Inoculation of Soybean Seedlings With Zoospores of Phytophthora megasperma var. sojae for Pathogenicity and Race Determination

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ABSTRACT

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Severe root disease resulted when 2-day-old soybean seedlings growing in vermiculite were inoculated with 10^4 zoospores per plant and incubated at 20–30 C. Severe disease also resulted when 2-day-old soybean seedlings were placed in petri dishes containing 10–20 g of steamed soil that had been flooded with 30 ml of distilled water containing 2×10^2 zoospores per plant and were incubated at 20–30 C. Nine differential cultivars of soybean were inoculated with zoospores of six races of the pathogen in vermiculite or flooded soil. Results in either case generally corresponded with those obtained by placing mycelia in wounded hypocotyls. Larger numbers of zoospores than those stated resulted in infection of some "resistant" cultivars. Two-day-old soybean seedlings baited the pathogen from flooded samples of several naturally infested soils.

Additional key words: Isolation, detection, Phytophthora root and stem rot.

Phytophthora root and stem rot, caused by *Phytophthora megasperma* (Drechs.) var. *sojae* Hildeb., is a widespread and damaging disease of soybeans in Michigan and elsewhere. The recent development of numerous races of this pathogen (19) has made necessary the development of reliable, convenient techniques for determining pathogenicity of isolates and for identifying sources of resistance. Conventionally this is done by inserting a small piece of mycelium into an incision in the hypocotyls of young soybean plants (7). Susceptible plants collapse and die within a few days. The hypocotyl inoculation method is time consuming, requires considerable amounts of space, and does not detect cultivar reactions designated as "field tolerance" (14, 15, 19). Moreover, the hypocotyl wound technique cannot be used for studies of the influence of environment on the infection process, inoculation experiments using natural propagules in relation to the natural infection court, or investigations of biological control.

Although *P. megasperma* var. *sojae* can be isolated readily from infected plant tissue, its isolation directly from soil has proved difficult (18). Although many other *Phytophthora* spp. have been detected in soil by use of host plants or fruits as baits (1-3, 5, 8), this method of detection apparently has not been evaluated for *P. megasperma* var. *sojae*.

The present investigation was undertaken to develop reliable inoculation methods using zoospores for pathogenicity tests; to identify resistance; and to determine the feasibility of employing soybean seedlings as baits to detect and isolate *P. megasperma* var. *sojae* from natural soil.

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MATERIALS AND METHODS

Maintenance of the fungus.—*Phytophthora megasperma* var. *sojae* races 1, 2, 3, 4, 5, and 6 were used. Isolates of races 1 to 5 were obtained from A. F. Schmitthenner, and isolates of races 1, 2, and 6 were obtained from J. H. Haas. Each isolate was maintained on V-8 juice agar slants (200 ml of V-8 juice, 3.0 g of $CaCO_3$, and 20 g of agar, all per liter). New cultures were prepared every 2–3 mo by placing soybean seedlings (cultivar Hark) on flooded cultures of the fungus on Difco lima bean agar. Three days later the fungus was reisolated from infected tissue.

Zoospores were produced from flooded cultures growing on lima bean agar (4).

Isolation.—Diseased hypocotyls of soybean seedlings were surface disinfested in 0.5% sodium hypochlorite for 10 sec. Small portions were placed on a selective medium (16) modified by substituting 0.02 g of Benlate (50% benomyl) for the antibiotics Pimaricin and Mycostatin®; cultures were incubated for 1–3 days at 23 ± 2 C. Mycelium from the diseased segments was transferred to V-8 juice agar containing antibiotics (16). After 24 hr, the agar was inverted, and when the fungus had grown up through the agar layer, it was transferred to 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 characteristic: pH 7.8, organic matter 2.96%, water-holding capacity 28%, clay 26%, silt 24%, and sand 50%. The soil was passed through a sieve with 2.5-mm openings, uniformly mixed, and stored air dried in polyethylene bags in the laboratory.

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 (23 C) in plastic trays 27 × 19 × 6 or 19 × 19 × 6 cm. The trays contained varying numbers of rows at least 2.5 cm apart, each with five to ten seeds. The seeds were covered with a 1.5-cm layer of dry vermiculite, and the trays were covered with polyethylene film to minimize evaporation. After 2 days at 23 C, the polyethylene was removed and water was added to saturate the vermiculite. The seedlings were inoculated by applying 2 ml of a zoospore suspension, usually containing 10^4 zoospores, over each nonemerged seedling using an automatic pipette syringe. Different trays were used for different races of the pathogen. After inoculation, the trays were incubated in a growth chamber under 17,000 lx for 12-hr photoperiods at 25 C, or on a laboratory bench under ambient fluorescent light (860 lx) at 23 C. Disease was evaluated 4–6 days after inoculation. A plant was considered susceptible if it failed to emerge or if the tap root and lateral roots were discolored and flaccid. The following cultivars were used: Altona, Hark, Harosoy 63, Higan, Kingwa, Mack, Sanga, Toku, and Tracy. These same cultivars also were used in the other inoculation methods described below.

Zoospore inoculation of soybean seedlings in the presence of soil.—Autoclaved Conover loam (10–20 g) was placed in autoclaved 10 cm diameter glass petri dishes and 30 ml of an aqueous suspension of zoospores (usually at the rate of 2×10^2 per plant) was added. In some cases, plastic trays 27 × 19 × 6 or 19 × 19 × 6 cm were used (100 g of soil and 150 ml of zoospore suspension, or 50 g of soil and 100 ml of zoospore suspension, respectively). Soybean seeds were allowed to germinate in moist vermiculite at 23 C for 2 days, and then were placed on the soil surface (five seedlings per plate, 20 to 45 seedlings per tray). The dishes were covered with glass tops, or the trays with polyethylene bags, and incubated at 23 C. Seedling infection was evaluated after incubation for 3 days. 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 lesions about 1 cm long. Cotyledons never showed symptoms of infection. The identity of the pathogen was confirmed by incubating the seedlings in distilled water for 6–24 hr, at which time sporangia of the pathogen were identified with a dissecting microscope. If sporangia were not present, the presence of the pathogen was confirmed by reisolation.

Hypocotyl inoculation method.—Soybeans were grown in 10 × 14 cm plastic pots (five to eight seeds per pot) in a potting mix (soil/peat/sand, v/v/v) in a greenhouse. Plants were inoculated when they were 8 days old by inserting mycelia from agar cultures into a longitudinal wound made in the hypocotyl (7). The wounds were covered with petroleum jelly, and pots were covered with plastic bags for 24 hr. Disease was evaluated after 5 days. Plants with lesion development resulting in collapsed hypocotyls were considered susceptible.

In all experiments, treatments were replicated at least three times, and experiments were repeated two to five times with similar results. Differences between means were detected using Tukey's *w* procedure following analysis of variance (17).

Soybean seedlings as baits for recovering P. megasperma var. sojae from soil.—Two-day-old soybean seedlings (cultivar Hark), previously germinated at 23 C in moist vermiculite, were placed in petri dishes (five seedlings per plate) containing 20 g of air-dried soil from fields with diseased soybeans, and 30 ml of distilled water. Two days later the seedlings were discarded, and a second set of 2-day-old seedlings was placed on the soil plates. Seedling infection in the second set was evaluated 3 days later, and the identity of the pathogen was confirmed by observing sporangia of the pathogen with a dissecting microscope. If sporangia were not observed, segments were plated on a selective medium (16) to verify the presence of the pathogen.

RESULTS

Zoospore inoculation of soybean seedlings grown in vermiculite.—The effect of seedling age on susceptibility to disease was studied using cultivar Hark. Seeds were planted 1, 2, 3, 4, and 6 days prior to inoculation. All seedlings were inoculated on the same day with zoospores of *P. megasperma* var. *sojae* race 1 (10^4 zoospores per seedling). Seedlings inoculated at 1, 2, 3, 4, and 6 days of age had 28, 100, 60, 51, and 23% diseased plants, respectively. Of the seedlings that were diseased, all those inoculated at 1 and 2 days old and half of those inoculated at 3 days old were killed. None of those inoculated at 4 and 6 days old were dead at the time of evaluation. Noninoculated seedlings of the same ages remained healthy.

To study the effect of temperature on disease incidence, seeds of the cultivar Hark were planted in trays of moist vermiculite, which after 2 days were flooded with distilled water adjusted to 15, 20, 25, 30, or 35 C. Each plant was inoculated with 5×10^3 zoospores in 2 ml of suspension, and the trays were incubated at corresponding temperatures. Maximum infection was obtained at 20, 25, and 30 C (Fig. 1). At 20 and 25 C, the infected plants failed to emerge, whereas at 30 C, many of the seedlings emerged even though they were severely infected. Noninoculated plants incubated at the same temperatures remained healthy.

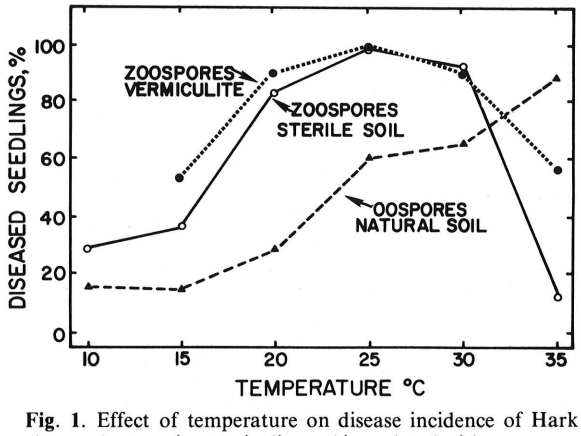

Fig. 1. Effect of temperature on disease incidence of Hark soybeans a) grown in vermiculite and inoculated with zoospores of *Phytophthora megasperma* var. *sojae* race 1 (5×10^3 per seedling), b) placed on soil and inoculated with zoospores (5×10^2 per seedling), or c) placed on soil naturally infested with *P. megasperma* var. *sojae*. Least significant ranges by Tukey's *w* procedure ($P = 0.05$) are 34.4, 23.3, and 32.2, respectively.

The effect of zoospore concentration on disease incidence was studied in the susceptible cultivar Hark and resistant cultivars Altona, Harosoy 63, and Toku. Although Altona is considered resistant, some individuals may be susceptible (A. F. Schmitthenner, *personal communication*). Two-day-old seedlings were each inoculated with 0, 10^4, 5×10^4, or 10^5 zoospores of race 1. All Hark seedlings were killed at all inoculum concentrations, but the three resistant cultivars responded differently to the various inoculum concentrations (Table 1). For example, at 10^5 zoospores, 47, 7, and 0% of the Altona, Harosoy 63, and Toku seedlings, respectively, exhibited disease.

A similar experiment was done using cultivars Hark, Corsoy, Wayne, and Williams inoculated with zoospores of race 1 at concentrations of 0, 10^2, 5×10^2, 10^3, and 5×10^3 per seedling. The lowest concentration giving consistent disease was 10^3 zoospores per plant. At 10^3 zoospores, Wayne, which is considered to have some field tolerance to Phytophthora root and stem rot, had significantly less disease (33%) than did Hark (83%), Corsoy (67%), or Williams (63%) (least significant range $[LSR][P=0.05]=22$). The disease incidence of Williams, another field-tolerant cultivar, was less (but not significantly less) than that of Hark. These differences were masked at higher inoculum concentrations.

Nine differential soybean cultivars were inoculated with zoospores of races 1–6 to determine if this method could be used to identify races of the pathogen. Two days after seeding, the seedlings were each inoculated with 10^4 zoospores per plant. The nine cultivars exhibited differential responses to the six races *P. megasperma* var. *sojae* that were in general accord with published data based on hypocotyl inoculation (19) (Table 2). Some individuals of some cultivars showed a susceptible reaction when a resistant reaction was expected. In Sanga (races 1, 3, 5, and 6), in which the proportion of "false" susceptible reactions tended to be high, poor seed quality with low germinability complicated disease evaluation. In Altona (races 1, 3, and 4), Higan (races 3 and 6), Kingwa (races 4 and 5), and Toku (races 3 and 6), however, the proportion of susceptible reactions was usually less than one-third, and did not complicate interpretation of results. Results using the conventional hypocotyl inoculation also were not completely consistent (Table 2).

Zoospore inoculation of soybean seedlings in the presence of soil.—Attempts failed to infect soybean

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

Zoospores/ seedling	Diseased seedlings of cultivar: Hark (%)	Altona (%)	Harosoy 63 (%)	Toku (%)
10^4	100	13	0	0
5×10^4	100	13	0	0
10^5	100	47	7	0

[a]Two-day-old seedlings were inoculated and disease was evaluated 5 days later. There were five seedlings per cultivar per tray and three trays per treatment. Least significant range by Tukey's *w* procedure ($P=0.05$) for interaction of concentration × cultivar = 25.8.

TABLE 2. Differential response of selected soybean cultivars to six physiologic races of *Phytophthora megasperma* var. *sojae* when seedlings were inoculated using the hypocotyl inoculation method, grown in vermiculite and inoculated with zoospores, or incubated on flooded soil and inoculated with zoospores

Cultivar	Diseased seedlings (%) resulting from inoculation with the races indicated: 1 H[a]	1 V[b]	1 S[c]	2 H	2 V	2 S	3 H	3 V	3 S	4 H	4 V	4 S	5 H	5 V	5 S	6 H	6 V	6 S
Altona	35	28	10 (R)[d]	10	0	0 (R)	55	32	0 (R)	0	40	50 (R)	75	96	70 (S)	96	68	100 (S)
Hark	100	100	100 (S)	100	76	100 (S)	70	100	100 (S)	90	100	100 (S)	100	84	100 (S)	100	100	100 (S)
Harosoy 63	0	0	0 (R)	4	0	0 (R)	90	72	100 (S)	72	92	100 (S)	100	96	85 (S)	100	80	100 (S)
Higan	0	0	0 (R)	0	8	0 (R)	0	20	0 (R)	100	96	90 (S)	83	92	65 (S)	0	28	5 (R)
Kingwa	0	8	0 (R)	0	0	0 (R)	0	0	0 (R)	67	20	0 (R)	...	28	0 (R)	38	92	75 (S)
Mack	...	12	0 (R)	...	0	0 (R)	0	4	0 (R)	100	...	100 (S)	...	...	85 (S)	...	...	0 (R)
Sanga	18	52	0 (R)	100	100	100 (S)	0	52	0 (R)	5	64	0 (R)	0	70	0 (R)	7	32	0 (R)
Toku	0	0	0 (R)	8	0	0 (R)	4	24	5 (R)	11	8	0 (R)	80	84	75 (S)	85	24	0 (R)
Tracy	0	0	0 (R)	0	0	0 (R)	0	0	0 (R)	0	...	0 (R)	0	0	0 (R)	0	...	0 (R)

[a]H = hypocotyl. Seedlings were inoculated at 8 days of age by hypocotyl wound method; disease was evaluated 5 days later. There were two replications, one containing five plants and one containing eight plants.

[b]V = vermiculite. Seedlings grown in vermiculite were inoculated at 2 days of age; disease was evaluated 5 days later. There were 45 seedlings per treatment.

[c]S = soil. Seedlings were inoculated at 2 days of age; disease was evaluated after 3 days' incubation on flooded soil. There were 20 seedlings per treatment.

[d]R = resistant, S = susceptible, based on published results obtained using hypocotyl inoculations (19).

seedlings using suspensions of zoospores in distilled water in petri dishes. Zoospores, however, readily infected seedlings in petri dishes containing 0.5–30 g of autoclaved soil flooded with distilled water. The effect of zoospore concentration on disease incidence in the presence of soil was evaluated. Ten grams of sterile air-dried Conover loam was placed in autoclaved glass petri dishes. Mean concentrations of 1, 3, 10, 30, 100, 500, and 1,000 zoospores of race 1 in 30 ml of distilled water were added to each plate. Resulting percentages of diseased seedlings were 0, 20, 24, 36, 64, 80, and 100, respectively (LSR [$P = 0.05$] = 23). A mean concentration as low as three zoospores per plate was sufficient to cause a low incidence (20%) of disease. Disease incidence increased as inoculum concentrations increased until 100% of the seedlings were infected at 1,000 zoospores per plate. Localized lesions about 1–2 cm in length were present on the hypocotyls of seedlings exposed to low (3–30) numbers of zoospores, whereas concentrations of 100 zoospores and higher produced the typical uniform browning of the hypocotyls. Noninoculated control seedlings remained healthy.

The effect of temperature on disease incidence in Hark seedlings was studied in petri dishes to each of which were added 2.5×10^3 zoospores of race 1 (5×10^2 per seedling), in the presence of soil. The distilled water used was adjusted to 10, 15, 20, 25, 30, or 35 C before use, and seedlings were placed in incubators at corresponding temperatures. Greatest numbers of diseased seedlings were obtained at 20, 25, and 30 C (Fig. 1). Temperatures higher and lower than this range resulted in significantly less disease incidence. Seedlings that were not inoculated and incubated at the same temperatures remained healthy.

The effect of zoospore concentration on disease incidence in four differential cultivars (Hark, Altona, Harosoy 63, and Toku) was studied. Suspensions containing 4×10^3, 2×10^4, 2×10^5, and 10^6 zoospores of race 1 per 100 ml of distilled water were added to soil in each tray (equivalent of 2×10^2, 10^3, 10^4, and 5×10^4 zoospores per seedling, respectively). Hypocotyls of Hark, a susceptible cultivar, were decayed severely at all inoculum concentrations (Table 3). The percentage of infected seedlings of the resistant cultivars Altona, Harosoy 63, and Toku increased as inoculum concentrations increased. The cultivars, however, exhibited different degrees of resistance as inoculum increased. For example, at 10^3 zoospores, 35, 20, and 5% of the seedlings, respectively, became diseased. 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. Seedlings that were not inoculated remained healthy.

TABLE 3. Effect of zoospore concentration on disease incidence in four differential soybean cultivars placed in trays containing sterilized soil and inoculated with *Phytophthora megasperma* var. *sojae* race 1[a]

Zoospores/ seedling	Diseased seedlings of cultivar: Hark (%)	Altona (%)	Harosoy 63 (%)	Toku (%)
2×10^2	100	10	0	0
10^3	100	35	20	5
10^4	100	50	65	35
5×10^4	100	100	100	100

[a]Two-day-old seedlings were incubated on flooded soil for 3 days before evaluation. There were five seedlings per cultivar per tray and four trays per treatment. Least significant ranges by Tukey's w procedure ($P = 0.05$) for interaction of concentration × cultivar = 14.4.

The experiment was repeated using 2- and 3-day-old seedlings of the same cultivars. Suspensions containing 8×10^3, 4×10^4, and 4×10^5 zoospores of race 1 per 150 ml of distilled water were added to each tray (equivalent of 2×10^2, 10^3, and 10^4 zoospores per seedling, respectively). At both ages, Hark exhibited 100% disease incidence at all inoculum concentrations. Disease in the resistant cultivars again tended to increase with inoculum concentrations. Three-day-old seedlings of Altona, Harosoy 63, and Toku, however, exhibited significantly ($P = 0.05$) less disease than did 2-day-old seedlings; specifically, 71 versus 20%, 47 versus 27%, and 24 versus 11%, respectively. Seedlings that were not inoculated remained healthy.

Nine differential soybean cultivars placed on flooded soil were inoculated with zoospores of races 1 to 6 to determine if this inoculation method could be used to differentiate races of the pathogen. Two-day-old seedlings of Altona, Hark, Harosoy 63, Higan, Kingwa, Mack, Sanga, Toku, and Tracy were added to trays after a suspension of 8×10^3 zoospores in 150 ml of distilled water (2×10^2 zoospores per seedling) was applied to the soil. The nine soybean cultivars exhibited differential responses to the six races of *P. megasperma* var. *sojae* in close accord with published data based on hypocotyl inoculations (19) (Table 2).

Detection of P. megasperma var. sojae in naturally infested soil using soybean seedlings as baits.—Twenty grams of air-dried Brookston loam, naturally infested with *P. megasperma* var. *sojae*, was placed into petri dishes; 30 ml of distilled water, which was first adjusted to the specific incubation temperatures of the treatment, was added. Plates were incubated for 4 days in the dark at 10, 15, 20, 25, 30, and 35 C. Disease incidence increased as the temperature increased (Fig. 1). Temperatures of 25–35 C produced significantly ($P = 0.05$) more disease than did lower temperatures.

Nine different natural soils were tested using soybean seedlings as baits. Five of the soils were from fields with soybean crops at the time of collection; the other four soils were from fields with crops other than soybean during the year that they were collected. *Phytophthora megasperma* var. *sojae* was baited successfully from all nine natural soils. *Pythium* spp. also were observed on seedlings in six of the soils. *Phytophthora megasperma* var. *sojae* also was baited from badly decayed roots of soybean plants killed by the pathogen in the field.

DISCUSSION

Methods are described for inoculating soybean seedlings with zoospores of *P. megasperma* var. *sojae*, and for detection of the pathogen in natural soil. The inoculation methods offer advantages over the hypocotyl-wound technique in that they are more rapid and easier to perform, and do not require a greenhouse.

The methods can be used for testing pathogenicity and virulence of isolates of the pathogen, and for evaluating varietal resistance. Since natural inoculum and infection courts are involved, the methods also can be used for studies of environmental factors that influence infection and for investigations on biological control.

In vermiculite, disease usually could be evaluated on a simple emergence versus nonemergence basis when 2-day-old seedlings were inoculated. Results were available within 6–8 days after inoculation. Soybean plants also were infected successfully when 2-day-old seedlings were incubated on flooded soil infested with zoospores of the pathogen. Results with this method were obtained within 3 days. Both inoculation methods yielded optimum results over a temperature range of 20 to 30 C and in various light conditions. Thus, neither a greenhouse nor carefully controlled temperature conditions were required. Control of inoculum concentrations was required, however, to discriminate between resistant and susceptible cultivars of soybean. Concentrations of about 10^4 zoospores per seedling in vermiculite and 2×10^2 zoospores per seedling in flooded soil were optimal. Lower concentrations sometimes failed to produce disease in susceptible cultivars, whereas excessive concentrations caused disease in some cultivars that were resistant by hypocotyl inoculation. Seedlings of resistant tobacco cultivars also were infected when inoculated with an excessive concentration of zoospores of *P. parasitica* var. *nicotianae* (6, 10). Standardization of seedling age at the time of inoculation also was important in differentiating resistant from susceptible cultivars. Seedlings 2 days old gave maximum discrimination with both methods. Seedlings older than 2 days rapidly became resistant.

These inoculation methods may be adaptable for the identification of "field tolerance," which cannot be detected by inoculating wounded hypocotyls with mycelium (14, 15, 19). These cultivars had lower disease incidences than did Hark, which is not known to be field tolerant, when inoculated with an appropriate concentration of zoospores (Table 1). Further work, however, will be required to evaluate this possibility.

Isolation of *P. megasperma* var. *sojae* directly from natural soil has proved difficult (18), but our results showed that soybean seedlings can be used as baits to detect the pathogen in soil. The fungus was identified microscopically without isolation and reinoculation, and pure cultures were obtained readily from diseased tissue. 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 *P. megasperma* using alfalfa seedlings as baits (8, 9, 11–13).

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