lower stem (16), and are assumed to provide a means for the fungus to overwinter.

The severity of Phytophthora rot is dependent upon soil moisture (18,20,37), temperature (22,36,39), bulk density (12,22), and porosity (22), as well as host-pathogen interaction. Yield loss may exceed 50 percent if a combination of these factors favor the fungus (18).

Although *P. megasperma* var. *sojae* exists as several physiological races (23), many commercially available soybean cultivars display tolerance or resistance to infection by one or more of these races. Recommendations to plant specific *Pms* resitant cultivars are not always useful, however, because the difficulty in determining *Pms* race distribution makes precise cultivar selection difficult.

The original goal of the research described here was to determine the race distribution of *Pms* in Kansas. This information could then be used when making recommendations to plant disease resistant cultivars, thus reducing crop loss caused by this fungus in the state. To accomplish this task, it was first necessary to become familiar with available techniques for isolating the fungus. These were explored using soil and plant samples known to contain *Pms*. This research is described in Part I. Inherent problems in fungus isolation using published techniques suggested that an improved technique for isolation and germination of individual oospores from field soil would be useful in a race distribution study, and that became the purpose of the research reported in Part II. Additional information on

antibiotic testing, selective medium development, and testing for synergistic and antagonistic effects when mixing *Pms* races using the zoospore injection method for inoculating soybean plants (34) will be found in Appendices A and B.

PART I. COMPARISONS OF PUBLISHED METHODS OF ISOLATING PHYTOPHTHORA MEGASPERMA VAR. SOJAE

Kaufmann and Gerdemann (20) routinely isolated *Pms* from margins of lesions on soybean stems. The research reported here often involved the use of soybean stems and roots that were contaminated with bacteria and fungi other than *Pms*. Schmitthenner (33) summarized the advantages of using antibiotics and fungal inhibitors in media for the selective isolation of *Pythium* and *Phytophthora* spp. from such tissue. If naturally infected plants are not available, *Pms* may be recovered by baiting the fungus from soil samples (3,7,11,29). The applicability of these isolation techniques to a race distribution study is described in this section.

MATERIALS AND METHODS

BPPS medium. A selective medium, termed BPPS, was prepared by adding to 500 ml of 1.5% lima bean agar (Difco), autoclaved and cooled to 50 C, 25 mg each of benomyl (Benlate 50% WP; E. I. du Pont de Nemours and Co.) and pentachloronitrobenzene (Terraclor 75% WP; Olin Mathieson Chemical Corp.), and 50 mg each of penicillin G potassium and streptomycin sulfate (United States Biochemical Corp.).

Isolation from infected plants. *Pms* was isolated from infected soybean stems using a variation of the method described by Kaufmann and Gerdemann (20). Stem pieces, 2 to 5 cm long and known to be infected with the fungus, were surface sterilized in 25 ml of 15% Chlorox for 2 minutes and rinsed in 15 ml of sterile distilled water for 1 minute. Tangential sections of tissue were cut from the stems,

placed on plates of Schmitthenner's medium (33), and incubated at 21 C in the dark.

Baiting techniques. Two published baiting techniques and a variation of a third were tested for their potential usefulness in determining race distribution.

METHOD 1 (3). Ten to 20 g of *Pms* infested soil was placed into a 100-ml beaker, wetted to field capacity with sterile distilled water, and incubated at 21 C for 7 days. Then 50 ml of sterile distilled water was added and 30-40 four mm discs cut from soybean leaves (cultivar Columbus) were floated on the surface of the water. After 3 hours the leaf discs were removed and placed on plates of BPPS medium.

METHOD 2 (11). Five, 2-day-old soybean seedlings (cultivar Columbus) from seeds germinated in vermiculite were placed in Petri dishes containing 20 g of *Pms* infested soil and 30 ml of sterile distilled water. Seedlings were removed after 3 days of incubation at 21 C in darkness, and placed on plates of BPPS medium.

METHOD 3 (29; a variation). A nurseryman's rooting tray with 12-cm-long plastic tubes, each 2.5 cm in diameter, was used to bait *Pms* from infested soil and infected plant debris. The drainage holes of 60 tubes were filled with cotton plugs, then 16 cc of vermiculite were placed in each tube. Six cc of infested soil were added to each of half of the tubes, and 0.5 g of infected plant tissue was added to each of the remaining tubes. All tubes were then filled to within 2 cm of the top with vermiculite. Three seeds of the cultivar Columbus, Amsoy 71, or Beeson were planted in each of ten tubes containing soil, and in each of ten tubes containing plant debris. The seeds were

covered with vermiculite and the tubes flooded from the bottom with tap water. The water level was maintained 2-3 cm below the sample layers in the tubes. The soybeans were germinated and plants grown at 28 C under continuous light in a controlled environment chamber. As symptoms developed (one to several weeks) plants were removed and small stem pieces placed directly onto BPPS medium.

RESULTS

Due largely to the inhibitory effects of chloramphenicol (Table 3), the growth habit of *Pms* on Schmitthenner's medium differed greatly from its growth habit on lima bean agar, the standard medium for the remainder of this work. The slow, dense growth of *Pms* on Schmitthenner's medium made the visual distinction between developing colonies of *Pms* and colonies of other fungi difficult. To minimize these growth anomalies, while retaining the advantages of a selective medium, the BPPS medium was developed (Appendix A, Table 4).

The BPPS medium was compared to Schmitthenner's medium in isolation of *Pms* from stem pieces of inoculated soybean plants. In Schmitthenner's medium, vancomycin, 0.1 g per 1,000 ml of medium, was substituted for neomycin and chloramphenicol, as he suggested (33). This substitution was made because vancomycin was less inhibitory than chloramphenicol to *Pms* (Table 3). Stems from these plants were surface sterilized as described above, and plated on both media (Table 1).

Recovery of *Pms* from plant tissue was equally effective using BPPS and Schmitthenner's medium. The BPPS medium facilitated the visual identification of *Pms* in this study because it did not alter the

TABLE 1. Comparison of the effectiveness of Schmitthenner's medium and a modification of Schmitthenner's medium (BPPS) in isolation of *Phytophthora megasperma* var. *sojae* (*Pms*) from plant tissue.

Medium	*Pms* colonies recovered[a]	Stems with bacteria[b]
Schmitthenner's	9	13
BPPS	10	15

[a] Three stem pieces were plated on each of five replicate plates.

[b] Number of stem pieces around which bacterial colonies formed.

growth habit of *Pms* relative to the growth of the fungus on lima bean agar. Using the BPPS medium, *Pms* was isolated from active lesions on naturally infected plants, but could not be isolated from inactive disease lesions or badly decayed plant tissue.

P. megasperma var. *sojae* was not recovered from infested soil using baiting methods 1 and 2, and only occasionally using method 3. With method 3, *Pms* was recovered from plants in 16 tubes, with equal success using infested soil and infected plant tissue. The cultivar Columbus was half as effective as the other cultivars for recovering the fungus from these samples.

DISCUSSION

When using the isolation of *Pms* from plant tissue to study the race distribution of the fungus, care must be taken in assessing the information obtained. Several isolations may be made from a single infected plant, but it will not be known if each is a sub-isolate of an infection caused by a single propagule, or if each represents an individual propagule. If maximum information about the race distribution of *Pms* in a field is to be obtained, it would be necessary to obtain single isolates from a large number of random samples. The time and effort required for sampling all soybean-producing areas of the state could easily become restrictive.

Once infection has been obtained, the use of baiting techniques for the isolation of *Pms* is analogous to isolation from plant tissue, with the advantage that it is always from active disease lesions. Baiting techniques are more time consuming and suffer the same

disadvantages as isolation from plant tissue when single-propagule isolates are desired. It is reasonable that if single oospores of the fungus could be recovered from soil and germinated, several of the problems involved in random sampling and single-propagule isolation could be eliminated.

PART II. ISOLATION AND GERMINATION OF PHYTOPHTHORA MEGASPERMA VAR. SOJAE OOSPORES FROM PLANT DEBRIS IN SOIL

Oospores in crop residue have been suspected as the overwintering stage and source of primary inoculum for infection by *Phytophthora megasperma* Drechs. var. *sojae* A. A. Hildeb. (*Pms*) (16), causal agent of Phytophthora rot of soybean (*Glycine max* [L.] Merr.) (18). *Pms* oospores can be observed microscopically by staining thin sections of naturally infected soybean stems and roots. *Pms* oospores grown in culture have been thoroughly studied and factors affecting their production and germination include a sterol requirement (8,15), temperature (9), and periods of exposure to both darkness (14) and light of wavelength 400-1,000 nm (1). It is not known to what degree, if any, these factors affect oospore production and germination under natural conditions.

Recovery of *Pms* from soil has, to date, involved the use of bait plants. Dance et al. (7) suggested the use of *Pinus radiata* or *Cedrus deodara* needles and *Lupinus angustifolius* radicles to bait *Pms* from soil. Paxton (29) consistently recovered *Pms* by germinating soybean seeds in flooded field soil. Eye et al. (11) used two-day-old soybean seedlings as baits. More recently Canaday and Schmitthenner (3) reported the development of a soybean leaf disc technique for isolating *Pms* from field soil. In diagnostic work, where soil samples are often taken from beneath infected or recently killed plants, *Pms* propagules other than oospores may exist. Baiting techniques are nonspecific, and may lead to the recovery of oospores, as well as viable hyphal fragments, chlamydospores and

zoospores, and therefore do not clearly define the importance of oospores in the disease cycle of *Pms*. Direct observation of oospore germination, following isolation from natural sources, is necessary to unquestionably demonstrate the role of oospores in this cycle, but methods for direct observation are lacking.

While *Pms* oospores may be numerous in heavily infested soil, they are a distinct minority amidst a myriad of other microorganisms, soil particles and organic debris (38). Furthermore, at any given time only a small percentage of naturally fromed oospores that may be recovered from soil can be expected to germinate (2). For these reasons, separation and concentration of oospores from a large soil sample would facilitate the direct observation of oospore germination.

Decanting and sieving, long used to recover nematodes from soil (5), are sometimes used to recover fungal propagules such as sclerotia (6,21,24,28), spores of mycorrhizal *Endogone* spp. (13) and chlamydospores of *Phytophthora cinnamomi* Rands. (26). Described herein is a soil sieving technique for the isolation of *Pms* oospores from field soil, and a medium on which these spores will germinate.

MATERIALS AND METHODS

Sampling. Soil was collected from field plots known to be infested with *Pms*. A Division of Plant Industry field subsampling tool (10) was used to take twelve, 15-cm-deep, 2.3-cm-diameter soil cores per sample (600-800 cc of soil). Soil was sampled when moist, but not wet. Samples were placed in polyethylene bags, which were

loosely folded and stored at room temperature until used.

Media. Difco lima bean agar (LBA) at a concentration of 1.5% was used as the routine growth medium for *Pms* (Difco Laboratories, Detroit, MI 48232).

Amended LBA (Am-LBA) was prepared by adding to 500 ml of 1.5% LBA, cooled to 50 C, 25 mg each of penicillin G potassium (1585 U/mg; United States Biochemical Corporation, Cleveland, OH 44122) and ampicillin (856 mg/g; Sigma Chemical Company, St. Louis, MO 63178). The Am-LBA had a pH of 5.6 after autoclaving.

A germination medium (Pms-G) was prepared by adding 1 ml of antibiotic stock solution to 30 ml of 1% agarose (U.S. Biochem. Corp.) which had been autoclaved and cooled to 45 C. The antibiotic stock contained 1,550 µg active ingredient/ml each of benomyl (Benlate 50% WP; E. I. du Pont de Nemours and Co., Biochemical Department, Wilmington, DE 19898) and pentachloronitrobenzene (Terraclor 75% WP; Olin Mathieson Chemical Corporation, Agricultural Division, Little Rock, AR 72203), and 3,100 µg product weight/ml each of penicillin G potassium and streptomycin sulfate (740 mg/g; U.S. Biochem. Corp.). After mixing, the Pms-G medium was pipetted into sterile 18 X 150 mm culture tubes, 9.5 ml per tube, and held (30-60 min.) as a liquid at 40 C until ready for use. The Pms-G medium had a pH of 5.4 after autoclaving.

Sieving. Sieves used in this work were 20.3-cm-diameter, 60, 200, and 400 mesh (250, 74, and 38 µm openings, respectively; The U.S. Tyler Company, Mentor, OH 44060), and 7.6-cm-diameter with 27 µm openings (Buckbee Mears Company, St. Paul, MN 55101).

Isolation and germination of *Pms* oospores. A soil sample, including root and stem debris, was thoroughly pulverized and mixed in a polyethylene bag. Three hundred cubic centimeters of this sample were placed in a 13 L plastic bucket and mixed thoroughly with 4 L of tap water. The mixture was allowed to settle for 10 seconds and then the liquid portion was poured through a 250 µm sieve. Initially liquid passing through the sieve (filtrate) was saved, and attempts were made to recover the oospores it contained by sieving and centrifugation. Silt particles in the water made the recovery difficult, so the 250 µm filtrate was discarded. The soil remaining in the bucket was washed with a smaller amount of tap water from a spray hose, allowed to settle for 10 seconds, and the liquid portion poured through the 250 µm sieve. This process was repeated until no obvious plant debris remained in the bucket. The soil remaining in the bucket was discarded. The debris (screenings) caught on the sieve was then washed to one side of the sieve and collected in a 1 L Waring Blendor jar with approximately 100 ml of tap water. This mixture was homogenized at top speed for 10 minutes. After each minute of homogenization the lid of the blender was removed and the lid and sides of the jar were washed with a stream of tap water. At the end of 10 minutes the blending jar was approximately half full.

The homogenized debris sample was poured through 75 and 38 µm sieves and the filtrates were collected in a bucket. Both screens were washed with a gentle spray of tap water, and the filtrate was combined with those in the bucket. The screenings were discarded. The filtrate was poured through a 27 µm sieve. The material caught

on this sieve consisted of plant tissue, silt particles, and fungal spores. Filtrate from the 27 µm sieve was discarded. The 27 µm screenings were backwashed with distilled water into a 13 X 100 mm test tube, and then centrifuged at 1,100 g for 5 seconds. The supernatant was discarded and the pellet was resuspended with sterile distilled water to a volume of 5 ml.

Two milliliters of glycerin (technical grade) were placed into a 13 X 100 mm test tube. The 5-ml debris sample was mixed thoroughly and 2 ml were layered above the glycerin. The tube was centrifuged at 270 g for 5 seconds. The water, plant debris and fungal spores remaining in the top layer were removed using a Pasteur pipette and placed into another 13 X 100 mm test tube. The glycerin and soil particles in the lower layer were discarded.

The glycerin was diluted from the debris by washing with 5 ml of sterile distilled water, centrifuging at 1,100 g for 5 seconds, and decanting. Washing, centrifugation and decanting were repeated two times. After the final centrifugation, the pellet was resuspended to a final volume of 2 ml with sterile distilled water.

The 2-ml sample was mixed thoroughly, and 0.5 ml of the mixture was then mixed with 9.5 ml of melted Pms-G medium. One-ml aliquots were pipetted into sterile 60 X 20 mm plastic culture dishes, taking care to cover the entire bottom of each dish. After the medium solidified, each plate was sealed with Parafilm and kept in the dark at 25 C.

After 4-6 days of incubation, plates were inverted and germinated oospores were located with a dissecting microscope at 80 X.

Using a laminar-flow hood and 60-80 X magnification, germinated oospores were removed from the Pms-G medium with a flattened dissecting needle and placed on Am-LBA. These plates were incubated at 25 C for 4 days, at which time *Pms* colonies from single-oospores were ready to use for race determination. The hypocotyl-slit method (20, method 8) or the zoospore injection method (34) was used to determine the race of the *Pms* isolates.

The methods described above were originally developed using mature soybean root and stem tissue that contained oospores. Two grams of root and stem pieces collected after harvest at the St. John Experiment field, near St. John, Kansas, were processed in the manner described above for soil, starting with the blending process. After germination in the Pms-G medium, oospores were transferred to LBA and incubated in darkness for 2 days at 21 C. A hyphal-tip was then transferred to a fresh LBA plate. Five days later this plate was flooded (11) and resultant zoospores used to determine race (34).

At the St. John Experiment field, field resistance of several soybean cultivars to *Pms* was being tested under various irrigation regimes. From these plots, samples of soil with plant residue were taken from plots of two cultivars, Amsoy 71 and Cutler 71, and processed as described above. One soil core was taken from each of the two center rows of six field treatment replicates and pooled. The number of oospores that germinated following isolation was recorded for each cultivar/irrigation treatment. The experimental field soil was Tabler loam.

RESULTS

During development of the techniques described above, the first oospore observed germinating on Pms-G did so by sending out a germ tube 60 µm long. After transfer to LBA, the germ tube branched and the new hypha produced a sporangium. This sporangium did not liberate zoospores, but instead produced many new hyphae. The isolate was determined to be race 5 (34). Using the same procedure, race 4 was recovered from a root and stem sample from a soybean field near Ottawa, Kansas.

The tendency for oospores to germinate directly as opposed to producing sporangia was tested using the procedure described above for soil samples. Germinating oospores were transferred to Am-LBA within 6 hours of germ tube formation. Seven of 20 transferred oospores produced sporangia in the nutrient-rich Am-LBA within 8 hours, while those left in the nutrient-deficient Pms-G medium required three or more days.

Disease index readings taken at the St. John field during July showed Amsoy 71 to be susceptible and Cutler 71 to be resistant to the races of *Pms* present. All of the 223 single-oospore isolates from this field were determined to be race 5. Both cultivars are susceptible to race 5 in the laboratory, but Cutler 71 displays field-tolerance to Phytophthora rot (27,34).

TABLE 2. *Phytophthora megasperma* var. *sojae* (*Pms*) oospores germinated from field samples of two soybean cultivars under varying irrigation schedules.

Treatment[a]	Disease index[b]	*Pms* oospores germinated[c]
Cutler 71-1	1.0	3.2
-2	1.0	5.2
-3	1.0	4.2
-4	1.0	1.0
Amsoy 71-1	3.5	5.5
-2	4.0	1.8
-3	5.0	13.8
-4	4.0	21.0
		LSD (P = 0.05) = 4.7

[a] 1, 2, 3, and 4 are irrigation treatments representing wetting for 6, 12, 36, and 72 hours, respectively, when irrigated.

[b] Plots were rated 7/17/79. 1 = no obvious symptoms; 5 = severely affected.

[c] Samples were collected 11/15/79. Values listed are means of four replicate plates. Germinated oospores were counted after four days incubation in darkness at 21 C.

DISCUSSION

P. megasperma var. *sojae* causes a destructive disease of susceptible soybean cultivars. It may attack soybean plants anytime during the growing season, causing a severe root and stem rot. Many soybean cultivars are resistant to one or more races of *Pms*, and selecting the proper disease-resistant cultivar provides good protection from high yield losses. Prior knowledge of the races of *Pms* present in a field can greatly facilitate this selection.

Until recently, recovery of *Pms* from field samples involved isolation of the fungus from actively growing disease lesions, or baiting from infested soil. During a season with unfavorable conditions for disease symptom development, the fungus may go undetected even though *Pms* propagules are present in the soil. In subsequent seasons, if conditions are favorable, disease losses may be high. Since disease development and severity are dependent on many environmental factors, a method to consistently recover *Pms* from infested soil, regardless of environmental considerations, would be useful. The methods described here to isolate, germinate, and identify to race *Pms* oospores from plant debris in the soil hold such a promise. However, to be most useful, the technique needs to be applicable to soil samples taken throughout the year. This is currently being tested.

P. megasperma var. *sojae* oospores, free from plant debris, were observed in infested soil, but their separation from silt particles has proven difficult. By screening oospore-infested plant debris from soil, interference by soil particles and microorganisms

in isolation plates is greatly reduced. Bacterial and fungal contaminants are further reduced by washing samples with sterile distilled water, and by the use of antibiotics in the isolation medium.

Spores of several fungi germinated on the Pms-G medium. The low level of nutrients in the medium resulted in sparse growth of all species, which allowed recovery of *Pms* in the presence of faster growing fungi, from plates up to 10 days old.

In the cultivar/irrigation experiment, disease index readings for Amsoy 71 (Table 2) suggest that *Pms* propagules were uniformly distributed in July in the Amsoy 71 plots, with no apparent difference due to irrigation levels. However, the number of germinable oospores recovered in November indicates that oospore production was greatest in field-susceptible cultivars grown under heavy irrigation.

In this study, *P. megasperma* var. *sojae* oospores isolated from field soil were found in all of the stages of development described by Erwin and McCormick (9). Oospores in the pre-germination stage were 30-35 µm in diameter. Their outer wall is uniformly roughened and a light golden brown in color (under bright field illumination). Positive identification could be made only after germ tube formation, since *Pms* oospores resemble oospores and chlamydospores of other species. *Pms* oospores typically produced a germ tube, one to two times as long as the diameter of the spore, with a rounded to bulbous end. *Pythium* spp. tended to produce longer, faster growing germ tubes which continued to grow, albeit sparsely, on the Pms-G medium. Spores of mycorrhizal *Endogone* spp., commonly associated

with soybeans (30,31,32), closely resemble germinated oospores. Attached hyphal fragments, characteristic of *Endogone* spp., could be distinguished from oospore germ tubes by their empty appearance and broken end.

CONCLUSION

The primary defense against soybean yield losses due to Phytophthora rot is to plant disease resistant cultivars. Since *Pms* exists as several physiological races, maximum control of the fungus cannot be achieved without accurately determining its race distribution. In the past, this process was usually restricted to the growing season, when conventional techniques to isolate the fungus are most effective. In this research, standard nematological and plant pathological methods have been adapted to construct a technique for the isolation of *Pms* oospores using plant debris screened from soil, and for the subsequent germination of these spores. This technique should facilitate diagnostic, ecological and control studies of *Pms* and related fungi.

The problems encountered using conventional isolation techniques have already been discussed along with the shortcomings of each method tested. The techniques reported here hold several advantages over conventional methods. First, since these techniques allow a large random sample to be taken when testing for the fungus, they should better represent the true race distribution of *Pms* in a field. Second, a large number of single-oospore isolations can be made from a single sample. Finally, samples can be processed and isolate races determined within 11-12 days (20). It is likely, though untested, that samples used in these techniques may be taken any time infected plant debris is present in the soil.

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APPENDIX A

Several selective media have been developed for the isolation of *Phytophthora* spp. from soil and plant tissue (1,2,3). However, one or more of the selective agents they contain may alter the mycelial growth of *Phytophthora megasperma* var. *sojae* (*Pms*) so that developing colonies of the fungus are not easily recognized. For that reason, several antibiotics and fungicides were tested for their effects on the mycelial growth of *Pms*. Those materials showing the least inhibitory effects were subsequently tested in selected combinations, in an attempt to determine a suitable combination for use in a selective medium for the isolation of *Pms*.

Materials tested were obtained from the following companies: ampicillin, bacitracin, chloramphenicol, gramicidin S hydrochloride, kanamycin, neomycin sulfate, nystatin, polymyxin B sulfate, and vancomycin from Sigma Chemical Co., St. Louis, MO 63178; benomyl from E. I. du Pont de Nemours and Co., Biochemical Department, Wilmington, DE 19898; dimethylsulfoxide from J. T. Baker Chemical Co., Phillipsburg, NJ 08865; hymexazol and pentachloronitrobenzene from Olin Mathieson Corp., Agricultural Division, Little Rock, AR 72203; penicillin G potassium and streptomycin sulfate from United States Biochemical Corp., Cleveland, OH 44122; and pimaricin from Gist-Brocades, N.V., Delft, Holland.

In all cases, 1.5% lima bean agar (LBA; Difco Laboratories, Detroit, MI 48232), 15 ml per plate, was used as the base medium. The LBA was autoclaved and cooled to 50 C before the antibiotics and fungicides were added. Mycelial plugs 3 mm in diameter were cut

from 4-5-day-old LBA cultures of *Pms* race 4, inverted, and placed on each plate as inoculum. The colony diameter was measured after 3 days incubation at 21 C in darkness.

Ampicillin, bacitracin, neomycin, penicillin, pentachloronitrbenzene (PCNB), polymyxin, and vancomycin were not significantly inhibitory to the growth of *Pms* mycelium at 100 parts per million (ppm). Benomyl and kanamycin were not inhibitory at 50 ppm. At some concentrations, bacitracin, neomycin, nystatin and penicillin actually increased the mycelial growth of *Pms* on LBA (Table 3).

P. megasperma var. *sojae* mycelium grows well on LBA amended with Tsao and Ocana's (3) combination of pimaricin and PCNB, when bacitracin and penicillin are substituted for vancomycin, as shown in Table 4. However, polyene antibiotics (pimaricin, nystatin, and hymexazol) are not thermostabile, and may be inactivated by light. For these reasons, Schmitthenner developed a medium containing a combination of benomyl and PCNB, as an alternative to the use of polyene antibiotics for selective isolation of *Pythium* and *Phytophthora* spp. (1). The medium containing this amendment combination (Table 4; B,E,F,H) was found as effective as Schmitthenner's medium for isolation of *Pms* from plant tissue (Table 1).

TABLE 3. Effects of selected antibiotic and fungicide amendments on the mycelial growth of *Phytophthora megasperma* var. *sojae* race 4 on lima bean agar (LBA).

Amendment	Purity	Concentration[a]	Growth Index[b]
Ampicillin	857 µg/mg	100	100.0
Bacitracin	50,000 U/ 0.935 g	25	103.6
		50	100.0
		100	100.0
Benlate	50% benomyl	100	100.0
		200	42.9
Chloramphenicol	———	5	75.5
		10	50.8
Dimethyl-sulfoxide	Baker analyzed	1,300	95.7[c]
Ethanol	95%	2,000	85.7
Gramicidin S hydrochloride	1,000 µg/mg	50	78.6
		100	64.3
		200	50.0
Hymexazol	70%	25	91.3[c]
		50	87.0[c]
		100	73.9[c]
Kanamycin	750 µg/mg	25	100.0
		50	94.7
		100	73.7
Neomycin sulfate	750 µg/mg	25	103.6
		50	100.0
		100	107.1
Nystatin	5,400 U/mg	5	107.1
		10	100.0
		15	92.9
		20	78.6
		25	78.3
		50	47.8
		100	34.8
		400	0.0
Penicillin G potassium	1585 U/mg	25	117.9
		50	110.7
		100	103.6

TABLE 3. (continued).

Amendment	Purity	Concentration[a]	Growth Index[b]
Terraclor	75% PCNB	67	92.9
		133	92.9
		267	78.6
Pimaricin	50%	25	78.6
		50	57.1
		100	42.9
Polymyxin B sulfate	8,000 U/mg	50	100.0
		100	96.4
Streptomycin sulfate	740 mg/g	25	89.5
		50	73.7
		100	57.9
Vancomycin	978 µg/mg	25	100.0
		50	96.4
		100	96.4

[a] Final amendment concentration, parts per million in 1.5% LBA.

[b] Colony diameter at 3 days as a percentage of the colony size on LBA without amendments; average of three replicate plates.

[c] Growth of fungus less dense and colony edge more irregular than on LBA without amendments.

TABLE 4. Combined effects of selected antibiotics and fungicides on the mycelial growth of *Phytophthora megasperma* var. *sojae* race 4 on lima bean agar (LBA).

Amendment combinations[a]	Concentrations, ppm[b]	Growth Index[c]
A,E	100,100	109.8
A,B,D	100,100,50	104.9
A,B,E	100,100,50	73.2
A,C,E	100,100,50	46.3
A,B,C,D	100,100,50,50	48.9
A,B,D,E	100,100,50,50	73.2
A,B,C,E	100,100,50,50	63.4
A,B,C,D,E	100,50,50,50,50	56.1
A,E,F	100,100,100	100.0
A,E,F,G[d]	100,100,100,2.5	100.0
A,E,F,G[d]	100,100,100,5	96.2
A,E,F,G[d]	100,100,100,10	96.2
B,E,F,H[e]	50,100,50,100	89.0
F,G,I[f]	100,10,200	30.8

[a] Amendment combinations in 1.5% LBA; A = bacitracin; B = benomyl; C = hymexazol; D = nystatin; E = penicillin G potassium; F = pentachloronitrobenzene; G = pimaricin; H = streptomycin; I = vancomycin.

[b] Final concentration of each amendment, parts per million in 1.5% LBA.

[c] Colony diameter at 3 days as a percentage of the colony size on LBA without amendments; average of three replicate plates.

[d] A modification of the amendment combination in Tsao and Ocana's $P_{10}VP$ medium (2).

[e] A modification of the amendment combination in Schmitthenner's medium (1).

[f] Tsao and Ocana's $P_{10}VP$ amendments substituting LBA as the base medium (2).

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APPENDIX B

In 1979, Schwenk et al. (3) described a method to inoculate soybean plants by injecting zoospores of *Phytophthora megasperma* var. *sojae* (*Pms*) to reduce the time and effort spent in screening soybean cultivars for resistance to the fungus. When zoospores from one race of *Pms* were used, this method gave results similar to those obtained by method 8 of Kaufmann and Gerdemann (2). A potential advantage of the zoospore injection method is that zoospores of several races of *Pms* could be mixed and injected simultaneously, further reducing screening time. To test for synergistic or antagonistic effects, zoospores of several races of *Pms* were mixed and injected into hypocotyls of seven soybean cultivars.

Races 1-6 of *Pms* were grown on 1.5% lima bean agar (LBA; Difco Laboratories, Detroit, MI 48232), 9-14 ml of medium per plate. Two to three-day-old cultures were flooded with three changes of sterile tap water (15 ml each flooding) at 40 minute intervals to produce zoospores (1). Zoospore concentration in the final flood water was determined (1), suspensions were diluted with sterile distilled water, and races mixed to give a final concentration for each race as listed in Table 5. Zoospore mixtures were used to inoculate 4-6-day-old soybean plants (3).

Seeds of the cultivars Columbus, Calland, D-60, Cutler 71, Mack, Altona, and Tracy were germinated in vermiculite and grown under continuous light at 28 C in controlled environment chambers (3). The vermiculite was fertilized once with tap water containing 1 g of 20-20-20 fertilizer/L. Resistance or susceptibility of the plants

was determined 3 days after inoculation. Plants which developed brown, constricted, or watersoaked lesions accompanied by wilting were rated as suceptible. Plants with no such sympoms were rated as resistant (Table 5).

At the zoospore concentrations tested for races 1-6, when a cultivar with known susceptiblilty to a race was injected with a zoospore mixture containing that race, a susceptible reaction was obtained. Conversely, inoculating with any mixture of races not normally pathogenic to a cultivar resulted in a resistant reaction. A notable exception in these two cases was the cultivar Altona, which did not exhibit a distinct resistance or susceptibility to mixed races of the fungus. Results when injecting Altona with any mixture of races ranged from moderately susceptible to susceptible.

TABLE 5. Reactions of selected soybean cultivars to hypocotyl injection with mixed zoospores of races 1-6 of *Phytophthora megasperma* var. *sojae*.

Races	Conc. X 10^5	Cultivar						
		Columbus	Calland	D-60	Cutler 71	Mack	Altona	Tracy
1,2	20	11/11[b]	0/11	9/9	-----	0/12	5/6	0/7
1,3	10	32/32	0/28	-----	-----	0/24	10/24	0/23
1,3	20	10/10	7/7	2/9	-----	0/12	8/8	0/5
1,5	10	19/19	14/14	-----	-----	12/12	12/12	2/15
1,5	30	7/7	19/19	-----	14/15	8/8	1/9	0/14
1,6	10	9/9	8/11	-----	-----	0/9	11/11	0/10
1,6	30	6/6	17/18	-----	10/11	0/9	12/12	0/13
2,3	10	45/45	7/22	-----	4/30	1/37	14/32	0/32
2,3	20	42/42	24/24	20/20	5/7	0/36	30/38	0/32
2,4	10	24/24	21/21	-----	8/8	20/20	12/19	1/18
2,4	20	29/32	22/29	25/26	-----	27/29	13/26	1/20
2,5	20	26/27	6/6	4/4	10/10	18/18	17/26	1/20
2,6	10	14/14	-----	-----	15/20	0/11	10/11	0/11
3,4	10	24/24	21/21	-----	8/8	20/20	9/18	0/18
3,4	20	14/14	15/15	10/14	-----	14/14	13/14	0/10
3,5	10	20/20	15/15	-----	-----	14/14	10/12	0/9
3,5	20	17/18	-----	-----	8/8	11/11	11/13	0/13
3,6	10	32/32	8/11	-----	17/17	0/25	22/24	3/19
3,6	20	6/7	9/11	0/8	-----	0/11	9/9	1/9
4,5	10	17/17	21/21	-----	17/18	21/21	10/23	0/21
4,6	20	18/18	25/25	-----	21/21	23/23	10/15	0/20
5,6	10	25/25	27/27	-----	17/17	19/19	6/13	0/12
5,6	30	8/8	13/13	-----	10/10	11/11	12/12	1/13
1,3,5	5	19/19	11/11	-----	-----	16/16	14/17	0/15
1,3,6	5	9/10	7/14	-----	-----	0/9	10/10	0/10
1,5,6	20	10/10	14/14	-----	11/11	12/12	11/13	0/13
2,3,4	5	14/14	13/14	-----	-----	15/15	11/16	0/12
2,3,5	20	15/15	-----	-----	10/10	13/13	14/15	0/11
4,5,6	10	32/32	46/47	-----	38/38	42/42	18/34	2/9
1,2,4,5,6	5	81/81	99/99	-----	75/75	66/67	43/68	1/63
1-6	10	25/25	29/29	-----	23/23	25/25	15/19	2/8
1-6	30	43/43	53/53	-----	23/23	25/25	15/19	2/18

[a] Zoospores of each race X 10^5 per ml of suspension; rounded to the nearest 5.

[b] Number of dead plants/ number of plants injected, 3 days after injecting 4-6-day-old plants.

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TECHNIQUES FOR ISOLATING PHYTOPHTHORA MEGASPERMA VAR. SOJAE FROM SOIL AND PLANT TISSUE

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B.A., DePauw University, 1977

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AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Plant Pathology

KANSAS STATE UNIVERSITY
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1980

Published methods for isolating *Phytophthora megasperma* Drechs. var. *sojae* Hildeb. from soil and plant material were investigated as a pretest to a race distribution study. A new technique was developed to isolate and germinate *P. megasperma* var. *sojae* oospores from soybean debris in field soil.

Oospores of the fungus were isolated from naturally infected soybean stem and root tissues recovered from 300 cc of soil by wet sieving. Oospores were freed from screened plant debris by homogenization in a Waring Blendor, separated from the debris by passing the homogenate through 250 and 75 µm sieves, and concentrated by collection on a 27 µm sieve.

Concentrated oospores could be germinated in a medium containing 1% agarose and 100 parts per million (ppm) each of penicillin G potassium and streptomycin sulfate, and 50 ppm each of benomyl and pentachloronitrobenzene. Germinated oospores were transferred to 1.5% lima bean agar amended with 100 ppm each of penicillin G potassium and ampicillin. Resultant colonies of the fungus were used to determine the race of the pathogen. Samples could be processed and isolate pathogenicities (races) determined within 11-12 days.

This technique has advantages over previously published methods. Large, random samples may be taken that should permit a more accurate assessment of the true race distribution in a field. Also, a large number of single-oospore isolates may be obtained.

This technique should be useful in diagnostic and ecological studies of *P. megasperma* var. *sojae* and related fungi.