Storage and Use of *Phytophthora megasperma* var. *sojae* Oospores as Inoculum

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ABSTRACT

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Chilling (−7 C), heating (40 C), and sonication were evaluated as methods of freeing oospores of *Phytophthora megasperma* var. *sojae* from viable mycelia. Hosts (*Glycine max*) were grown in soil infested with 1,000 oospores per gram (OPG) of soil from each treatment. Percentages of plants infected were 100, 100, 93, and 69 for untreated oospores, chilled, sonicated, and heated, respectively. When chilled suspensions were used to infest soil at 0, 10, 100, 250, 500, and 1,000 OPG, percentages of soybean seedlings infected were 0, 27, 65, 81, 98, and 100, respectively. Chilled oospores were evaluated for pathogenicity at 2-wk intervals from 0 to 6 wk of storage. Relative root reduction of inoculated seedlings at 1,000 OPG were 37.8, 30.8, 22.3, and 10.8 for 0, 2, 4, and 6 wk, respectively.

Oospores of *Phytophthora* spp. are believed to be important overwintering structures (8) and therefore are important propagules in the disease cycle of many *Phytophthora* spp. (19). *Phytophthora megasperma* Drechs. var. *sojae* A. A. Hildeb. (PMS) causes a serious root and stem rot on soybeans, *Glycine max* (L.) Merr. Only limited work has been done on the importance of PMS oospores in the disease cycle.

Sterols are necessary for the formation of oospores in culture (4,6,7). After oospores are formed, they eventually germinate, but the percentages that germinate during a given time are low and variable (19). Schmitthenner (14) considered this dormancy to be constitutive. Oospore germination varies with temperature under which the oospores are produced (5). Oospores produced at 27 C had a higher rate of germination than oospores produced at other temperatures. Continuous light in the developmental stage suppressed oospore production (11).

Oospore density in soil and its influence on disease incidence have been investigated in several *Pythium* spp. (9,12). Oospore density studies require the use of oospores free from viable mycelial fragments. Methods used to kill mycelial tissue without destroying oospores include freezing (10), feeding mycelial mats to snails (15), treating suspensions of oospores and mycelia with enzymes (17), forcing cultures through a 50-μ sieve (5), and sonication of culture suspensions (9). Sauve and Mitchell (13) reported that freezing reduced oospore germination in *Phythium aphanidermatum* and *P. myriotylum*.

This research was done to evaluate methods of freeing PMS oospores from viable mycelium, to examine the influence of oospore inoculum density on disease incidence, and to study the viability of PMS oospores in frozen storage.

MATERIALS AND METHODS

The fungus used in all tests was PMS, race 1. All stock cultures were grown on V8 agar (177 ml of V8 juice, 823 ml of water, 3 g of $CaCO_3$, and 15 g of agar). For each experiment, two 5-mm disks from the margin of actively growing colonies were transferred into 50 ml of liquid medium. PMS was grown on V8 broth (177 ml of V8 juice, 823 ml of water, and 3 g of $CaCO_3$). The medium was dispensed in 50-ml portions into 180-ml prescription bottles, which were then autoclaved for 25 min.

Soybean seedlings of the susceptible cultivar Harosoy were used as host. Commercial 240-ml polystyrene cups were used as containers. Approximately 350 g (dry wt) of steamed sand was added to each container. Various levels of inoculum were mixed with dry sand for infestation treatments. One 4-day-old seedling was transplanted from sterile sand into each container. Unless stated otherwise, all plants were grown in a growth chamber with 14-hr days and 10-hr nights at 27 and 22 C, respectively. Daytime light intensities were 450 microeinsteins m^{-2} sec^{-1} from fluorescent light and 45 microeinsteins m^{-2} sec^{-1} from incandescent light.

PMS was isolated from root sections by surface sterilizing 1-cm sections in 0.5% NaOCl for 60 sec and placing them on antibiotic medium made from V8 agar containing 100 μg/ml each of streptomycin sulfate, neomycin sulfate, and pentachloronitrobenzene. This medium is used routinely in this laboratory for isolation of PMS. Plates were incubated for 5 days at 27 C and observed for growth of PMS.

Three methods were investigated to free oospores from viable mycelium. These included chilling at −7 C, heating at 40 C, and sonication using a Bronson model W200P ultrasonic system. Cultures were grown on V8 broth incubated with shaking for 8 days and then for 6 days without shaking at 27 C. After washing and grinding, 25 ml bottles containing 15 ml of culture suspension were exposed to various lengths of each treatment. After suspensions were treated, 1 ml of each culture was plated on the antibiotic medium and incubated at 27 C. Plates were evaluated after 3 and 7 days of incubation for growth of PMS.

Oospores from treatments that yielded no growth on plates were incorporated into steamed sand to give a final concentration of 1,000 OPG. Seven days later, roots from seedlings grown in infested and noninfested treatments were plated on the antibiotic medium. Data taken were percentage of plants infected and percentage of the single plant root system that was colonized.

The effect of oospore inoculum density on disease incidence was investigated by adding chilled and untreated cultures to steamed sand. Cultures were grown by the shake method described above. A single seedling was transplanted into sand containing 0, 10, 100, 250, 500, and 1,000 OPG and grown for 7 days. Sand was kept moist but well drained. Roots were plated on antibiotic medium and the percentages of plants and root systems colonized were calculated.

Viability of oospores in frozen storage was evaluated after 0–6 wk storage periods. Samples were removed at 14-day intervals and

incorporated into steamed sand to yield 1,000 OPG. Seedlings were transplanted into infested and noninfested sand and grown for 7 days; then roots were dried at 70 C for 24 hr and weighed.

RESULTS

Mycelium-free oospores. Heating and chilling of PMS cultures for 30 and 120 min, respectively, or longer, resulted in suspensions that contained only oospores as viable propagules. The point at which mycelium failed to grow on plates after exposure to cold temperatures was associated with the formation of crystalline ice in the culture suspension. Sonication (Bronson model W200P) of PMS cultures at 30% of maximum intensity for up to 90 sec did not reduce resultant mycelial growth on plates noticeably. When cultures were exposed to 60 or 90 sec of sonication at 60% of maximum intensity, resultant mycelial growth was only 50 and 5% of untreated suspensions, respectively. Soybeans grown in soil infested with chilled oospores had a higher percentage of plants and roots infected than did plants grown in soil infested with oospores that had been heated or sonicated (Table 1).

Oospore inoculum density. The percentage of infected plants and roots increased as soil oospore density increased. Roots of plants grown in soil containing 500 and 1,000 OPG showed extreme discoloration and produced only limited lateral roots. Plants grown in soil infested with the untreated culture suspension showed a higher percentage of plants and roots colonized than did plants grown in soil infested with the equivalent frozen suspension (Fig. 1A).

Oospore storage. Storage of frozen oospores for prolonged periods resulted in a decrease in apparent pathogenicity to soybean seedlings (Table 2).

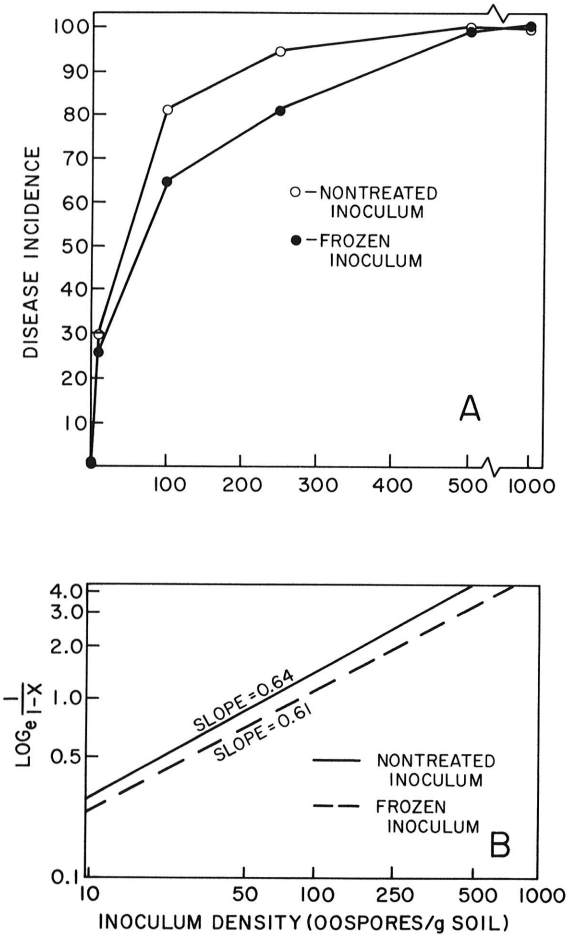

Fig. 1. Relationship of disease incidence in soybean seedlings to the density of oospores of *Phytophthora megasperma* var. *sojae* in infested soil. **A**, Arithmetic plot. **B**, Log-log transformation (x = disease severity).

DISCUSSION

Chilling of cultures was the best method evaluated for freeing oospores from viable mycelium. Blackwell (3) advocated lowering the temperature to 1 C to break dormancy of oospores of *P. cactorum;* however, Zentmyer and Erwin (19) found no increase in the percentage of germination of PMS oospores exposed to 1 C. The requirement for high temperatures and high intensity of sonication for prolonged periods to destroy viable mycelia may result in destruction of some potentially viable oospores. This probably accounts for the lower disease incidence in susceptible seedlings exposed to oospores from these treatments.

Disease incidence increased directly with inoculum density. The slope of the log-log regression line for frozen oospores is 0.61 (R^2 = 0.97) (Fig. 1B). This is similar to the slope of 0.67, which Baker et al showed to fit both model I and model II systems (1). Both of these systems assume that the inoculum source is fixed, ie, nonmotile. This fact leads to speculation that oospores of PMS germinate directly, either in direct contact with the root (model I, fixed infection court, rhizoplane effect) or in response to the growing root tip (model II, motile infection court). Oospore germination directly in the presence of root exudates has been suggested by several workers (2,9,18). This assumption allows the study of oospores as primary infection units. The slope of the log-log regression line of untreated inoculum was 0.64 (R^2 = 1.00). It parallels the regression line of frozen oospores but has been shifted to the left, because the viable mycelia were not considered as contributing to inoculum density. From the regression line of the frozen oospores, the inoculum density required to produce 50% diseased plants (ID_{50}) was 41.7 OPG, greater than the value obtained by Mitchell (9) for *P. myriotylum.*

The percentage of infected plants was positively correlated with the percentage of the root system infected. Some root sections that were discolored and contained abundant oospores did not produce fungal growth on the medium. Mycelia in older infected root tissue may become dormant or die after formation of oospores. It is also possible that this older mycelium is inhibited by the antibiotics contained in the medium. Root staining procedures similar to that

TABLE 1. Effect of oospore treatment on the percentage of soybean plants infected and the percentage of the root system colonized by *Phytophthora megasperma* var. *sojae* in Harosoy seedlings grown in soil containing 1,000 oospores per gram[y]

Treatment	Percent infection	
	Plants	Root system
Noninoculated control	0 d[z]	0 c
Untreated inoculated	100 a	12.5 a
Freezing	100 a	9.7 a
Sonication	93 b	6.1 b
Heating	69 c	4.6 b

[y]Values are mean of two experiments containing 28 plants of each treatment.
[z]Values in columns with different letters are significantly different (P = 0.05) by Duncan's multiple range test.

TABLE 2. Effect of storage time of frozen oospores of *Phytophthora megasperma* var. *sojae* on the relative root reduction of soybean seedlings grown in soil containing 1,000 oospores per gram[y]

Time (wk)	Relative root reduction (%)	
	Experiment 1	Experiment 2
0	31.4 a[z]	44.1 a
2	29.5 a	32.1 b
4	21.6 b	23.0 c
6	15.0 c	6.5 d

[y]Values are the mean of four plants in each experiment.
[z]Values in columns with different letters are significantly different (P = 0.05) by Duncan's multiple range test.

of Slusher and Sinclair (16) may aid in understanding the dynamics of the PMS oospore inoculum system. After inoculum density relationships have been evaluated, the influence of external factors such as fungicide dosage, environmental components, biological control agents, and host disease tolerance can be quantitatively measured. This study presents techniques and the results of initial studies that contribute to a better understanding of the PMS oospore as an infection propagule.

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