Evaluation of Several Actinomycetes and the Fungus *Hyphochytrium catenoides* as Biocontrol Agents for Phytophthora Root Rot of Soybean

A. B. FILONOW, Former Research Associate, and J. L. LOCKWOOD, Professor, Department of Botany and Plant Pathology, Michigan State University, East Lansing 48824-1312

ABSTRACT

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Soybean seed (cultivar Corsoy) were coated with *Actinoplanes missouriensis, A. utahensis, Amorphosporangium auranticolor, Micromonospora* sp., and *Hyphochytrium catenoides* in 1% carboxymethyl cellulose + 1% soluble starch (w/v). Treated seed were planted in a loam soil naturally infested with *Phytophthora megasperma* f. sp. *glycinea* (*P. m. glycinea*) in a greenhouse. In some experiments, the soil was supplemented with 400 oospores per gram. After 1 mo, stands of plants from seed coated with *A. missouriensis, A. utahensis,* or *Micromonospora* sp. were significantly ($P = 0.05$) greater than those from uncoated seed in three of four experiments. Surviving plants from seed coated with these microorganisms in many cases also had increased root and shoot weights and reduced root rot severity. *Amorphosporangium auranticolor* was effective in one experiment, but *H. catenoides* provided no protection against Phytophthora root rot. Aqueous suspensions of the five microorganisms were also applied to soil and incubated for 1 and 3 wk. Two-day-old soybean (cultivar Corsoy) seedling baits for *P. m. glycinea* were incubated in 10-fold dilutions of treated and untreated soil. Seedling decay at the greater soil dilutions was significantly ($P = 0.05$) reduced by *A. missouriensis* and *A. utahensis* in one experiment and by all five hyperparasites in a second experiment compared with that in untreated soil, suggesting that the hyperparasites had reduced *P. m. glycinea* inoculum in soil.

Soybean production in Michigan and in other soybean-producing states is often severely limited by Phytophthora root rot (PRR) caused by *Phytophthora megasperma* Drechs. f. sp. *glycinea* Kuan & Erwin (*P. m. glycinea*). Chemical control is often not practical because of excessive costs or lack of efficacy, and resistant cultivars are subject to recurring attacks by new races of *P. m. glycinea*. The overwintering structure, the oospore, is parasitized by various microorganisms in soil (6,11,12). Because *P. m. glycinea* survival and inoculum carryover from season to season largely depend on the oospore population in soil, their destruction by hyperparasites should result in reduced disease. Recently, Hsu and Lockwood (8) showed that *Hyphochytrium catenoides* Karling, a hyperparasite of *P. m. glycinea* oospores (2,11), was effective in reducing Phytophthora root rot when applied to soil in greenhouse tests. In preliminary experiments, Sutherland and Lockwood (12) found that soybean seed treated with *Actinoplanes missouriensis* Couch or *Humicola fuscoatra* Traaen. produced plants with reduced root rot. These same authors (12) also found that the actinomycetes *Actinoplanes utahensis* Couch, *Amorphosporangium auranticolor* Couch, and *Micromonospora* sp. parasitized oospores of *P. m. glycinea*, but they did not conduct biological control experiments with these organisms. We evaluated the effectiveness of the above five hyperparasites of *P. m. glycinea* oospores as soybean seed coatings in greenhouse experiments and attempted to determine whether the hyperparasites reduced oospore populations in soil.

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Present address of first author: Department of Plant Pathology, Oklahoma State University, Stillwater 74078.

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MATERIALS AND METHODS

Hyperparasites. Microorganisms used were the actinomycetes *Actinoplanes missouriensis, A. utahensis, Micromonospora sp.*, and *Amorphosporangium auranticolor* and the fungus *Hyphochytrium catenoides*. Isolates used in this study were previously shown to parasitize *P. m. glycinea* oospores (11,12). Subcultures of these hyperparasites were maintained on yeast extract-starch agar (5) in the dark at 23–25 C. Hyperparasite inoculum was grown in 2.8-L Fernback flasks containing 250 ml of liquid yeast extract-starch medium (5) and incubated in the diffuse light of the laboratory for 4–6 wk before use. Cultures were comminuted for 1 min in a Sorvall Omni Mixer at 8,000 rpm, sedimented by centrifugation at 3,100 × *g* for 10 min, and washed twice with dilute (1:100, v/v) Pfeffer's salts solution (pH 6.5) (3) by centrifugation. The pellet was then resuspended with distilled water and an aliquot taken to estimate hyperparasite density in the suspension. Serial dilutions of the aliquot were plated on yeast extract-starch agar, and colony-forming units (cfu) per milliliter were determined after 5 days of incubation.

Coated seed experiments. Five to 10 ml of hyperparasite suspensions at densities of 6×10^3 to 1×10^{10} cfu/ml were pelleted by centrifugation (3,100 × g for 10 min). The pellet was resuspended in 5–10 ml of 1% (w/v) soluble starch (Difco Laboratories, Detroit, MI) + 1% carboxymethyl cellulose (w/v) in distilled water to give a hyperparasite density of about 1×10^9 cfu/ml. The suspension was poured into a glass petri dish (9 cm in diameter) containing about 100 soybean seeds (cultivar Corsoy) and the seeds were gently mixed for 1 min in the suspension. Coated seeds were placed on cheesecloth and quickly dried (10–15 min) with a hair dryer, taking care not to allow the air temperature at the surface of the soybeans to exceed 35 C. Seeds were planted 2–18 hr after coating.

Coated seeds were planted in a Conover loam soil naturally infested with *P. m. glycinea* (race not determined). In some experiments, soil was supplemented with 400 oospores of *P. m. glycinea* (race 1) per gram of soil. Oospores of *P. m. glycinea* were produced in cultures grown for 1 mo in the dark in Roux bottles containing 150 ml of V-8 juice broth (1) with 30 μg/ml of β-sitosterol. Cultures were comminuted in an Omni Mixer for 1 min at 8,000 rpm, then mixed with soil and incubated for at least 1 mo to allow the lysis of mycelial fragments (8). Styrofoam containers (19 × 14 × 6 cm)

with 1 kg of soil per container were used, and 15 seeds were planted in each container. A randomized complete block design was used with seven replicates per treatment, except for one experiment with three replicates. Controls consisted of uncoated seed in steamed and unsteamed soil and vehicle (starch plus CMC)-coated seed in steamed and unsteamed soil. Soil was steamed for 1 hr at 100 C on two successive days.

Plants were grown in the greenhouse (28 ± 2 C) under natural light and were watered daily. After 1 mo, stands were counted and plants were removed from soil. Roots of plants were rated for disease severity with a disease index of 1–5, where 1 = healthy, no apparent infection (no discoloration of roots, long taproot, many lateral roots); 2 = very light infection (<25% of root area discolored, long taproot with some lateral roots); 3 = slight infection (25–50% of roots discolored, moderately long taproot, no lateral roots); 4 = moderate infection (50–75% of roots discolored, short taproot with no lateral roots); and 5 = severe infection (>75% of roots discolored, no taproot, completely decayed seed or seedling). Infection was confirmed by observing sporangia of *P. m. glycinea* in diluted soil or after transferring seedlings to distilled water. Segments of seedlings were plated on a *P. m. glycinea*-selective medium (10) if no sporangia were observed. Weights of roots and shoots of surviving plants were determined after drying for 40 hr at 60 C.

Experiments with hyperparasites added directly to soil. Aqueous suspensions of hyperparasites at 1–3 × 10^9 cfu/ml, as determined by serial dilutions plated on yeast extract-starch agar, were mixed into 100–800 g of naturally infested soil to give concentrations of 1–5 × 10^8 cfu/g of soil. Soils were incubated for 3 wk in plastic bags or in plastic containers (7.5 cm high × 15 cm in diameter) sealed with Parafilm to maintain soil moisture at about 80–90% of water-holding capacity. At 1 and 3 wk, subsamples of soil were taken and a sufficient weight of soil (previously determined) to give 50 g oven-dry weight was added to 50 ml of sterile distilled water. The soil and water were blended for 1 min at moderate speed and the soil slurry was diluted to give 0.5, 0.05, and 0.005 g of soil per milliliter.

Ten milliliters of each dilution, and in addition, 1 ml of the 0.005-g dilution were pipetted into sterile glass petri dishes (9 cm in diameter) to give 5.0, 0.5, 0.05, and 0.005 g of soil per dish. Sterile water was then added to each dish to give a final volume of 30 ml. There were four or five dishes per dilution. After 3 days of incubation at 24–25 C under the diffuse light of the laboratory to allow for zoospore release, five 2-day-old soybean seedlings (cultivar Corsoy) from surface-disinfested (1% NaOCl for 3 min and then rinsed with sterile water) seed were placed in each dish (6). The seedlings were incubated 5–7 days, then rated for disease severity on a scale of 1–5 as described. Presence of *P. m. glycinea* in infected seedlings was also confirmed by plating, when necessary, as described before.

Statistical analysis. Each experiment was done at least twice. Significant differences between treatment means were determined by Duncan's multiple range test at $P = 0.10$ and $P = 0.05$. Orthogonal polynomial analyses also were done with the data from the experiments adding hyperparasites to soil.

RESULTS

Coated seed experiments. Results of experiments done in *P. m. glycinea*-infested soil at low (natural) or high (supplemented) oospore density are given in Table 1. Results of repeated experiments were similar. In soil with low oospore density, mean stands of soybean from seed coated with *A. missouriensis, A. utahensis,* or *Micromonospora* sp. were increased significantly ($P = 0.05$) over those from uncoated seed. *Amorphosporangium auranticolor* significantly ($P = 0.05$) increased stand in one experiment (Table 1) but not in a repeated experiment. Stands from seed coated with these actinomycetes were increased twofold to 2.6-fold and in some instances to levels equivalent to the stand in steamed soil. *H. catenoides* did not increase stand in either experiment. Seed coated with vehicle only had no significant ($P = 0.10$) effect on plant stands in steamed or unsteamed soil.

Treatments giving increased stands tended to have reduced root rot severity (Table 1). For example, disease indices were significantly ($P = 0.05$) reduced by *A. missouriensis, A. utahensis,* or *Micromonospora* sp., which were also most effective in increasing stands. Hyperparasites that increased plant stands also generally increased root and shoot weights. However, this was not always the case. For instance, plants from seeds treated with *Amorphosporangium auranticolor* and *Micromonospora* sp. had increased stands, but surviving plants showed no significant increase in root and shoot weights over those from uncoated seeds.

In soil with a high inoculum density, fewer hyperparasites increased plant stands. Only *A. utahensis* and *Micromonspora* sp. in one experiment (Table 1) and *A. missouriensis* in a repeated experiment were effective ($P = 0.05$). In these experiments, neither root disease indices nor root and shoot heights of surviving plants were significantly ($P = 0.10$) increased.

Effect of hyperparasites on the inoculum density of *P. m. glycinea* in soil. Soybean seedlings incubated in suspensions of *P. m. glycinea*-infested soil showed progressively less disease with increasing dilutions of soil (Table 2). For example, at 5.0, 0.5, 0.05, and 0.005 g of soil per dish, disease indices in experiment 1 were 4.9, 4.1, 3.3, and 3.0, respectively. Treatment of *P. m. glycinea*-infested soil with hyperparasites for 1 wk resulted in significant ($P = 0.05$) reductions in disease in soil treated with *A. utahensis* (0.05 and 0.005 g of soil per dish) and *A. missouriensis* (0.005 g of soil per dish).

Table 1. Effect of oospore hyperparasites applied as seed coatings and planted in soil naturally infested with *Phytophthora megasperma* f. sp. *glycinea*, or the same soil with a supplemented density, on Phytophthora root rot of soybean seedlings in greenhouse tests[v]

Treatment	Soil with natural inoculum density				Soil with supplemented inoculum density[w]			
	Stand (%)	Disease index[x]	Shoot weight[y]	Root weight[y]	Stand (%)	Disease index[x]	Shoot weight[y]	Root weight[y]
Uncoated	35 a	3.5 c	2.8 a	0.9 a	31 a	2.6 b	3.8 a	1.2 a
Actinoplanes missouriensis	91 c	1.6 a	4.6 c	1.8 b	35 a	1.9 ab	2.7 a	1.5 a
A. utahensis	87 c	1.7 a	4.1 bc	2.9 c	47 b	2.9 b	3.7 a	1.3 a
Amophosporangium auranticolor	71 b	3.0 bc	2.2 a	1.2 a	35 a	2.0 b	2.5 a	1.5 a
Hypochytrium catenoides	53 a	2.8 bc	3.7 b	1.2 a	27 a	1.8 ab	3.1 a	1.8 a
Micromonospora sp.	80 c	2.1 b	2.4 a	0.9 a	65 c	3.2 b	5.3 a	2.0 a
Steamed soil[z]	100 c	1.2 a	5.0 c	2.3 bc	93 c	1.3 a	9.7 b	5.2 b

[v] Data were taken 1 mo after seeding. There were seven replicate containers per treatment with 15 seeds each. Means in a column followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.
[w] Supplemented with 400 oospores per gram of soil.
[x] Disease index was based on a scale of 1–5, where 1 = healthy plants and 5 = severely infected or dead plants.
[y] Dry weights of surviving plants after 48 hr at 60 C.
[z] Uncoated seed in steamed soil.

Table 2. Effect of oospore hyperparasites applied to soil infested with *Phytophthora megasperma* f. sp. *glycinea* and incubated for 1 wk on decay of soybean seedlings subsequently incubated in different dilutions of the soil[w]

Treatment	Disease index[x]									
	Experiment 1					Experiment 2				
	5.0 g	0.5 g	0.05 g	0.005 g	F^y	5.0 g	0.5 g	0.05 g	0.005 g	F^y
Untreated	4.9 b	4.1 b	3.3 b	3.0 c	15.9	4.6 b	4.4 c	4.0 d	4.0 c	2.2
Actinoplanes missouriensis	5.0 b	4.3 b	3.0 b	2.3 ab	34.4	4.7 b	3.2 b	2.1 b	1.8 ab	42.7
A. utahensis	5.0 b	3.7 b	1.9 a	2.0 ab	42.4	5.0 b	3.9 bc	2.4 bc	2.0 ab	49.1
Amorphosporangium auranticolor	4.4 b	4.4 b	3.1 b	2.6 abc	17.7	4.3 b	3.7 bc	3.7 d	2.5 b	13.0
Micromonospora sp.	5.0 b	3.8 b	3.0 b	2.7 bc	20.6	4.5 b	3.8 bc	3.1 cd	2.8 b	15.0
Hyphochytrium catenoides	4.6 b	4.5 b	3.4 b	2.7 bc	18.3	5.0 b	3.8 bc	3.5 cd	2.6 b	25.1
Sterile soil control[z]	1.9 a	...	...	...	...	1.0 a	...	...	...	...

[w] Two-day-old seedlings (cultivar Corsoy) were incubated in dishes (five seedlings per dish, four or five dishes per treatment) containing 5.0, 0.5, 0.05, or 0.005 g of soil in 30 ml of water per dish for 5–7 days before disease rating. Means in a column followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

[x] Scale of 1–5, where 1 = healthy and 5 = complete decay of seedlings.

[y] F values for linear components in orthogonal polynomial analyses.

[z] Autoclaved (1 hr) soil in sterile distilled water.

Decreasing disease with increasing dilution of soil also occurred in experiment 2, but the overall decrease in disease index in untreated soil (4.6–4.0) was less than in experiment 1. Seedlings incubated in soil treated with *A. utahensis* or *A. missouriensis* for 1 wk showed significantly ($P = 0.05$) less disease than seedlings in untreated soil at dilutions of 0.05 g of soil per dish; at 0.005 g of soil per dish, disease indices of seedlings in all of the hyperparasite-treated soils were significantly ($P = 0.05$) lower (1.8–2.8) than those of seedlings incubated on untreated soil (4.0). Seedlings in diluted soils treated for 3 wk with *A. missouriensis* or *A. utahensis* also showed significantly ($P = 0.05$) reduced infection at 0.05 and 0.005 g of soil per dish compared with untreated soil, but disease was not reduced beyond that shown after 1 wk of treatment.

Since the F ratio was significant for both experiments, orthogonal polynomial analyses were done to determine whether a linear, quadratic, or cubic equation best fit the data points. Only the linear component was significant in 11 of the 12 sets of data. Thus, the data tended to lie along a straight line. Neither of the three components was significant for the control treatment in experiment 2, which showed virtually no change in amount of disease with soil concentration. The F statistic reflects the steepness of the slope connecting the data points, and therefore, these values confirmed the results of the analysis of variance in that those hyperparasites showing significant differences from the controls also tended to have steeper slopes (Table 2).

DISCUSSION

Plant stands in *P. m. glycinea*-infested soil were increased most consistently by *A. missouriensis, A. utahensis,* and *Micromonospora* sp., which increased stands, sometimes as much as 2.6-fold, in three of four experiments. Hyperparasite treatments were more effective in soil with a low inoculum density of *P. m. glycinea* than in inoculum-supplemented soil. Natural population densities of *P. m. glycinea* oospores in soil are not known because of the absence of methods for enumerating this pathogen. However, populations of other *Phytophthora* spp. in soil are generally low, i.e., from 1 to 60/g (4,9). Treatments that increased stands in low-inoculum soil also tended to produce plants with decreased root rot severity. In some instances, plant stands and root health of surviving plants approached (but never equaled) those of plants growing in steamed soil. The improved plant health was frequently reflected in increased shoot and root weights of surviving plants, but this did not always occur. Where it did not occur, it is likely that growth of the surviving plants was suppressed because of competitive effects. Sutherland and Lockwood (12) also found that *A. missouriensis*-treated soybean seed planted in *P. m. glycinea*-infested soil increased stands up to fourfold in two of three greenhouse tests. Reduction in Phytophthora root rot of soybean by hyperparasites has so far been shown only with young plants. Whether hyperparasites will afford any protection against the later occurring stem-canker phase of the disease is not known.

In this study, only *H. catenoides* as a seed coating failed to reduce Phytophthora root rot in any experiment. However, *H. catenoides* reduced disease when added to soil on a vermiculite carrier (8) or when added as an aqueous suspension to soil in a laboratory bioassay previously (12) and in the present work. *H. catenoides* may not be as effective per unit of hyperparasite inoculum as some of the actinomycetes; thus, a greater concentration of the fungus on seed would be required for control. Moreover, these results point out the need for evaluating different application modes for biological control because success in one mode may not ensure success in another.

All of the microorganisms used in this study are known hyperparasites of *P. m. glycinea* oospores (7,11,12). Although direct observation of parasitized oospores was not made in this study, soybean seedling baits in dilutions of soil that had been treated with hyperparasites showed less disease than baits in untreated soil. Thus, it is likely that oospore hyperparasitism in the spermospheres of the soybean seed accounts for the success of *A. missouriensis, A. utahensis,* or *Micromonospora* sp. as seed treatments. Other mechanisms of action, such as antibiosis or nutrient competition in the spermosphere, appear to be less likely but are not ruled out by our work.

Seed coating was the only method of application of hyperparasites tested in our work, since this would appear to be an acceptable and economical practice. The partial control of Phytophthora root rot achieved by *A. missouriensis, A. utahensis,* or *Micromonospora* sp. as seed treatments is encouraging and suggests that these microorganisms merit further study. Future research should be done on such factors as strain selection, concentration of inoculum on seed, carriers of hyperparasite inoculum, and culture medium for inoculum production to better assess their potential efficacy. In addition, field evaluations, which so far have been hampered by insufficient disease development (12), also need to be made.

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LITERATURE CITED

1. Ayers, W. A., and Lumsden, R. D. 1975. Factors affecting the germination of oospores of three *Pythium* species. Phytopathology 65:1094-1100.
2. Ayers, W. A., and Lumsden, R. D. 1977. Mycoparasitism of oospores of *Pythium* and *Aphanomyces* species by *Hyphochytrium catenoides*. Can. J. Microbiol. 23:38-44.
3. Bristow, P. R., and Lockwood, J. L. 1975. Soil fungistasis: Role of spore exudates in the inhibition of nutrient-independent propagules. J. Gen. Microbiol. 90:140-146.
4. Chuang, T. Y., and Ko, W. H. 1981. Propagule density: Its relation to population density of microorganisms in soil. Soil Biol. Biochem. 13:185-190.
5. Emerson, R. 1958. Mycological organization.

Mycologia 50:589-621.
6. Eye, L. L., Sneh, B., and Lockwood, J. L. 1978. Inoculation of soybean seedlings with oospores of *Phytophthora megasperma* var. *sojae* for pathogenicity and race determination. Phytopathology 68:1769-1773.
7. Humble, S. J., and Lockwood, J. L. 1981. Hyperparasitism of oospores of *Phytophthora megasperma* var. *sojae*. Soil Biol. Biochem. 13:355-360.
8. Hsu, S. C., and Lockwood, J. L. 1984. Biological control of Phytophthora root rot of soybean by *Hyphochytrium catenoides* in greenhouse tests. Phytopathol. Z. 109:139-146.
9. Mitchell, D. J., and Kannwischer-Mitchell, M. E. 1983. Relationship of inoculum density of *Phytophthora* species to disease incidence in various hosts. Pages 259-269 in: *Phytophthora*: Its Biology, Taxonomy, Ecology, and Pathology. D. C. Erwin, S. Bartnicki-Garcia, and P. H. Tsao, eds. American Phytopathological Society, St. Paul, MN. 392 pp.
10. Schmitthenner, A. F. 1973. Isolation and identification methods for *Phytophthora* and *Pythium*. Pages 94-100 in: First Woody Ornamental Disease Workshop. University of Missouri, Columbia.
11. Sneh, B., Humble, S. J., and Lockwood, J. L. 1977. Parasitism of oospores of *Phytophthora megasperma* var. *sojae*, *P. cactorum*, *Pythium* sp. and *Aphanomyces euteiches* in soil by oomycetes, chytridiomycetes, hyphomycetes, actinomycetes and bacteria. Phytopathology 67:622-628.
12. Sutherland, E. D., and Lockwood, J. L. 1984. Hyperparasitism of oospores of some Peronosporales by *Actinoplanes missouriensis* and *Humicola fuscoatra* and other actinomycetes and fungi. Can. J. Plant Pathol. 6:139-145.