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METHODS FOR INOCULATION OF SOYBEAN SEEDLINGS WITH ZOOSPORES AND OOSPORES OF PHYTOPHTHORA MEGASPERMA VAR. SOJAE FOR PATHOGENICITY AND RACE DETERMINATION

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METHODS FOR INOCULATION OF SOYBEAN SEEDLINGS WITH ZOOSPORES AND OOSPORES OF PHYTOPHTHORA MEGASPERMA VAR. SOJAE FOR PATHOGENICITY AND RACE DETERMINATION

By

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ABSTRACT

METHODS FOR INOCULATION OF SOYBEAN SEEDLINGS WITH ZOOSPORES AND OOSPORES OF PHYTOPHTHORA MEGASPERMA VAR. SOJAE FOR PATHOGENICITY AND RACE DETERMINATION

By

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Factors affecting zoospore production by Phytophthora megasperma var. sojae in culture were studied so that large numbers of zoospores could be produced for use in soybean seedling inoculation experiments. Two inoculation methods were developed and evaluated for their reliability in producing disease and for identifying races of the pathogen using differential cultivars of soybean (Glycine max (L.) Merr.).

Cultures of P. megasperma var. sojae grown on lima bean agar were flooded with distilled water to produce zoospores. Maximum numbers of 4×10^5 zoospores/ml were obtained when 6-day-old cultures of race 1 of the pathogen received five water changes (15 ml/plate/change) at 30 minute intervals, followed by incubation in the dark for 8 to 18 hours at 20°C. Isolates of races 2 to 6 also produced large numbers of zoospores using this method.

One inoculation procedure involved inoculating soybeans planted in moist vermiculite with a zoospore suspension when seedlings were 2 to 6 days old. Disease was most severe when 2-day-old seedlings were inoculated. Concentrations of 500 zoospores/seedling did not produce disease; 10^4 zoospores/seedling differentiated susceptible from

resistant cultivars, but 10^5 zoospores/seedling caused disease in some cultivars which were resistant when wounded hypocotyls were inoculated with mycelium. Disease development differed little when seedlings were incubated for 5 to 6 days at temperatures of 20 to 30°C from the time of inoculation. Zoospores from isolates of six races of the pathogen were used to individually inoculate nine differential cultivars of soybean. Susceptible cultivars usually rotted before emergence, whereas resistant cultivars remained healthy. Results corresponded with those obtained by hypocotyl inoculation.

The second inoculation procedure involved placing 2-day-old soybean seedlings in petri dishes containing 10 to 20 g steamed soil that had been flooded with 30 ml distilled water and infested with zoospores. Some seedlings were infected when soil was infested with as few as 10 zoospores/petri dish (5 seedlings/petri dish); 5×10^3 zoospores/petri dish caused disease in some cultivars which were resistant by hypocotyl inoculation. Illumination did not affect disease when zoospores were used as inoculum. However, disease incidence was higher in naturally infested soil or soil artificially infested with oospores kept in darkness than under continuous fluorescent light. Temperatures of 20 to 30°C did not affect disease development. However, temperatures above or below this range decreased disease incidence. Nine differential cultivars of soybean were inoculated individually with zoospores (2×10^2/seedling) of six races of the pathogen in flooded soil. Results corresponded with those obtained by hypocotyl inoculation.

Soybean seedlings were evaluated as baits for recovering *P. megasperma* var. *sojae* from naturally infested soil. Two-day-old

seedlings were placed in petri dishes containing 20 g soil and 30 ml distilled water. Seedling infection was evaluated after 3 days incubation, and sporangia were identified by direct examination with a dissecting microscope. Using this method, the pathogen was recovered from several field soils. The pathogen was similarly recovered from diseased soybean root and stem tissue placed on flooded sterile soil.

To Doug

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INTRODUCTION

Phytophthora root and stem rot, caused by Phytophthora megasperma (Drechs.) var. sojae Hildeb., is a very widespread and damaging disease of soybeans in Michigan. Although there are no comprehensive figures available as to the extent of economic losses, it is considered to be the major disease problem in the over 600,000 acres under soybean cultivation. It is also an important disease in other parts of the U.S. and in Ontario.

Phytophthora root and stem rot of soybeans was first observed in Ohio in 1951 (82). A number of different sources of resistant germ plasm were found by 1954 (9), and resistant cultivars were developed that controlled the disease until new races of the pathogen appeared that caused disease in soybean cultivars which were formerly resistant. In 1965 a second physiological race of the fungus was identified in Mississippi (40). Since 1972 races 3, 4, 5, 6, 7, 8, and 9 have been identified (25, 47, 69, 72). The occurrence of numerous races of the pathogen has made necessary the development of reliable race-identification techniques.

Hypocotyl inoculation is the conventional method of evaluating resistance in soybeans. A small piece of mycelium is inserted into an incision in the hypocotyl, and susceptible plants collapse and die within a few days (37).

However, the hypocotyl inoculation method has shortcomings. The method is tedious to perform, requires considerable amounts of

greenhouse space, is susceptible to changes in environmental conditions such as temperature fluctuations, may yield inconsistent results, and does not detect types of resistance designated as 'field tolerance'. Moreover, the hypocotyl-wound technique is of no use for studies of environmental factors on the infection process, inoculation experiments using natural propagules, i.e., oospores and zoospores in relation to the natural infection court, or for investigations of biological control. An alternative method utilizing spores of the pathogen and the plant root as the site of penetration and infection would therefore be desirable.

Phytophthora spp. can readily be isolated from plant tissue, but isolation even with the aid of selective media directly from soil has proven difficult. Susceptible host plants or fruits have been relied upon to detect and isolate these pathogens directly from naturally infested soils (6, 11, 13, 14, 21, 50, 58, 62, 63, 64, 83). Baiting methods allow rapid detection and identification of the pathogen, often without the necessity for isolation and inoculation procedures.

The present investigation was undertaken to i) develop methods for abundant zoospore production, ii) develop reliable inoculation methods using natural propagules which could be used for race identification, and iii) determine the feasibility of employing soybean seedlings as baits to detect and isolate P. megasperma var. sojae from natural soil.

LITERATURE REVIEW

The Pathogen

Phytophthora root and stem rot, incited by *Phytophthora megasperma* (Drechs.) var. *sojae* Hildeb., is a relatively new but important disease of soybeans (*Glycine max* (L.) Merr.). It attacks soybean plants in all stages of growth, causing pre-emergence and post-emergence damping-off of seedlings, and a root and stem rot that results in wilting and death of plants from the primary-leaf stage to maturity (31, 37). It was first observed in Ohio in 1955 by Suhovecky and Schmitthenner (82) who reported that the disease was caused by a species of *Phytophthora*. Subsequently, the pathogen was referred to, incorrectly, as *Phytophthora cactorum* (Leb. & Cohn) Schroet. (29). Kaufmann and Gerdemann (37) considered the organism distinct from described species of *Phytophthora* and proposed the name *Phytophthora sojae*. The current trinomial, based on morphological similarity to *Phytophthora megasperma* and host specificity, was proposed by Hildebrand in 1959 (31).

After the first report of this root rot of soybean in Ohio, the disease appeared in many other areas throughout the soybean-producing regions of the Midwest, South, and Ontario (25, 40, 47, 69, 72). In 1954 resistant cultivars were identified, with resistance of the cultivar Mukden controlled by a single dominant gene (9, 56). The Mukden-type resistance, assigned the gene symbol *Rps*, was used to

develop several resistant cultivars for the Midwest. In 1965 a second physiological race of P. megasperma var. sojae was identified in Mississippi, and an additional allele, rps^2, recessive to Rps but dominant to rps, was discovered (27, 40). In 1972 a third and more virulent race was found in Ohio (69). Cultivars with the Mukden-type resistance were susceptible to race 3, whereas Arksoy and its derivative, Mack, were resistant to race 3. In 1974 a strain obtained from Kansas was designated race 4; it was pathogenic to cultivars with both the Arksoy and Mukden types of resistance (72). Those cultivars with the $rps^2 rps^2$ allele are resistant to races 1, 3, and 4, but susceptible to race 2 (57). Cultivars Altona, Kingwa, and Toku are resistant to races 1, 2, 3, and 4 (57). In 1976 races 5 and 6, to which Altona is susceptible, were reported from Ontario, Canada (25). The two races were differentiated by the susceptibility of Mack to race 5 and its resistance to race 6. In 1977 races 7, 8, and 9 were reported from Indiana (47). The three races were differentiated as follows: Harosoy, Harosoy 63, and Altona were susceptible and Sanga and Mack were resistant to all the Indiana isolates; P.I. 103.091 was resistant and P.I. 171.442 was susceptible to race 7; but P.I. 103.091 was susceptible and P.I. 171.442 was resistant to race 8; both P.I. 103.091 and P.I. 171.442 were resistant to race 9. Races 5 and 9 were differentiated by the susceptibility of Mack to race 5 and its resistance to race 9. The cultivar 'Tracy' is resistant to all nine races of the pathogen and studies have shown that three different dominant genes are involved in the resistance (4). In July 1975 at Harrow, Ontario plant pathologists and plant breeders agreed to adopt a uniform set of soybean cultivars for differentiating races 1 through

9. These consisted of Harosoy, Sanga, Harosoy 63, Mack, Altona, P.I. 103.091, and 171.442 (47).

Oospores of Phytophthora spp. have a constitutive dormancy period (7, 17, 78) which may be broken by various treatments. Germinability of P. cactorum oospores in vitro was increased by prior incubation in natural soil (78), and that of P. megasperma var. sojae was increased by preincubation in water for 48 hours at 36°C (49). Water or water agar is usually used as a medium for oospore germination, but soil extract agar (7) has given improved germination of oospores of P. cactorum. Germination of oospores in soil has not been studied.

Visible light has been shown to stimulate oospore germination of Phytophthora spp. in vitro (7, 8, 17, 49, 68). The blue and far-red regions of the light spectrum have been shown to increase oospore germination in P. cinnamomi, P. megasperma var. sojae, and P. capsici (68). However, the significance of light as a factor in oospore germination in natural soil apparently has not been studied.

Zoospore production by P. megasperma var. sojae apparently has been studied very little. Previous workers found that sporangia never developed on solid media, but could be induced to form in variously treated media, e.g., i) placing mycelial wefts in Petri's solution (75), ii) treating corn meal culture squares with Petri's solution and then with distilled water (31), and iii) placing mycelial plugs in non-sterile stream water (33, 35). However, the factors affecting zoospore production have not been systematically investigated.

Inoculation Methods

Hypocotyl inoculation is the conventional method of testing pathogenicity of P. megasperma var. sojae, and evaluating resistance in soybeans (4, 27, 31, 37, 40, 45, 47, 56, 69, 72). A small piece of mycelium is inserted into a longitudinal incision in the hypocotyl midway between the soil line and the cotyledons. Kaufmann and Gerdemann (37) were the first to use this method for studies of soybean resistance to P. megasperma var. sojae.

Some investigators have sought a more natural host-parasite system for evaluating resistance. Inoculation methods have been worked out whereby plants were grown in either sand or soil (31, 36, 38, 56, 69) or liquid culture (38, 40) infested with a mycelial suspension. Studies comparing these methods showed no clear advantage of one method over another (31, 40, 56). Recently Keeling (38) compared i) hypocotyl inoculation, ii) seed planted in sand infested with mycelia, and iii) plants grown in liquid medium infested with mycelia. He used plants representing a wide range of germ plasm and two races of the pathogen. He concluded that the hypocotyl inoculation method was more reliable and less subject to experimental variation due to inoculum concentration, environmental influence, and differences in seed or seedling vigor.

Isolation from Soil

Selective media have been developed for isolation of Phytophthora spp. from soils and plant tissue. These media were based mainly on the selective inhibition of non-pythiaceous fungi by the use of the tetraene antibiotics, pimaricin and mycostatin (15, 16, 24, 59, 60, 84,

85) or gallic acid (18), and of bacteria by penicillin, polymixin, chloramphenicol, and vancomycin. The literature on this subject was summarized by Tsao (84). Using these selective media, Phytophthora colonies from soil usually can be identified under low magnification after a short incubation period. However, all these media have allowed the growth of Pythium spp. and most Mortierella spp. which are common in soils and infected tissues, and often interfere with the direct isolation and enumeration of Phytophthora spp. Sneh (76) reported a selective agar medium containing corn oil which permitted P. cactorum colonies to become visible macroscopically on plates free from other organisms. Very recently, HMI (3 - hydroxy - 5 methylisoxazole), was reported to greatly inhibit the growth of Pythium spp. and Mortierella spp., but was virtually noninhibitory to germination of various kinds of spores of selected Phytophthora spp. (52, 86). A selective agar medium supplemented with HMI was used to successfully isolate Phytophthora spp. from artificially and naturally infested soils containing high populations of Pythium spp. and Mortierella spp. The medium may have potential for the direct isolation and enumeration of Phytophthora spp. from soil.

Because Phytophthora is one of the most difficult genera of pathogenic fungi to isolate from soil, its detection, isolation, and enumeration have usually relied upon the use of susceptible host plants or fruits as baits. Phytophthora cactorum has been isolated from naturally infested soil by baits such as apple fruit (58, 74, 88), pear fruit (74), and blue lupine seedlings (62). The apple technique is not specific for P. cactorum and also has been used for isolation of other Phytophthora species such as P. cinnamomi (11, 58), P. citricola

(58, 74), P. cryptogea (58), and P. syringae (53, 58, 74). Alfalfa seedlings have been used as baits to detect P. megasperma in infested soils and to study the ecology of the fungus (50, 51, 63, 64). Pine needles (Pinus radiata and Cedrus deosara) have been used to bait P. boehmeriae, P. cactorum, P. cambivora, P. cinnamomi, P. citricola, P. crytogea, P. drechsleri, P. hibernalis, P. megasperma var. megasperma, P. nicotianae var. nicotianae, P. nicotianae var. parasitica, and P. rachardiae from naturally infested soils (13, 21). Laboratory tests with zoospore suspensions have established that P. citrophthora, P. heveae, P. megasperma var. sojae, and P. palmivora were also able to infest needles of Pinus radiata. Radicles of lupin (Lupinus angustifolius) were shown to have the same spectrum of susceptibility as pine needles (13).

Tsao (83) described a lemon fruit baiting technique for Phytophthora citrophthora and P. parasitica which allowed estimation of inoculum levels in soils by assaying them in a series of dilutions with uninfested soil. Marks and Mitchell (50) used the "serial dilution end-point" method to estimate inoculum levels of P. megasperma in soil by using alfalfa seedlings as baits. Duncan (14) used rooted runners of a clone of Fragaria vesca as baits to detect P. fragariae in infested soils and assessed inoculum levels using diluted soil suspensions and the 'most probable number' method.

Baiting methods allow detection and enumeration of Phytophthora spp. directly from naturally infested soils. However, identification of species may require isolation on agar since sexual structures may not be sufficiently visible (and measurable) on the baits. Race

identification of many Phytophthora spp. may also be possible using differential bait plants without the need for isolation and inoculation procedures.

METHODS AND MATERIALS

Maintenance of Fungus

Phytophthora megasperma var. sojae, races 1, 2, 3, 4, 5, and 6 were used. Isolates of races 1 to 5 were obtained from Dr. A. F. Schmitthenner (Ohio Agricultural Research and Development Center, Wooster), and isolates of races 1, 2, and 6 were obtained from Dr. J. H. Haas (Agriculture Canada Research Station, Ontario, Canada). Race 1, obtained from Dr. Schmitthenner, was used in all experiments in which the factors affecting zoospore production were evaluated. All the isolates were used in experiments to evaluate inoculation methods. Each race of the fungus was maintained as a stock culture on V-8 juice agar slants (per liter: 200 ml V-8 juice, 3.0 g $CaCO_3$, and 20 g agar). New cultures were prepared every 2 to 3 months from soybean seedlings (cultivar 'Hark') placed on flooded cultures of the fungus on Difco lima bean agar. Three days later the fungus was reisolated as described in 'Isolation Methods'.

Production, Collection, and Preparation of Inocula

Oospores of the fungus were produced on V-8 juice broth in petri dishes [per liter: 100 ml V-8 juice, 1.0 g $CaCO_3$, and 30 µg cholesterol (dissolved in 1.5 ml ethanol over heat)] (5). The plates were incubated in the dark at room temperature (23 $\pm$ 2°C). When the cultures were 1 to 6 weeks old, the mycelial mats containing oospores were placed on disks of nylon mesh (54 µm) and excess moisture was

removed by placing the disks on the base of a membrane filter apparatus, and applying a slight vacuum. The mycelial mats were then air-dried for 24 to 36 hours to kill the mycelium. When dried material was placed on V-8 juice agar for four days, there was no evidence of mycelial growth. Oospore suspensions were prepared by grinding the dried mycelial mats with a glass tissue homogenizer. Concentrations were determined using a hemacytometer.

When the effect of light on oospore germination was studied, a suspension of 5-week-old oospores was applied to Nuclepore (polycarbonate) membrane filters (13 mm diameter, 0.4 µm pore size; General Electric Corp., Pleasantville, California). About 10^3 oospores were applied per membrane. The membranes were incubated on flooded sterile soil for seven days. For determination of germination, the oospores were transferred to water agar (77).

Zoospores were produced as follows: mycelial disks were cut with a 5 mm diameter cork borer from the periphery of 4- to 6-day-old cultures grown on Difco lima bean agar. Individual disks were transferred to the center of 10 cm diameter petri dishes containing 7 to 16 ml of agar, and incubated at 23 $\pm$ 2°C for 2 to 10 days. The plates were then flooded with 15 ml distilled water or other flooding solutions (See Results) which submerged all mycelia. The water was changed 0 to 8 times at 2.5 to 60 minute intervals. Incubation temperatures of 10 to 35°C were used. The plates were left uncovered throughout the washing sequence. After the final water change, the plates were flooded once more, covered, and placed under various light conditions (See Results) for 4 to 24 hours. The volume of water recovered from each plate was recorded as the zoospores were collected.

Volumes varied from 13 to 17 ml. A second and third crop of zoospores could be harvested from the same plates when flooding was repeated, once for each crop. Zoospore concentration was determined either by counting the spores in five random fields of a hemacytometer or by counting the spores of five different 2 µl aliquots (44). Before counting, zoospore motility was stopped by adding a drop of 0.1% cotton blue in lactophenol (w/v) to 1 ml of zoospore suspension.

Isolation Methods

The pathogen was isolated from diseased hypocotyls of soybean seedlings. The cotyledons were removed, and the hypocotyls were surface-disinfested by immersion in 0.5% sodium hypochlorite for 10 seconds. The tissue was then washed with water and dried on filter paper. Small portions of the tissue were excised by making cross-sectional cuts, and placing them, cut surface down, on a modified selective medium (76) (per liter: 0.1 g $CaSO_4$, 0.02 g thiamine-HCl, 5.0 g sucrose, 7 ml corn oil, 0.1 ml Triton B 1965, 15 g agar, 20 mg benomyl, 60 mg penicillin G (K^+), 60 mg polymixin B sulfate, and 60 mg chloramphenicol). The plates were incubated for 1 to 3 days at 23 $\pm$ 2°C. Mycelial growth from the diseased segments was transferred, as soon as visible, to V-8 juice agar containing antibiotics (per liter: 200 ml V-8 juice, 3.0 g $CaCO_3$, 20 g agar, 60 mg penicillin G (K^+), 60 mg polymixin B sulfate, and 60 mg chloramphenicol). These plates were incubated for 24 hours and then the agar was inverted. Three to five days later, when the fungus had grown up through the agar layer, it was transferred to Difco lima bean agar.

Source and Preparation of Soils

Conover sandy clay loam was collected from the Michigan State University Botany and Plant Pathology Farm. It possessed the following characteristics: pH 7.8, organic matter 2.96%, water holding capacity 28%, clay 26%, silt 24%, and sand 50%. This soil was found to be free of the pathogen, P. megasperma var. sojae, and was used in its natural state or was steamed at 100°C for 30 minutes. Brookston loam soil, also collected from the Michigan State University Botany and Plant Pathology Farm, was found to be infested with P. megasperma var. sojae. It possessed the following characteristics: pH 7.5, organic matter 6.46%, water holding capacity 48%, clay 19%, silt 33%, and sand 48%. The soils were screened through a sieve with 2.5 mm openings, uniformly mixed, and stored air-dried in polyethylene bags in the laboratory.

In some experiments, steamed Conover sandy clay loam was artificially infested with an aqueous suspension of oospores of P. megasperma var. sojae. The oospores had been concentrated by centrifugation, and the oospore suspension was mixed with the air-dried soil by hand.

Extract from natural soil was prepared by suspending 1 kg of air-dried Conover sandy clay loam in 1 liter distilled water for 24 hours. The supernatant soil suspension was coarsely filtered through 3 layers of cheesecloth under a slight vacuum, and was centrifuged at 5000 rpm for 10 minutes. The soil extract, either used as prepared, diluted, or autoclaved, was employed to test its effect on disease incidence in soybean seedlings inoculated with zoospores and oospores.

Zoospore Inoculation of Soybean Seedlings Growing in Vermiculite

Soybean seeds were planted on the surface of a 4 cm-deep layer of distilled water-saturated vermiculite in plastic trays 27 X 19 X 6, 19 X 19 X 6, or 19 X 9 X 6 cm. The trays contained varying numbers of rows at least 2.5 cm apart each with 5 to 10 seeds. The seeds were covered with a 1.5 cm layer of dry vermiculite and trays were covered with polyethylene bags to minimize evaporation. After 2 days at 23$\pm$ 2°C, the polyethylene bags were removed and distilled water was added to saturate the vermiculite. The seedlings were inoculated by applying 2 ml of a zoospore suspension, usually containing 10^4 zoospores, at the base of each seedling using a Cornwall automatic pipette syringe. The zoospore suspension was constantly agitated with a magnetic stirrer throughout inoculation of seedlings. After inoculation, the trays were incubated in a growth chamber under light (17,000 lux) for 12 hour photoperiods at 23 $\pm$ 2°C. In controlled temperature experiments, trays were placed in non-illuminated incubators. The standing water in the trays evaporated during the first 24 to 36 hours. After that time, water was added as needed to keep the vermiculite moist. Disease was evaluated 4 to 6 days after inoculation, when the uninoculated control seedlings had produced their first true leaves.

Disease was evaluated on the basis of emergence and by examining the root systems of emerged seedlings. A plant was considered susceptible if it failed to emerge or if the taproot and lateral roots were discolored and flaccid. Roots free of lesions or with small (<1 mm), localized lesions were considered resistant to the pathogen. The pathogen could be isolated from these lesions.

Races 1, 2, 3, 4, 5, and 6 of P. megasperma var. sojae were used to inoculate the following cultivars of soybean: Altona, Hark, Harosoy 63, Higan, Kingwa, Mack, Sanga, Toku, and Tracy (Table 1).

In July of 1975 plant pathologists and plant breeders agreed to adopt a uniform set of soybean cultivars for differentiating races of P. megasperma var. sojae (47). These are Harosoy, Harosoy 63, Sanga, Mack, Altona, P.I. 103.091, and P.I. 171.442. This author used as many of these cultivars as were available but substituted Hark for Harosoy, Higan for Mack in many cases, and Kingwa for P.I. 103.091. The substituted cultivars had the same differential reactions as the cultivars they replaced (25, 47).

Zoospore or Oospore Inoculation of Soybean Seedlings Placed on Soil

Weighed amounts of soil were placed into autoclaved 10 cm diameter glass petri dishes (0.5 to 60 g/petri dish) and 30 ml of distilled water were added. In some cases plastic trays 27 X 19 X 6 or 19 X 19 X 6 cm were used (100 g soil and 150 ml distilled water, or 50 g soil and 100 ml distilled water, respectively). When soil was to be artificially infested with zoospores or oospores, the inocula were added with the water. Soybean seeds were germinated in moist vermiculite at 23 $\pm$ 2°C in trays covered with polyethylene bags. When the seedlings were 2 days old, with hypocotyls 0.5 to 2 cm long, the seedlings were removed from vermiculite, washed, sorted by length, and placed on the soil surface with hypocotyls submerged in water (5 seedlings/petri dish, 20 to 45 seedlings/tray). The petri dishes were covered with glass tops or in the case of trays with polyethylene bags to prevent desiccation, and incubated on the laboratory bench at

Table 1. Differential response of soybean cultivars to races 1 to 6 of Phytophthora megasperma var. sojae[a]

Cultivar	Race					
	1	2	3	4	5	6
Hark	S	S	S	S	S	S
Sanga	R	S	R	R	R	R
Harosoy 63	R	R	S	S	S	S
Mack	R	R	R	S	S	R
Higan	R	R	R	S	S	R
Altona	R	R	R	R	S	S
Toku	R	R	R	R	S	R
Kingwa	R	R	R	R	R	R
Tracy	R	R	R	R	R	R

[a]Reactions: S = susceptible; R = resistant.

23 ± 2°C, usually under ambient light conditions (130 lux). When the effect of light on disease incidence was studied, petri dishes were incubated under fluorescent light (4800 lux provided by 4 Sylvania Gro-Lux WS 40 watt lamps placed 35 cm above the plates), or incubated on the laboratory bench under ambient light (130 lux). Petri dishes for dark treatments were wrapped in aluminum foil.

Seedling infection was evaluated after 3 days' incubation. The plants were removed from the soil surface and placed in distilled water. Six to 24 hours later, sporangia of the pathogen were identified by direct examination with a dissecting microscope. If sporangia were not present, the presence of the pathogen was confirmed by re-isolation of the fungus as previously described in 'Isolation Methods'.

Infected seedlings were stunted and failed to develop lateral roots. The hypocotyls were either totally brown in color and flaccid, or contained one or two severe localized lesions about 1 cm long. Cotyledons never showed signs of infection.

Nine differential cultivars of soybean and six races of *P. megasperma* var. *sojae* were used to evaluate this method (Table 1).

Soybean Seedlings as Baits for Recovering *P. megasperma* var. *sojae* from Soil

Two-day-old soybean seedlings (cultivar 'Hark'), previously germinated at 23 ± 2°C in moist vermiculite, were placed in petri dishes (5 seedlings/plate) containing 20 g air-dried natural soil and 30 ml distilled water. Two days later the seedlings were removed and discarded. A second set of two-day-old seedlings was then placed on the soil. Disease developed more consistently in the second set of seedlings than the first. Seedling infection was evaluated after 3

days' incubation by direct examination for sporangia of the pathogen with a dissecting microscope. If sporangia were not observed, hypocotyl segments were plated on a selective medium (76) to verify the presence of the pathogen.

Disease symptoms of seedlings incubated on natural soils were similar to those occurring on seedlings artificially inoculated in soils. However, a much higher incidence of these seedlings had 1 or 2 individual lesions (0.5 to 1 cm) and fewer had totally brown hypocotyls. Pythium spp. in the soil could also cause these symptoms on soybean seedlings, but upon microscopic examination, the sporangia and mycelia of Pythium spp. could be differentiated from those of P. megasperma var. sojae.

Hypocotyl Inculation Method

Soybeans were planted in 10 X 14 cm plastic pots (5 to 8 seeds/pot) in a potting mix (soil:peat:sand; 1:1:1; v/v/v). Plants were inoculated when they were 8 days old or had their first true leaves. A longitudinal incision was made with a razor blade in the hypocotyl midway between the soil line and the cotyledons. A 2 X 5 mm mycelial segment from a 5-day-old culture of P. megasperma var. sojae was placed in the wound. Petroleum jelly was used to seal the wound to prevent desiccation and each pot was covered with a polyethylene bag for 24 hours. The pots remained in the greenhouse at 26 $\pm$ 4°C. Disease was evaluated after 5 days (37). Plants which had collapsed hypocotyls both above and below the wound were considered susceptible. Those which developed restricted lesions around the point of incision but did not collapse, were considered resistant. In some cases, lesion

development was severe and caused collapse of the seedling above the inoculation site. These questionable reactions were considered resistant reactions, and may have resulted from wounding the hypocotyl too deeply.

Nine differential cultivars of soybean and six races of P. megasperma var. sojae were used (Table 1).

In all experiments treatments were replicated at least three times, and experiments were repeated 2 to 5 times with similar results. Differences between means were detected using Tukey's 'w' procedure following analysis of variance (81).

RESULTS

Zoospore Production

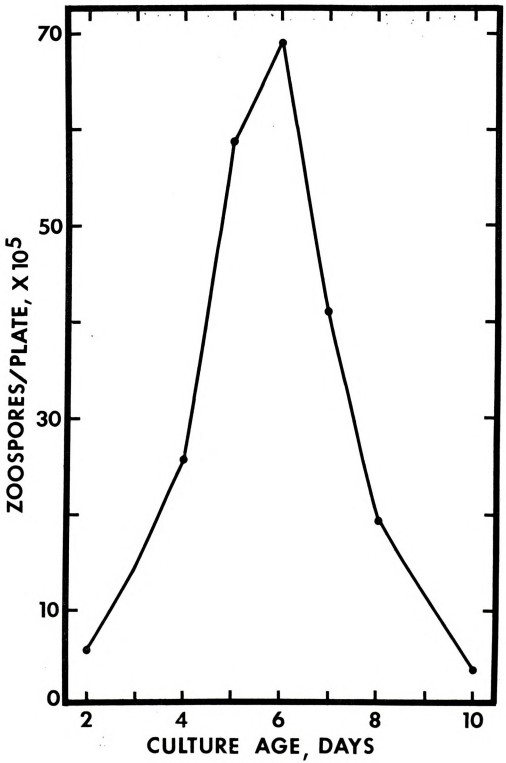
A number of factors affecting zoospore production of *P. megasperma* var. *sojae* in culture was studied in order to optimize conditions for zoospore production for use in inoculation experiments.

Effect of culture age on zoospore production. Cultures 2 to 10 days old were evaluated for zoospore production. Cultures were washed 4 times at half-hour intervals, then reflooded, covered, and incubated at 23 ± 2°C for 20 hours.

Maximum numbers of zoospores were produced from 6-day-old cultures (Fig. 1). At this age, the culture completely covered the surface of the agar, and probably contained the greatest amount of young and vigorous mycelia. Cultures older than 6 days showed a decline in zoospore numbers probably due to aging and loss of vigor of mycelium.

Effect of mycelial age on zoospore production. Three cultures were started by placing a 5 mm diameter disk of agar inoculum in the center of each agar plate. Each day's growth was measured, and the growth ring marked with ink on the underside of the petri dishes. After six days incubation, 7 mm diameter disks ranging from 1 to 5 days old were cut from the rings of mycelium. Five mycelial disks of each age were transferred to sterile petri dishes and flooded with 15 ml of distilled water for one-half hour, flooded once more with 10 ml, then incubated for 20 hours at 23 ± 2°C.

Fig. 1. Effect of culture age on zoospore production by Phytophthora megasperma var. sojae race 1. Least significant range by Tukey's 'w' procedure is 9.9 ($P = 0.05$).


70
50
30
10
0
ZOOSPORES/PLATE, X 10^5
2
4
6
8
10
CULTURE AGE, DAYS

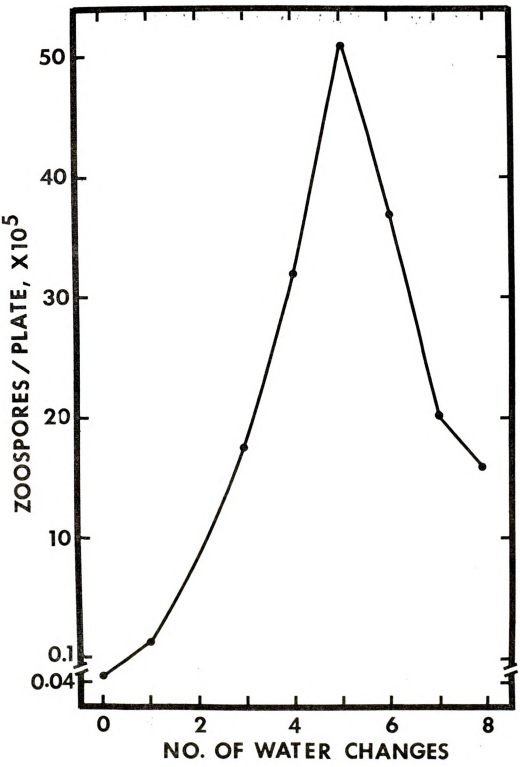
The younger the mycelium, the greater was the zoospore production. One-day-old mycelia produced 1.4 X 10^4 zoospores/disk, which was four times greater than that produced by 5-day-old mycelium (3.7 X 10^3 zoospores/disk). Two-, 3-, and 4-day-old disks produced 7.5 X 10^3, 6.1 X 10^3, and 4.2 X 10^3 zoospores, respectively.

Effect of number of water changes on zoospore production. Five-day-old cultures were washed at one-half hour intervals. The effect of zero to 8 water changes on zoospore production was examined. After 16 hours incubation at 23 $\pm$ 2°C, zoospores were collected and concentrations determined.

A single flooding, without any water change, was sufficient to cause sporangial development and release of low numbers of zoospores (4 X 10^3/plate, 2.7 X 10^2/ml) (Fig. 2). However, many more zoospores were obtained with additional changes of water until maximum numbers of zoospores (5.2 X 10^6/plate, 3.6 X 10^5/ml) occurred with 5 water changes. Zoospore production decreased after more than 5 water changes. Apparently, removal of nutrients from the culture enhances zoospore production, but excessive washing may impose an excessive stress on the mycelium.

Effect of flooding interval between water changes on production of zoospores. Since a sequence of washings of the culture promoted zoospore production, an experiment was designed to determine the optimum time interval between changes of water on zoospore production. Four-day-old and 6-day-old cultures were flooded for intervals of 2.5 minutes to 60 minutes. Each culture received 5 water changes. Zoospores were collected and counted after 18 hours incubation. The times of flooding were so arranged that washing in all treatments was

Fig. 2. Effect of the number of times the culture was flooded with water (30 minutes each) on zoospore production by Phytophthora megasperma var. sojae race 1. Least significant range by Tukey's 'w' procedure is 7.3 ($P = 0.05$).


ZOOSPORES / PLATE, X10^5
50
40
30
20
10
0.1
0.04
0
2
4
6
8
NO. OF WATER CHANGES

terminated at the same time.

The flooding interval had a significant effect on zoospore production (Fig. 3). Numbers of zoospores increased as the time interval between washings lengthened up to 30 minutes. However, a 60 minute interval did not produce significantly greater numbers of zoospores than the 30 minute interval. Cultures 4 and 6 days old had similar trends.

Effect of incubation temperature on production of zoospores. Five-day-old cultures were washed four times at half-hour intervals with water at room temperature (23 $\pm$ 2°C). The water used for the fifth and final flooding was adjusted to the specific incubation temperature for each treatment, and the plates were incubated at these same temperatures in darkness by covering the plates with aluminum foil. Incubation temperatures were 10, 15, 20, 25, 30, and 35°C. Eighteen hours later, the zoospores were collected and counted.

Plates incubated at 20°C yielded the greatest numbers of zoospores (2.5×10^6/plate, 1.7×10^5/ml) (Fig. 4). This treatment also appeared to result in the highest proportion of actively swimming zoospores, though precise determinations were not made. Plates incubated at 30°C contained many dehiscent sporangia (35) in which the zoospores remained trapped. Many zoospores germinated within the sporangia, and produced germ tubes which penetrated the sporangial wall. At 35 °C no sporangial production was observed.

The effect of temperature from 10 to 35°C on colony growth was also examined. The incubation temperature for optimal colony growth was 25°C. These results are substantiated by other researchers (32, 33, 75). The optimum temperature for colony growth was higher than

Fig. 3. Effect of flooding interval between water changes on production of zoospores by 4- and 6-day-old cultures of *Phytophthora megasperma* var. *sojae* race 1. Least significant range by Tukey's 'w' procedure is 4.3 ($P = 0.05$) for 4-day-old cultures and 2.3 ($P = 0.05$) for 6-day-old cultures.

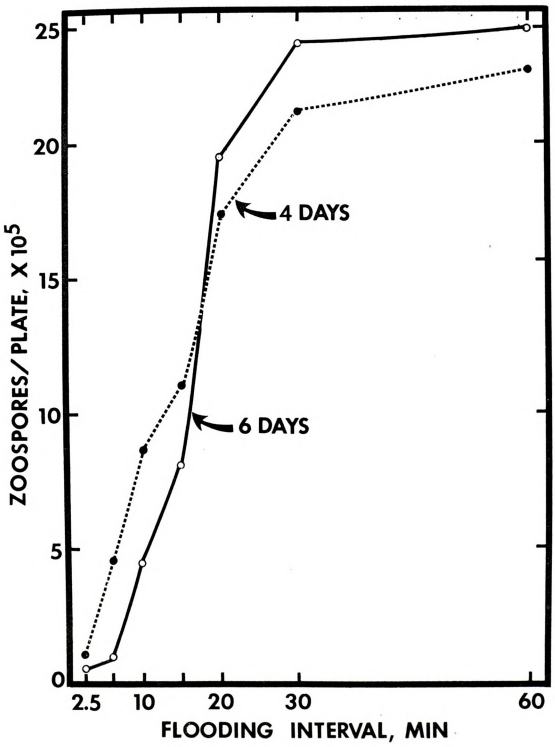
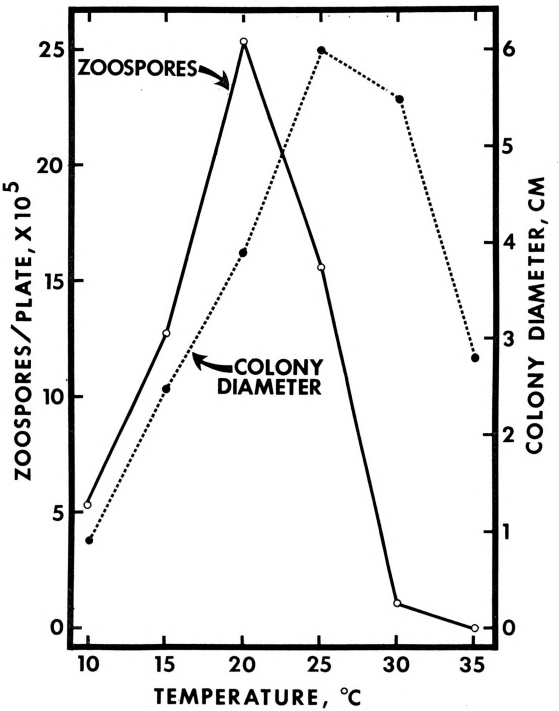

25
20
15
10
5
0
ZOOSPORES/ PLATE, X 10^5
4 DAYS
6 DAYS
2.5
10
20
30
60
FLOODING INTERVAL, MIN

Fig. 4. Effect of incubation temperature on colony growth and on zoospore production (after the final flooding) by *Phytophthora megasperma* var. *sojae* race 1. Least significant range by Tukey's 'w' procedure is 3.1 ($P = 0.05$) for zoospore production and 0.4 ($P = 0.05$) for colony diameter.


ZOOSPORES
COLONY DIAMETER
ZOOSPORES/PLATE, X 10^5
COLONY DIAMETER, CM
TEMPERATURE, °C
0
5
10
15
20
25
0
1
2
3
4
5
6
10
15
20
25
30
35

that for sporangial development and zoospore production.

Effect of light on zoospore production. Five-day-old cultures were washed at half-hour intervals four times with water at room temperature (23 ± 2°C). Water, adjusted to 20°C, was used to flood the plates the fifth and final time. All cultures were then incubated at 20°C for 20 hours. Treatments included cultures incubated: i) under fluorescent light (4800 lux) for 20 hours, ii) under fluorescent light for 10 hours followed by 10 hours in darkness, and iii) in darkness for 20 hours. Dark treatments were prepared by wrapping the plates in aluminum foil. The temperature inside foil-covered and uncovered plates did not differ.

Cultures incubated in the dark produced 10 times more zoospores than those incubated in the light. The 20-hour dark treatment produced 2.0×10^6 zoospores/plate (1.3×10^5/ml), whereas the 20-hour light treatment only produced 1.8×10^5 zoospores/plate (1.2×10^4/ml). The sequential light/dark treatment produced 9.4×10^5 zoospores/plate (6.3×10^4/ml). Dark incubation of flooded plates enhanced zoospore production, but the length of the dark period did not appear to be critical since the light/dark treatment produced almost as many zoospores as the totally dark treatment.

Effect of amount of agar on zoospore production. Five-day-old cultures were washed at half-hour intervals five times. The cultures were incubated on the laboratory bench at 23 ± 2°C for 18 hours before zoospores were harvested. Treatments consisted of plates containing 7, 10, 13, and 16 ml of lima bean agar.

Before the plates were washed, colony diameters were measured. The diameters were 7.8 cm for 7 ml agar, 7.5 cm for 10 ml agar, and

7.3 cm for both 13 and 16 ml treatments. Zoospore numbers per ml were 3.0×10^5, 5.6×10^5, 1.5×10^5, and 4.9×10^4 for 7, 10, 13, and 16 ml agar, respectively. Ten ml was chosen for future work since it gave large numbers of zoospores, was convenient to dispense, and easily covered the petri dish.

Effect of different flooding solutions on zoospore production. Five-day-old cultures were flooded five times at half-hour intervals. Tapwater, single distilled water, double distilled water, vermiculite extract (approximately 1 g vermiculite was shaken in 100 ml distilled water, then passed through cheesecloth), and a salt solution (per liter: $CaCl_2$, 1.75×10^{-3} M; KCl, 10^{-3} M; $MgSO_4$, 10^{-3} M) were tested for their effect on zoospore production. It had been observed that zoospores diluted in a vermiculite extract retained motility longer than those diluted in distilled water. The salt solution has been used for zoospore production in Aphanomyces euteiches (55). Fifteen ml of distilled water was used in each of the five washings for each treatment, but 10 ml of each test solution was used in the final flooding. The plates were incubated at 23 $\pm$ 2°C for 20 hours.

Zoospore numbers were greatest with double and single distilled water. Double distilled water produced 1.6×10^5, single distilled water 1.1×10^5, salt solution 3.0×10^4, vermiculite extract 2.4×10^4, and tapwater 8.0×10^3 zoospores/ml.

Length of time required for zoospore production. Six-day-old cultures were flooded six times at half-hour intervals. Cultures were examined and zoospores harvested at 2, 4, 6, 8, and 18 hour periods after the washing procedure was completed. Plates were incubated on the laboratory bench at 23 $\pm$ 2°C.

After 2 hours incubation in a flooded condition, sporangia had begun to appear, but no zoospores had been released (Table 2). However, at 4 hours, 8.6×10^5 zoospores/plate (5.7×10^4/ml) were collected. Numbers continued to increase with incubations of 6, 8, and 18 hours. After the supernatant fluid was collected for each treatment (except the 18 hour treatment), the plates were flooded once more and allowed to stand overnight. All plates, regardless of treatment, produced a total of 5.1 to 6.2×10^6 zoospores in the two harvests. In subsequent experiments, the incubation period was usually 18 to 20 hours.

Evaluation of zoospore production method with P. megasperma var. sojae races 2, 3, 4, 5, and 6. Once the optimum conditions for zoospore production were established with an isolate of race 1 of the pathogen, isolates of races 2, 3, 4, 5, and 6 were used to evaluate the zoospore production method. Six-day-old cultures of the races grown on 10 ml agar/plate were flooded six times at half-hour intervals with 15 ml of distilled water. The plates were then incubated in darkness at 20°C for 18 hours. High zoospore counts were obtained with all races using the optimum conditions established with race 1 of the pathogen. Race 2 produced 1.0×10^5; race 3, 1.4×10^5; race 4, 1.2×10^5; race 5, 9×10^4; and race 6, 2.1×10^4 zoospores/ml.

Inoculation of Soybean Seedlings Grown in Vermiculite with Zoospores

Several factors were examined in order to develop a reliable method for the inoculation of soybeans grown in vermiculite with zoospores of P. megasperma var. sojae.

Effect of seedling age on disease incidence. Seeds of soybean cultivars 'Hark' and 'Harosoy 63' were planted in water-saturated

Table 2. Effect of incubation period, after the final flooding with distilled water, on zoospore production by Phytophthora megasperma var. sojae race 1

Incubation period, hr[a]	Zoospores/plate[b]		Total production of two harvests
	First harvest	Second harvest	
2	0	5.8×10^6	5.8×10^6
4	8.6×10^5	5.3×10^6	6.2×10^6
6	1.1×10^6	4.0×10^6	5.1×10^6
8	2.4×10^6	3.3×10^6	5.7×10^6
18	5.3×10^6		

[a] Incubation period after the washing procedure was completed.

[b] Mean values for three replicate plates. After the initial zoospore harvest, plates were flooded again and zoospores were collected at 18 hours.

vermiculite in plastic trays 19 X 9 X 6 cm. Each tray contained one row of each cultivar with 6 seeds/row. Seeds were planted 1, 2, 3, 4, and 6 days prior to inoculation and all seedlings were inoculated on the same day with zoospores of race 1 of the pathogen. Controls were uninoculated seedlings of the same ages.

Hark seedlings were most susceptible when inoculated after 2 days incubation when their hypocotyls were 1 to 3 cm long. Those inoculated after one day, or older than 2 days had consistently less disease (Table 3). All 1- and 2-day-old seedlings which were diseased were dead. Over half of the diseased 3-day-old seedlings emerged and continued to develop after inoculation, and had not died at the time of evaluation. The diseased seedlings in 4- and 6-day-old treatments continued to grow, but the root systems were browning and becoming flaccid. Harosoy 63, a cultivar resistant to race 1 of the pathogen, showed no disease when 2- to 6-day-old seedlings were inoculated, but some of the 1-day-old seedlings were infected. Uninoculated seedlings remained healthy.

Effect of temperature on disease incidence. Seeds of soybean cultivar 'Hark' were planted in water-saturated vermiculite in plastic trays 19 X 9 X 6 cm. Each tray contained 2 rows of 5 seeds each. When seedlings were 2 days old, the trays were flooded with distilled water which had been adjusted to the specific incubation temperature of each treatment. Inoculum concentrations of 10^3 and 5 X 10^3 zoospores in 2 ml of suspension were then aplied to each seedling. The trays were incubated in darkness at 15, 20, 25, 30, or 35°C. Control plants were incubated at the same temperatures but were not inoculated. Disease was evaluated in the 25, 30, and 35°C treatments

Table 3. Effect of seedling age on disease incidence of soybeans grown in vermiculite and inoculated with zoospores of Phytophthora megasperma var. sojae race 1

Age at inoculation, days[b]	Diseased seedlings (%)[a]	
	Hark	Harosoy 63
1	28	13
2	100	0
3	60	0
4	51	0
6	23	0

[a]Seedlings were evaluated for disease 5 days after inoculation. There were 6 seedlings/age/cultivar/tray and 4 trays/age. Hark was susceptible and Harosoy 63 resistant to race 1.

[b]Each seedling was inoculated with 10^4 zoospores in 2 ml of suspension.

after 5 days. Since the uninculated plants incubated at 15 and 20°C had not emerged in 5 days, the trays were removed from the incubators and allowed to remain one day at room temperature (23 $\pm$ 2°C) before disease evaluation. It was also necessary to decant excess water from the trays incubated at 15 and 20°C 24 to 36 hours after inoculation.

The greatest numbers of diseased seedlings were obtained at temperatures of 20, 25, and 30°C (Table 4). All diseased seedlings failed to emerge at 25°C. Incubation at 20°C slowed development and neither inoculated or control seedlings emerged. However, at 30°C many of the seedlings emerged even though they were severely infected. Uninoculated plants remained healthy.

Effect of zoospore concentration on disease incidence. Seeds of soybean cultivars 'Hark', 'Corsoy', 'Wayne', and 'Williams' were planted in water-saturated vermiculite in plastic trays 17 X 19 X 6 cm, containing one row of each of the four cultivars with 10 seeds/row. Zoospores of race 1 of the pathogen at concentrations of 10^2, 5 X 10^2, 10^3, 5 X 10^3, and 10^4 in 2 ml volumes were applied to each seedling. Controls consisted of uninoculated plants.

The lowest concentration giving consistent disease was 10^3 zoospores/seedling (Table 5). At 10^4 zoospores/seedling all four cultivars exhibited 100% disease. There was some indication of differences among the cultivars in susceptibility to the pathogen at an inoculum level of 10^3 zoospores. The cultivars 'Wayne' and 'Williams' are considered to have field tolerance to race 1 of P. megasperma var. sojae. They resist Phytophthora root rot in the field even under favorable disease conditions. These differences tended to be masked at inoculum concentrations above 10^3 zoospores/seedling. Controls

Table 4. Effect of temperature on disease incidence in 'Hark' soybeans grown in vermiculite and inoculated with zoospores of Phytophthora megasperma var. sojae race 1[a]

Temperature, °C	Diseased seedlings (%) at indicated zoospore concentration[b]	
	10^3	5×10^3
15	16	30
20	88	88
25	88	100
30	84	88
35	40	56
LSR[c]	33.6	34.4

[a]Two-day-old seedlings were inoculated, and disease was evaluated 5 days later for 25, 30, and 35°C treatments, and 6 days later for 15 and 20°C treatments. There were 10 seedlings/tray and 4 trays/concentration/temperature.

[b]Zoospores/seedling.

[c]Least significant range by Tukey's 'w' procedure ($P = 0.05$).

Table 5. Effect of zoospore concentration on disease incidence of soybeans grown in vermiculite and inoculated with zoospores of Phytophthora megasperma var. sojae race 1

Zoospores/ seedling	Diseased seedlings (%)[a]				
	Corsoy	Hark	Wayne	Williams	Mean[b]
0	0[d]	0	0	0	0.0
10^2	0	0	0	13	3.3
5×10^2	0	0	0	0	0.0
10^3	67	83	33	63	61.5
5×10^3	90	100	87	93	92.5
10^4	100	100	100	100	100.0
Mean[c]	42.8	47.2	36.7	44.8	

[a]Two-day-old seedlings were inoculated and disease was evaluated 5 days later. There were 10 seedlings/cultivar/tray and 3 trays/ treatment.

[b]LSR ($P = 0.05$) = 8.9.

[c]LSR ($P = 0.05$) = 10.9.

[d]LSR ($P = 0.05$) for interaction of inoculum concentration X cultivar = 21.8.

remained healthy.

Effect of zoospore concentration on disease incidence in resistant cultivars. Cultivars 'Hark', 'Altona', 'Harosoy 63', and 'Toku' were chosen for this experiment because their susceptibilities to race 1 of the pathogen differed. Hark is susceptible, Altona is resistant [but some individuals are susceptible (A.F. Schmitthenner, personal communication)], and Harosoy 63 and Toku are homogeneously resistant.

Seeds were planted in water-saturated vermiculite in plastic trays 19 X 19 X 6 cm containing one row each of the four cultivars with 5 seeds/row. Seedlings were inoculated with 10^4, 2.5 X 10^4, 5.0 X 10^4, or 10^5 zoospores in 2 ml of suspension.

The three resistant cultivars Altona, Harosoy 63, and Toku exhibited different reactions to the various inoculum concentrations (Table 6). For example when 10^5 zoospores were applied, 47%, 7%, and 0%, of the seedlings, respectively, exhibited the typical severe decay of the hypocotyl. The percentage of severely infected Altona seedlings tended to increase as inoculum concentrations increased. Resistance apparently can be overcome with sufficient inoculum. There was no significant interaction between the cultivars and inoculum concentrations. In addition to the severe infections, hypocotyls of some plants of each of these resistant cultivars frequently developed numerous, tiny (<1mm), brown lesions which remained localized (Table 6). Control seedlings remained healthy.

Differential response of soybean cultivars to six races of P. megasperma var. sojae. Nine differential soybean cultivars growing in vermiculite were inoculated with zoospores of races 1 to 6 of the

Table 6. Effect of zoospore concentration on disease incidence in differential soybean cultivars grown in vermiculite and inoculated with Phytophthora megasperma var. sojae race 1[a]

Zoospores/ seedling	Diseased seedlings (%)[b]				Mean
	Hark	Altona	Harosoy 63	Toku	
10^4	100	13 (7)	0	0	28.3
2.5 X 10^4	100	13 (40)	0 (7)	0	28.3
5.0 X 10^4	100	0 (27)	0 (27)	0	25.0
10^5	100	47 (31)	7 (33)	0 (27)	38.5
Mean	100	18.3	1.8	0	

[a]Two-day-old seedlings were inoculated and disease was evaluated 5 days later. There were 5 seedlings/cultivar/tray and 3 trays/ treatment.

[b]Hark is susceptible to race 1; the other three cultivars are resistant. Values are percent of seedlings which exhibited severe susceptible reaction. Values in parentheses are percentage of additional seedlings which exhibited only a slight browning of the taproot or small localized lesions on the hypocotyls. Least significant ranges by Tukey's 'w' procedure ($P = 0.05$): Means, 12.9; interaction of inoculum concentration X cultivar, 25.8.

pathogen to determine if this inoculation method could be used to identify races of the pathogen.

Soybeans were planted in trays 27 X 19 X 6 cm containing one row of each of the nine cultivars with 5 seeds/row. Two days after sowing, the seedlings were inoculated with 10^4 zoospores each. Controls were uninoculated seedlings of each cultivar.

The nine soybean cultivars selected exhibited differential responses to the six races of P. megasperma var. sojae generally in accord with published data based on hypocotyl inoculation (Fig. 5, Table 7) (25, 47). Some individuals of some cultivars showed a susceptible reaction when a resistant reaction was expected. In Sanga (races 1, 3, 4, 5, and 6) where the proportion of susceptible reactions tended to be high, seed contaminated with fungi capable of rotting seedlings complicated disease evaluation. However, in Altona (races 1, 3, and 4), Higan (races 3 and 6), Kingwa (races 4 and 5), and Toku (races 3 and 6) the proportion of susceptible reactions was usually less than one-third, and did not complicate interpretation of results. False susceptible reactions in the latter three cultivars was attributed to poor seed quality or moldy seed.

Differential response of cultivars to six races of P. megasperma var. sojae in seedlings inoculated using the hypocotyl inoculation method. Nine differential cultivars of soybean were inoculated by placing mycelium of races 1 to 6, individually, into an incision in the hypocotyls. One replication (pot) consisted of 5 seedlings and the other 8 seedlings. Control plants of each cultivar were wounded but uninoculated.

Fig. 5. Differential soybean cultivars (left to right) Altona, Hark, Harosoy 63, Higan, Sanga, Toku, and Tracy in vermiculite and inoculated with zoospores (10^4/seedling) of race 4 of Phytophthora megasperma var. sojae. Hark, Harosoy 63, and Higan are susceptible to race 4 and failed to emerge after inoculation.

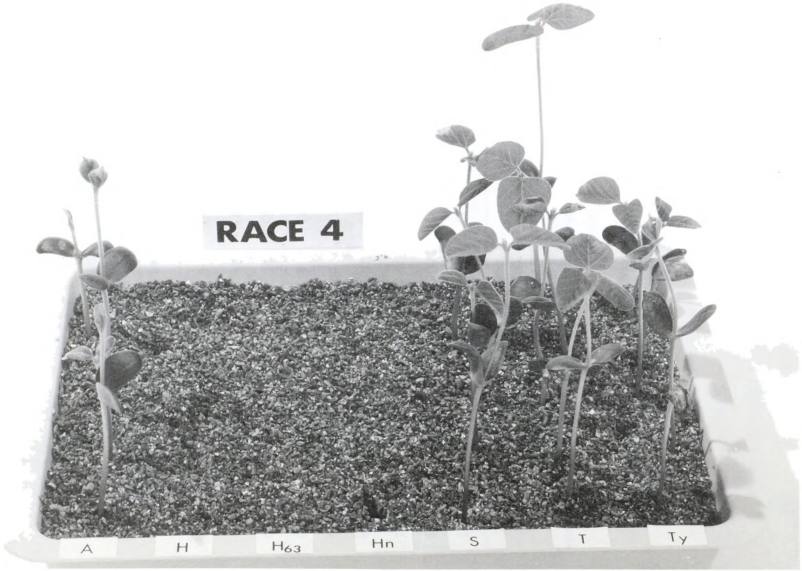

RACE 4
A
H
H63
Hn
S
T
Ty

Table 7. Differential response of selected soybean cultivars to six physiologic races of Phytophthora megasperma var. sojae when seedlings were grown in vermiculite and inoculated with zoospores[a]

Cultivar	Diseased seedlings (%) resulting from inoculation with the races indicated					
	1	2	3	4	5	6
Altona	28 (R)	0 (R)	32 (R)	40 (R)	96 (S)	68 (S)
Hark	100 (S)	76 (S)	100 (S)	100 (S)	84 (S)	100 (S)
Harosoy 63	0 (R)	0 (R)	72 (S)	92 (S)	96 (S)	80 (S)
Higan	0 (R)	8 (R)	20 (R)	96 (S)	92 (S)	28 (R)
Kingwa	8 (R)	0 (R)	0 (R)	20 (R)	28 (R)	92 (S)
Mack	12 (R)	0 (R)	4 (R)	- (S)	- (S)	- (R)
Sanga	52 (R)	100 (S)	52 (R)	64 (R)	70 (R)	32 (R)
Toku	0 (R)	0 (R)	24 (R)	8 (R)	84 (S)	24 (R)
Tracy	0 (R)	0 (R)	0 (R)	- (R)	0 (R)	- (R)

[a]Two-day-old seedlings were inoculated and disease evaluated 5 days later. There were 5 seedlings/cultivar/tray and 9 trays/treatment. R = resistant; S = susceptible, based upon data obtained using hypocotyl inoculations. Missing data are indicated by -.

The soybean cultivars exhibited differential responses to the six races of the pathogen generally in accord with published data based on hypocotyl inoculation (Table 8) (25, 47). With this method also, some individuals of some cultivars showed a susceptible reaction when a resistant reaction was expected. In almost all cases discrepancies with expected results with this method occurred with the same cultivar-race combinations as was the case with zoospore inoculation. Mack and Kingwa exhibited poor emergence.

Inoculation of Soybean Seedlings with Zoospores in Soil

A number of factors was examined to evaluate the effectiveness of inoculating soybean seedlings with zoospores in the presence of sterilized soil.

Effect of amount of soil or of several aqueous media on disease incidence. In preliminary experiments zoospores produced disease readily in soil extract or in flooded sterile soil but no disease was produced in aqueous suspensions of zoospores with no soil. Therefore, weighed amounts of sterile air-dried Conover sandy clay loam (0, 0.5, 1.5, 2.5, 5, 10, 20, 30, 40, and 60 g) were placed in petri dishes and 3×10^3 zoospores of race 1 were added in 30 ml distilled water to each plate. Several aqueous solutions in the absence of soil also were tested for their effect on seedling infection. Three thousand zoospores in 30 ml of tapwater, vermiculite extract, or soil extract were added to petri dishes. The soil extract was either i) used as prepared, ii) autoclaved, iii) centrifuged, or iv) centrifuged and autoclaved. Controls were uninoculated seedlings exposed to the same conditions.

Table 8. Differential response of selected soybean cultivars to six physiologic races of Phytophthora megasperma var. sojae when seedlings were inoculated using the hypocotyl inoculation method[a]

Cultivar	Diseased seedlings (%) resulting from inoculation with the races indicated					
	1	2	3	4	5	6
Altona	35 (R)	10 (R)	55 (R)	0 (R)	75 (S)	96 (S)
Hark	100 (S)	100 (S)	70 (S)	90 (S)	100 (S)	100 (S)
Harosoy 63	0 (R)	4 (R)	90 (S)	72 (S)	100 (S)	100 (S)
Higan	0 (R)	0 (R)	0 (R)	100 (S)	83 (S)	0 (R)
Kingwa	0 (R)	0 (R)	0 (R)	67 (R)	- (R)	38 (S)
Mack	- (R)	- (R)	0 (R)	100 (S)	- (S)	- (R)
Sanga	18 (R)	100 (S)	0 (R)	5 (R)	0 (R)	7 (R)
Toku	0 (R)	8 (R)	4 (R)	11 (R)	80 (S)	85 (R)
Tracy	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)

[a]Seedlings were inoculated at 8 days of age; disease was evaluated 5 days later. There were two replications, one containing 5 plants and one containing 8 plants. R = resistant; S = susceptible, based upon published results obtained using hypocotyl inoculations. Missing data are indicated by -.

Seedling infection was 100% or nearly so using soil in a range of 0.5 to 30 g soil/petri dish (Table 9). At 40 and 60 g, the soil was no longer saturated and disease incidence declined. Seedlings incubated in the variously prepared soil extracts had 50% disease incidence, whereas those incubated in distilled water, tapwater, or vermiculite suspension did not become infected. Uninoculated seedlings remained healthy.

Effect of temperature on disease incidence. Ten grams of sterile air-dried Conover sandy clay loam were placed into petri dishes to each of which 3×10^3 zoospores of race 1 were added in 30 ml of distilled water. Distilled water used to flood the soil was adjusted to the specific incubation temperature of the treatment. All petri dishes were wrapped in aluminum foil before being placed in incubators at temperatures of 10, 15, 20, 25, 30, and 35°C. Controls were uninoculated seedlings incubated at the same temperatures. Petri dishes incubated at temperatures of 10 and 15°C for 3 days were placed at 23 ± 2°C for one day before evaluation.

Greatest numbers of diseased seedlings were obtained at temperatures of 20, 25, and 30°C (Table 10). Temperatures higher or lower than this range significantly decreased disease incidence. The appearance of symptoms was delayed at temperatures of 10 and 15°C.

Effect of zoospore concentration on disease incidence. Ten grams of sterile air-dried Conover sandy clay loam were placed into autoclaved glass petri dishes. Concentrations of 1, 3, 10, 30, 100, 500, and 1000 zoospores of race 1 in 30 ml of distilled water were added to each petri dish. Controls were uninoculated seedlings.

Table 9. Effect of amount of soil on disease incidence in 'Hark' soybeans placed in petri dishes and incubated with zoospores of Phytophthora megasperma var. sojae race 1[a]

Soil, g/plate	Diseased seedlings (%)
0	0
0.5	94
1.5	100
2.5	100
5	100
10	100
20	100
30	100
40	80
60	66

[a]Two-day-old seedlings in petri dishes containing soil and 30 ml of water were infested with 3 X 10^3 zoospores/petri dish. Disease was evaluated 3 days later. There were 5 seedlings/petri dish and 3 petri dishes/treatment.

Table 10. Effect of temperature on disease incidence of 'Hark' soybeans incubated on flooded sterilized soil artificially infested with zoospores of Phytophthora megasperma var. sojae or on soil naturally infested with the pathogen[a]

Temperature, °C	Diseased seedlings (%)	
	Zoospore infested soil	Naturally infested soil
10	28	16
15	36	16
20	80	24
25	100	60
30	88	68
35	12	88
LSR[b]	23.3	32.2

[a]Two-day-old seedlings were placed on 10 g zoospore infested soil (3×10^3 zoospores/plate) or 20 g naturally infested soil in petri dishes. Disease was evaluated 4 days later. there were 5 seedlings/petri dish and 5 petri dishes/treatment.

[b]Least significant range by Tukey's 'w' procedure ($P = 0.05$).

This method appeared to be a very sensitive inoculation method since only 3 zoospores/petri dish caused disease in one of five seedlings (Table 11). Disease incidence increased as inoculum concentrations increased until 100% of the seedlings were infected at 1000 zoospores/petri dish. Localized lesions about 1 to 2 cm in length were present on the hypocotyl of seedlings exposed to low (3 to 30) numbers of zoospores, whereas concentrations of 100 zoospores and higher produced the typical uniform browning of the hypocotyls. Uninoculated control seedlings remained healthy.

Effect of zoospore concentration on disease incidence in differential soybean cultivars. Fifty grams of steamed Conover sandy clay loam were placed in trays 19 X 19 X 6 cm. Each tray contained one row each of Hark, Altona, Harosoy 63, and Toku with 5 seedlings/row. Suspensions containing 4×10^3, 2×10^4, 2×10^5, and 10^6 zoospores of race 1/100 ml of distilled water were added to trays (equivalent of 2×10^2, 10^3, 10^4, and 5×10^4 zoospores/seedling, respectively). Controls were uninoculated seedlings.

Hypocotyls of Hark, a susceptible cultivar, were severely decayed at all inoculum concentrations (Table 12). The percentage of infected seedlings of the resistant cultivars Altona, Harosoy 63, and Toku increased as inoculum concentrations increased. However, the cultivars exhibited different degrees of resistance as inoculum increased. For example, when 10^3 zoospores were applied, 35%, 20%, and 5%, of the seedlings, respectively, became diseased, but when 5×10^4 zoospores were applied, 100% of the seedlings of all three cultivars became diseased. Thus, resistance apparently can be overcome with sufficient inoculum. In addition to the severe infections, hypocotyls

Table 11. Effect of zoospore concentration on disease incidence in 'Hark' soybeans placed in petri dishes containing 10 g autoclaved soil and inoculated with zoospores of Phytophthora megasperma var. sojae race 1[a]

Zoospores/plate	Diseased seedlings (%)[b]
0	0
1	0
3	20
10	24
30	36
100	64
500	88
1000	100
LSR[c]	22.6

[a]Two-day-old seedlings were inoculated, and disease was evaluated 3 days later. There were 5 seedlings/petri dish and 5 petri dishes/ treatment.

[b]Symptoms on plants inoculated with 3, 10, and 30 zoospores/plate were restricted to lesions of 1 to 2 cm in length, whereas those inoculated with higher concentrations were completely decayed.

[c]Least significant range by Tukey's 'w' procedure ($P = 0.05$).

Table 12. Effect of zoospore concentration on disease incidence in differential soybean cultivars placed in trays containing sterilized soil and inoculated with Phytophthora megasperma var. sojae race 1[a]

Zoospores/ seedling	Diseased seedlings (%)[b]				Mean
	Hark	Altona	Harosoy 63	Toku	
2 X 10^2	100	10	0	0	27.5
10^3	100	35	20	5	40.0
10^4	100	50	65	35	62.5
5 X 10^4	100	100	100	100	100
Mean	100	48.8	46.3	35.0	

[a]Two-day-old seedlings were incubated on flooded soil for 3 days before evaluation. There were 5 seedlings/cultivar/tray, and 4 trays/treatment.

[b]Least significant ranges by Tukey's 'w' procedure ($P = 0.05$): Means, 7.2; interactions of inoculum concentration X cultivar, 14.4.

of some plants frequently developed numerous, small (<1 mm), brown lesions which remained localized. Some Altona seedlings were diseased at all inoculum concentrations. Control seedlings remained healthy.

Since it appeared that resistance could be overcome with sufficient inoculum, the experiment was repeated using seedlings of two ages. One hundred grams of steamed Conover sandy clay loam was placed in trays 27 X 19 X 6 cm, each containing one row each of 2- and 3-day-old seedlings of Hark, Altona, Harosoy 63, and Toku with 5 seedlings/row. Suspensions containing 4 X 10^3, 2 X 10^4, snd 2 X 10^5 Zoospores of race 1/150 ml distilled water were added to each tray (equivalent of 2 X 10^2, 10^3, and 10^4 zoospores/seedling, respectively). Controls were uninoculated seedlings.

The susceptible cultivar, Hark, at both ages, exhibited 100% disease at all inoculum concentrations (Table 13). Here again, disease in the resistant cultivars usually increased with inoculum concentrations. However, 3-day-old seedlings of Altona, Harosoy 63, and Toku exhibited less disease than 2-day-old seedlings at all inoculum concentrations. Apparently, resistance can be overcome with sufficient inoculum, and the younger the seedlings are at the time of inoculation, the greater their susceptibility. Control seedlings remained healthy.

Differential response of soybean cultivars to six races of P. megasperma var. sojae. Nine differential soybean cultivars placed on flooded soil were inoculated with zoospores of isolates of races 1 to 6 of the pathogen to determine if this inoculation method could be used to identify races of the pathogen.

One hundred grams of steamed Conover sandy clay loam was placed in trays 27 X 19 X 6 cm. Each tray contained one row each of Altona, Hark,

Table 13. Effect of zoospore concentration on disease incidence of differential soybean cultivars 2 and 3 days old placed on soil and inoculated with Phytophthora megasperma var. sojae race 1

Zoospores/ seedling	Diseased seedlings (%)[a]								Mean[b]
	Hark		Altona		Harosoy 63		Toku		
	2	3	2	3	2	3	2	3	
2 X 10^2	100[e]	100	47	13	0	0	0	0	32.5
10^3	100	100	87	27	80	47	20	7	58.5
10^4	100	100	80	20	60	33	53	27	59.1
Mean[c]	100	100	71.3	20.0	46.7	26.7	24.3	11.3	
Mean[d]	100		45.7		36.7		17.8		

[a]Seedlings were incubated on flooded soil for 3 days before disease evaluation. There were 5 seedlings/cultivar/tray and 3 trays/treatment. Means for 2- and 3-day-old seedlings were 60.6 and 39.5, respectively ($P < 0.01$).

[b]LSR ($P = 0.05$) = 8.4.

[c]LSR ($P = 0.05$) = 6.9.

[d]LSR ($P = 0.05$) = 7.7.

[e]LSR ($P = 0.05$) for interaction of cultivars X concentrations X ages = 24.0.

Harosoy 63, Higan, Kingwa, Mack, Sanga, Toku, and Tracy with 5 seedlings/row. A suspension containing 9 X 10^3 zoospores/150 ml of distilled water was added to each tray (equivalent of 2 X 10^2 zoospores/seedling). Controls were uninoculated seedlings.

The nine soybean cultivars selected exhibited differential responses to the six races of P. megasperma var. sojae in close accord with published data based on hypocotyl inoculations (Fig. 6, Table 14) (25, 47). Ten and 50% of Altona seedlings inoculated with race 1 and race 4, respectively, showed a susceptible reaction when a resistant reaction was expected. Also, Higan showed 5% (1 out of 20 seedlings) disease when inoculated with race 6 and Toku showed 5% disease when inoculated with race 3.

Inoculation of Soybean Seedlings with Oospores in Soil

Some factors were examined to evaluate the effectiveness of inoculating soybean seedlings with oospores in artificially infested sterilized soil. Attempts also were made to isolate the pathogen from naturally infested soils using soybean seedlings as baits.

Effect of different soil media on disease incidence. Four soil media were evaluated for their effect on seedling infection by 5-week-old oospores. Twenty grams of natural Conover sandy clay loam, steamed Conover loam, potting mix, or sand were placed in petri dishes. Concentrations of 10^3 and 10^4 oospores in 30 ml distilled water were added to the first three treatments, and the same concentrations of oospores in 30 ml of soil extract (conover sandy clay loam) were added to the sand. All petri dishes were incubated in darkness at 23 $\pm$ 2°C. Two-day-old Hark seedlings were incubated on the plates

Fig. 6. Differential soybean cultivars (left to right) Altona, Hark, Harosoy 63, Higan, Kingwa, Sanga, Toku, and Tracy inoculated with zoospores (2×10^2/seedling) of race 5 of Phytophthora megasperma var. sojae in the presence of steamed soil. Altona, Hark, Harosoy 63, Higan, and Toku are susceptible to race 5; the hypocotyls turned brown and failed to develop after inoculation.


A
H
H63
Hn
K
S
T
Ty

Table 14. Differential response of selected soybean cultivars to six physiologic races of Phytophthora megasperma var. sojae when seedlings were incubated on flooded soil and inoculated with zoospores[a]

Cultivar	Diseased seedlings (%) resulting from inoculation with the races indicated					
	1	2	3	4	5	6
Altona	10 (R)	0 (R)	0 (R)	50 (R)	70 (S)	100 (S)
Hark	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)	100 (S)
Harosoy 63	0 (R)	0 (R)	100 (S)	100 (S)	85 (S)	100 (S)
Higan	0 (R)	0 (R)	0 (R)	90 (S)	65 (S)	5 (R)
Kingwa	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	75 (S)
Mack	0 (R)	0 (R)	0 (R)	100 (S)	85 (S)	0 (R)
Sanga	0 (R)	100 (S)	0 (R)	0 (R)	0 (R)	0 (R)
Toku	0 (R)	0 (R)	5 (R)	0 (R)	75 (S)	0 (R)
Tracy	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)

[a]Seedlings were inoculated at 2 days of age; disease was evaluated after 3 days incubation on flooded soil. There were 5 seedlings/cultivar/tray and 4 trays/treatment. R = resistant; S = susceptible, based upon data obtained using hypocotyl inoculations.

for 2 days, then replaced with a new set of seedlings. Disease was evaluated 3 days later.

Disease incidence was 100% at both oospore concentrations in steamed Conover loam, potting mix, and sand. There was no seedling infection with 10^3 oospores/petri dish in the natural Conover loam, and disease was 80% with 10^4 oospores/petri dish. All plants in the first and second set of seedlings were diseased in all treatments except for natural Conover loam. Uninoculated seedlings remained healthy.

Effect of temperature on disease incidence using naturally infested soil. Twenty grams of air-dried Brookston loam, naturally infested with P. megasperma var. sojae were placed into petri dishes and 30 ml of distilled water added. Distilled water used to flood the soil was adjusted to the specific incubation temperature of the treatment. All plates were wrapped in aluminum foil before being placed in incubators at temperatures of 10, 15, 20, 25, 30, and 35°C.

In the naturally infested soil, disease incidence increased as the temperature increased (Table 10). Temperatures of 25 to 35°C produced significantly more disease than lower temperatures. Seedling development was retarded at temperatures below 20°C. Attempts to cause seedling infection using soil artificially infested with oospores failed.

Effect of light on oospore germination and on disease incidence. Twenty grams of air-dried Brookston loam, naturally infested with P. megasperma var. sojae, 20 g sterile Conover loam infested with 10^3 oospores/petri dish, or 10 g sterile Conover loam artificially infested with 10^3 zoospores/petri dish were added in 30 ml of distilled water to separate petri dishes. Petri dishes were incubated under fluorescent

light (1800 lux) with either a 24 hour or 12 hour photoperiod or in continuous darkness. For dark treatments plates were wrapped in aluminum foil. Two-day-old soybean seedlings were incubated on the flooded soil for 2 days, then replaced with a new set of seedlings. Disease was evaluated 3 days later. Controls were uninoculated seedlings exposed to the same light conditions. To study the effect of light on oospore germination, a suspension of 5-week old oospores was applied to membrane filters (ca. 10^3/membrane). The membranes were incubated on flooded, sterile Conover loam for seven days. For determination of germination oospores were transferred to water agar (77).

Germination of oospores was greatest in darkness, whereas germination of those incubated under a 12 hour photoperiod or continuous light was significantly decreased. Development of mature sporangia on relatively longer germ tubes was characteristic of oospores incubated in darkness, whereas short germ tubes and only a few sporangia were characteristic of those incubated under light.

Disease incidence of seedlings in soils naturally or artificially infested with oospores of the pathogen was significantly greater in darkness than under continuous light. With a 12 hour photoperiod, results were intermediate. Apparently, light had no effect on the susceptibility of the seedlings, since they were uniformly infected at all photoperiod treatments when inoculated with zoospores. In another experiment, 500 zoospores/petri dish also gave 100% disease in all three light conditions. Uninoculated controls remained healthy.

Soybean seedlings as baits for recovering P. megasperma var. sojae from soil. Two-day-old soybean seedlings (cultivar 'Hark') were placed in petri dishes (5 seedlings/petri dish) containing 40 g

Table 15. Effect of light on oospore germination and soybean seedling infection by Phytophthora megasperma var. sojae race 1 in naturally infested soil and in soil artificially infested with oospores or zoospores of the pathogen

Photoperiod, hours/day[b]	Oospore germination, %[c]	Diseased seedlings (%)[a]		
		Naturally infested	Oospore infested (10^3/plate)	Zoospore infested (10^3/plate)
24	8	4	8	100
12	23	28	20	100
0	36	60	44	100
LSR[d]	8	24	31	0

[a]Two-day-old soybean seedlings were incubated on flooded soil for 2 days, then replaced with new seedlings (5 seedlings/petri dish and 5 petri dishes/treatment).

[b]Petri dishes were incubated under fluorescent light providing 4800 lux.

[c]Five-week-old oospores were incubated on flooded soil for 7 days before evaluation; 20-36% were infected with hyperparasites, which were included among those nongerminated.

[d]Least significant range by Tukey's 'w' procedure ($P = 0.05$).

air-dried natural soil and 40 ml distilled water. Seedling infection was evaluated after 3 days' incubation by direct examination for sporangia of the pathogen with a dissecting microscope. Segments of infected tissue were also plated on a selective medium to verify the presence of the pathogen.

Nine different natural soils were tested with soybean seedling baits. Five of the soils were taken from fields under soybean cultivation; the other four soils were from fields with crops other than soybean.

P. megasperma var. sojae was successfully baited from all nine natural soils even when they had been stored air-dried. Pythium spp. were also observed on seedlings in six of the soils.

DISCUSSION

Methods were described for inoculating soybean seedlings with zoospores and oospores of Phytophthora megasperma var. sojae, and for detection and isolation of the pathogen in natural soils. These methods offer advantages over those presently available. The methods are more rapid and easier to perform, less susceptible to variable environmental conditions, gave more consistent results, and are less artificial than the hypocotyl-wound technique. These methods also can be used for testing pathogenicity and virulence of isolates, and for studies on field tolerance, biological control, and environmental factors.

Large numbers of zoospores of the pathogen were readily produced in flooded cultures on lima bean agar, provided that culture age, number and frequency of water changes, temperature, and light conditions were appropriately standardized. The optimal temperature in this study for zoospore production by P. megasperma var. sojae was found to be 20°C. Previous workers (35) found 25°C to be better than 15°C. The number of zoospores produced on plates incubated in darkness was ten times greater than on plates incubated under continuous light. Light has been variously reported i) to have no influence on the production of sporangia (23, 89), ii) to be stimulatory (2, 18), and iii) to be inhibitory (91) to several species of Phytophthora. The effect of light on sporangial development or zoospore production by P. megasperma var. sojae has not previously been studied. The method

yielded high numbers of zoospores (10^5/petri dish) in as few as 4 hours post-washing; previous methods have required 24 to 72 hours post-treatment before harvest (31, 32, 33, 75). Further, the new method may yield a second and third harvest of zoospores from the same petri dish after reflooding.

Only one isolate of race 1 of the fungus was used in this study, so it is not known whether the factors tested may affect other isolates differently. However, the method was verified using isolates of races 2, 3, 4, 5, and 6 each of which produced more than 10^5 zoospores/ petri dish. Moreover, additional isolates of races 1, 3, 4, 5, and 6 all produced numerous zoospores in further experiments (Lockwood and Cohen, unpublished results).

Hypocotyl inoculation is the conventional method for determining pathogenicity of the fungus and evaluating resistance in soybeans. Two techniques were successfully developed for the inoculation of soybean seedlings with zoospores of P. megaseprma var. sojae. In vermiculite, disease could usually be evaluated on a simple emergence vs. non-emergence basis when inoculation took place before seedlings were three days old. Results were available within 6 to 8 days after inoculation. This inoculation method yielded optimal results over a temperature range of 20 to 30°C. Experiments done on the laboratory bench under ambient light (130 lux) and fluorescent light (1800 lux), and in a growth chamber (17,000 lux), for varying photoperiods all gave similar disease responses.

Inoculation of 2-day-old seedlings of nine cultivars with six races of P. megasperma var. sojae at 10^4 zoospores/seedling gave results

corresponding with those obtained using hypocotyl inoculation (25, 47). Concentrations of 500 zoospores/seedling failed to produce disease in susceptible cultivars while 10^5 zoospores/seedling caused disease in some cultivars which were resistant by hypocotyl inoculation. Seedlings of "resistant" tobacco cultivars have also been infected when inoculated with an excessive concentration of zoospores of P. parasitica var. nicotianae (23, 54). For example, increasing zoospore concentration from 10^4/ml to 10^5/ml resulted in black shank symptoms on seedlings of resistant cultivars (54).

In some cases, resistant soybean cultivars showed a susceptible reaction when inoculated with 10^4 zoospores/seedling. These 'false' susceptible reactions were usually less than 30%, and did not complicate interpretation of results. The decreased germination was attributed to poor seed quality. However, in Sanga the proportion of 'false' susceptible reactions tended to be higher and apparently was due to the seed being contaminated with fungi capable of rotting the seedlings. Seed quality is an uncontrollable factor in this inoculation method because germination cannot be checked prior to the pre-emergence inoculation.

Soybean seedlings were also successfully infected when incubated on flooded soil infested with zoospores of P. megasperma var. sojae. Seedlings were selected for use after germination which eliminated the problem of seed quality control. As with vermiculite inoculations, disease incidence was optimal in the temperature range of 20 to 30°C. Variable light conditions did not affect results. The soil system required fewer zoospores than did vermiculite. As few as three zoospores were found capable of causing infection in a seedling despite

zoospore motility loss in the process of dilution (35).

Differentiation of resistant and susceptible cultivars with this method was most consistent in 2-day-old seedlings inoculated with 2×10^2 zoospores/seedling. Inoculation of nine cultivars with six races of P. megasperma var. sojae very closely corresponded with results obtained using hypocotyl inoculation. However, resistant cultivars showed disease symptoms at higher zoospore concentrations. This susceptibility of 'resistant' cultivars to infection decreased with increasing age of seedlings.

These inoculation methods may distinguish resistance from 'field tolerance' which cannot be detected using hypocotyl inoculation. Those seedlings of field tolerant cultivars which did develop hypocotyl infections tended to be less severely affected than those of susceptible cultivars.

Oospores of P. megasperma var. sojae have a constitutive dormancy period (17). Attempts to induce germination in these resting structures have in large part failed (8, 67, 68). In this study most attempts to cause seedling infection using oospores produced in culture and added to soil proved unsuccessful. In other work from our laboratory, oospores were found to be parasitized by a diversity of soil microorganisms which have the potential to reduce populations of the pathogen in soil (80). It was suggested that culturally produced oospores may be more susceptible to parasitism than those produced in and protected by plant tissue (80). Parasitized oospores may explain my lack of success in a number of experiments to induce disease in seedlings when oospores were added to natural soil.

Light has been shown to stimulate oospore germination in vitro (8, 26, 48, 68). Contrary to these reports, when oospores were incubated on natural soil, more germination occurred in darkness than in light. Moreover, in the presence of soil, germ tube elongation and sporangium formation were greater in darkness than in light. Additionally, seedling disease incidence was greater in darkness than under continuous light when seedlings were incubated on naturally infested soil or soil artificially infested with oospores. Since disease incidence was not affected by varying light conditions when zoospores were used as the source of inocula, it appears that light conditions directly affected oospores and their germination.

Isolation of P. megasperma var. sojae directly from soil has proved to be difficult (84), but my results showed that soybean seedlings could be used as baits to detect the presence of P. megasperma var. sojae in soil. The fungus was microscopically identified without isolation and reinoculation and pure cultures were readily obtained from disease tissue. The baiting method was sensitive enough to detect the pathogen in soils with no disease history or in soils not under soybean cultivation. In other work (Cohen and Lockwood, unpublished), several races of the fungus were identified directly from soil when differential soybean cultivars were used as baits. The ease with which the pathogen was detected and isolated directly from soil by means of soybean seedling baits suggests that the method could be adapted to study the occurrence and behavior of the fungus in soil as has been done with Phytophthora megasperma using alfalfa seedling baits (50, 51, 63, 64, 65). In

addition, the method provides a natural system by which the effect of environmental factors on the infection process can be investigated.

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