Potential for Biocontrol of *Sclerotinia sclerotiorum* Through Colonization of Sclerotia by *Trichoderma harzianum*

G. R. KNUDSEN and D. J. ESCHEN, Department of Plant, Soil and Entomological Sciences, L. M. DANDURAND, Department of Bacteriology and Biochemistry, and L. BIN, Department of Plant, Soil and Entomological Sciences, University of Idaho, Moscow 83843

ABSTRACT

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In the laboratory, *Trichoderma harzianum* strain ThzID1, formulated as mycelial fragments in alginate pellets with or without wheat bran incorporated, colonized sclerotia of *Sclerotinia sclerotiorum* in raw or steamed soil. Incidence of colonization was higher in steamed soil than in raw soil, higher at 25 C than at 15 C, and higher at −500 kPa than at −50 kPa but was not affected by bran in pellets. Some sclerotia were colonized by indigenous *Trichoderma* spp. Pellets containing *T. harzianum* were added to soil in a pea (*Pisum sativum*) field or field microplots at densities of 4×10^2–2×10^4 pellets per square meter, along with sclerotia of *S. sclerotiorum*. Recovery of *Trichoderma* spp. from soil samples was not significantly increased by adding pellets. Addition of *T. harzianum* at high density (1×10^4 or 2×10^4 pellets per square meter) significantly increased the proportion of sclerotia colonized in microplots.

Sclerotinia sclerotiorum (Lib.) de Bary is a widely distributed and destructive pathogen of many crops. As with many soilborne plant pathogens, chemical control is sometimes infeasible or not economical. The efficacy of crop rotation as a control measure is reduced by the wide host range of this pathogen and its ability to survive long periods as sclerotia. Strains of *Trichoderma harzianum* Rifai were reported as antagonists (including mycoparasites) of mycelium or sclerotia of numerous plant pathogens, including *Pythium* spp. (5), *Rhizoctonia solani* Kühn (5,7,11), *Sclerotium cepivorum* Berk. (1), *Sclerotinia minor* Jagger (2), and *Sclerotium rolfsii* (14) Sacc. An isolate of *T. harzianum* (ThzID1), obtained from field soil at Moscow, ID, parasitized sclerotia of *S. sclerotiorum* on agar and sterilized sand (G. R. Knudsen and D. J. Eschen, *unpublished data*). Isolate ThzID1 grew and sporulated profusely from surface-disinfested (15 min, 0.5% NaOCl) sclerotia, and hyphae of *T. harzianum* were viewed microscopically in thin sections from sclerotia. The ability of mycoparasitic fungi to attack sclerotia, thus reducing pathogen inoculum or source of inoculum in the soil, is a potential means of biocontrol. To our knowledge, *T. harzianum* has not been investigated extensively as a biocontrol agent for *S. sclerotiorum*.

Methods to apply biocontrol agents to soil must ensure delivery of a viable agent capable of mycelial growth and/or sporulation in situ. A recent development for application of biocontrol agents is the incorporation of spores or hyphal biomass into alginate pellets, with or without an additional nutrient source or other additives (4,7,9). Advantages of this method include a relatively high viability and long shelf life of the formulated agent and ease of application (4,7).

The objective of this study was to test the ability of *T. harzianum*, formulated in alginate pellets, to colonize sclerotia

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of *S. sclerotiorum* under laboratory and field conditions.

MATERIALS AND METHODS

Formulation of pellets of *T. harzianum*. Isolate ThzID1 of *T. harzianum* was grown on potato-dextrose agar (PDA). Alginate pellets containing ThzID1 were made as described previously (6). Isolate ThzID1 was grown at 22 C for 1 wk in potato-dextrose broth in flasks on a rotary shaker, with 12 hr light and 12 hr dark per day. The hyphal biomass was strained, rinsed with sterile water, added to a 1% aqueous sodium alginate solution, and added as drops to 0.25 M aqueous $CaCl_2$. In one treatment, 2 g of wheat bran was added to each 100 ml of ThzID1-alginate mixture before pelletizing. Pellets formed in the $CaCl_2$ solution were removed within 10 min, rinsed with distilled water, allowed to air-dry on waxed paper, and stored in glass beakers at approximately 22 C and 30–50% relative humidity (RH). Hyphal biomass per pellet was estimated to be 1.8 mg (oven-dried weight). If added, the mean weight of bran per pellet was 2.5 mg. Pellets with bran weighed approximately twice as much as pellets without bran. ThzID1 grew from all pellets placed on PDA, even after six or more months of storage. Pellets used in all experiments were 2–8 wk old.

Production of sclerotia. An isolate of *S. sclerotiorum* obtained from a diseased lentil (*Lens culinaris* Medik.) plant was maintained on PDA. Mycelial disks were transferred to either plates of PDA (laboratory experiments and 1988 field experiment) or sterilized sliced carrots contained in Erlenmeyer flasks (1989 field experiment). After approximately 4 wk of incubation at 22 C, sclerotia were harvested, rinsed with water, air-dried, and stored at 22 C for 2–8 wk before use.

Colonization of sclerotia under laboratory conditions. A quantity of a Palouse silt loam soil was obtained from a pea (*Pisum sativum* L.) field near Moscow, ID, air-dried, and sieved through a 2-mm mesh. For one set of treatments, the soil (2 kg vol) was steamed at 90–110 C for 1 hr. This reduced resident fungal populations to low levels, although occasionally low numbers of some fungi (*Rhizopus*, *Penicillium*, or *Trichoderma* spp.) either survived the steam treatment or recolonized the steamed soil. Raw or steamed soil was adjusted gravimetrically, according to a previously derived soil moisture release curve, to a moisture level of either −50 or −500 kPa. Soil pH in soil/water (2:1) solution was approximately 6.0. Soil (90 g) was added to glass screw-cap jars (9 cm diameter × 9 cm deep). Ten sclerotia (from PDA) were placed on the soil surface. A single ThzID1 pellet was placed in the center of every jar except for control treatments, and an additional 30 g of soil was added, so that jars were about half full. Soil bulk density was approximately 1.2 g/cm^3. Jars were closed loosely, sealed with plastic film (Parafilm, American Can Co., Greenwich, CT), and incubated in the dark at 15 or 25 C. In preliminary experiments, soil moisture loss was less than 1% by weight over a 2-wk period.

Jars were incubated at either 15 or 25 C, so that experimental variables were: 1) soil—raw or steamed; 2) matric potential—−50 or −500 kPa; 3) temperature—15 or 25 C; and 4) pellets—none, pellets without bran, pellets with bran. Thus, there were a total of 24 treatments. After 1, 2, or 3 wk, jars were opened and sclerotia were recovered by wet-sieving. Sclerotia from each treatment were surface-disinfested by gently stirring for 5 min in a solution of 10 ml of 0.5% NaOCl + 10 ml of ethanol + 80 ml of water and rinsing with sterile water. Sclerotia were then placed on PDA and, after 2 wk of incubation at 25 C, were observed to determine if *S. sclerotiorum*, *Trichoderma* spp., other fungi, or no fungi were growing from them. The experiment was performed with five replicates per treatment for each sample time and repeated once.

For each treatment in the first two experiments, the proportion of sclerotia colonized by *Trichoderma* spp. at each sample time was recorded. Data were arcsine transformed (12), and the experiment was analyzed as a split-plot design with sample time as a main plot effect (12,13). Analysis of variance and orthogonal contrasts (12) were used to compare effects of temperature, matric potential, bran, and *T. harzianum*.

1988 field experiment. Peas were planted near Moscow, ID, in a field which was previously planted to winter wheat (*Triticum aestivum* L.) that had been harvested 8 mo earlier. The soil was the same Palouse silt loam soil mentioned previously. Peas were planted on 16 May at a rate of 28 kg/ha in plots that were seven rows wide and 3.5 m long. Plots were separated by four border rows of peas on either side and by 2-m alleys on either end. Treatments were applied on 6 June. In all plots, sclerotia of *S. sclerotiorum* (from PDA) were mixed with about 2 kg of field soil and applied in a strip 2.5 cm wide × 3.5 m long × 2 cm deep to each of the center four rows in each plot, at a density of 360 sclerotia per square meter. In some treatments, pellets with ThzID1 were also mixed with sclerotia and soil. Treatments included: 1) no ThzID1; 2) ThzID1 without bran, 400 pellets per square meter (10 kg/ha); 3) ThzID1 without bran, 1,000 pellets per square meter (25 kg/ha); 4) ThzID1 plus bran, 400 pellets per square meter (20 kg/ha); and 5) ThzID1 plus bran, 1,000 pellets per square meter (50 kg/ha). Treatments were arranged in a nested design with four replicates randomly assigned within sample dates and treatments randomized within blocks.

After 1, 2, 3, 5, 7, and 9 wk, sclerotia were sampled from each plot. Soil was dug from a randomly selected section (1 m long × 5 cm wide × 4 cm deep) within each treatment. Soil samples were then washed over a 1.4-mm mesh screen and 10 sclerotia per plot were selected from the remaining debris with forceps. Only rarely were fewer than 10 sclerotia obtained. Sclerotia were then surface-disinfested and placed on PDA, and the proportion colonized by *Trichoderma* spp. was recorded after a 2-wk incubation period.

Before sieving, subsamples of soil (approximately 5 g) were taken, diluted serially in sterile water, and placed on the semiselective medium described by Papavizas (8). The medium was modified slightly in that chloroneb was not included. After 10–14 days of incubation at 20 C, colonies of *Trichoderma* spp. were counted. *Trichoderma* spp. were identified by a combination of growth on the selective medium, colony color, and morphology of conidia and conidiophores; however, colonies were not identified to species. In addition to the above sampling times, initial populations were obtained in the same manner on the day that plots were infested.

Rain fell on the plots immediately before and 3 days after application of treatments. Plots were irrigated with an overhead sprinkler on 15 June and 22 and 23 June to field capacity.

Analysis of variance and orthogonal contrasts were used to compare effects of bran and ThzID1 on the two dependent variables—proportion of sclerotia colonized (after arcsine transformation) and $\log_{10}$ (colony-forming units [cfu] + 1 *Trichoderma* spp. per gram of soil).

1989 field experiment. In 1989, a second field experiment was conducted. Instead of a conventional planting operation, treatments were applied in microplots. Microplots were made by burying plastic buckets (25 cm diameter × 30 cm deep) to their rims in holes dug by a large tractor-mounted posthole digger. Buckets each had four 3-cm^2 drainage holes near the bottom. Each bucket was filled to within 5 cm of the top with sieved (5-mm mesh) Palouse silt loam soil dug from a field border area with no immediate cropping history. Microplots were separated from one another by approximately 0.75 m of turf.

On 1 June, 12 pea seeds (cv. Columbia) were spread on the soil surface. Sclerotia and ThzID1 (where appropriate) were mixed with more soil and added in a 2-cm layer. Finally, buckets were filled with additional soil and watered. Combinations of sclerotia and ThzID1 formulations were as in 1988, but the densities of both were increased in an attempt to increase colonization over levels observed in 1988 and to sample larger

numbers of sclerotia.

Sclerotia were applied to all microplots at a rate of 1,000 sclerotia per square meter (50 sclerotia per microplot). Treatments included: 1) no ThzID1; 2) ThzID1 without bran, 1×10^4 pellets per square meter (500 per microplot, 250 kg/ha); 3) ThzID1 without bran, 2×10^4 pellets per square meter (1,000 per microplot, 500 kg/ha); 4) ThzID1 plus bran, 1×10^4 pellets per square meter (500 per microplot, 500 kg/ha); and 5) ThzID1 plus bran, 2×10^4 pellets per square meter (1,000 per microplot, 1,000 kg/ha). Treatments were arranged in a nested design with four replicates randomly assigned within sample dates and treatments randomized within blocks. Three microplots were used per treatment per replicate, with one-half of a microplot sampled by removal at each time interval.

After emergence, pea seedlings were thinned to six per microplot. Soil matric potential was estimated daily for the first 2 wk of the experiment and weekly thereafter, from measurements with gypsum blocks and a datalogger (Campbell Scientific Co., Logan, UT). Blocks were buried 4 cm deep in three microplots. Over the first 2 wk of the experiment, plots were watered equally to maintain high soil moisture (approximately −50 kPa). Relatively uniform soil moisture was obtained by timing the duration of watering for each microplot. The turf between microplots was not watered. After 2 wk, plots were no longer watered, and observed soil moisture levels fell and remained below −1,500 kPa.

After 1, 2, 3, 5, 7, and 9 wk, sclerotia were sampled as described previously, except that 20 sclerotia per plot were recovered, plated, and examined for colonization by *Trichoderma* spp. Population densities of *Trichoderma* spp. in soil from microplots also were determined as previously described. Experimental data (proportions of colonized sclerotia, populations of *Trichoderma* spp.) were analyzed as in 1988.

Although apothecial production from sclerotia was observed in field trials in both years, weather conditions were not conducive to disease development, thus, disease was not rated in either 1988 or 1989.

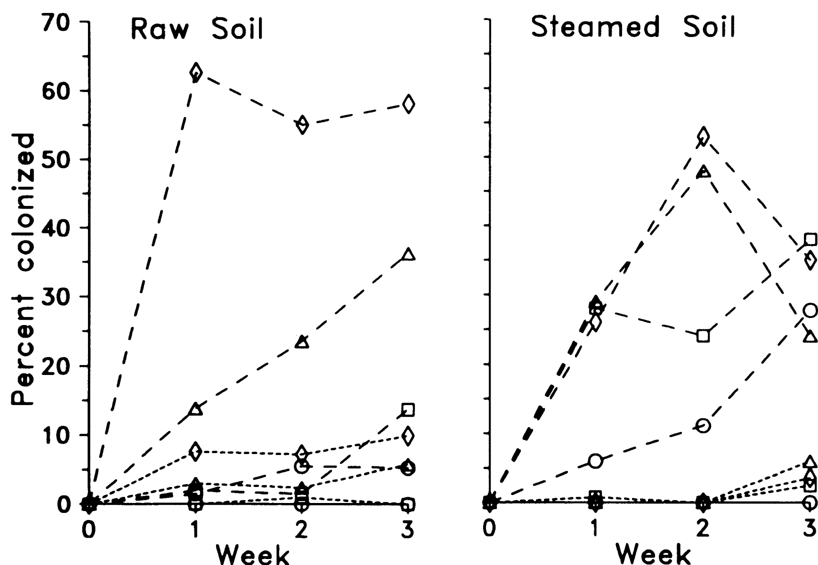

Fig. 1. Mean percentages of sclerotia of *Sclerotinia sclerotiorum* colonized by *Trichoderma* spp. in raw or steamed soil, when pellets of *T. harzianum* strain ThzID1 were added (---) or not (- - -). (○) = 15 C, −50 kPa; (□) = 25 C, −50 kPa; (△) = 15 C, −500 kPa; (◇) = 25 C, −500 kPa.

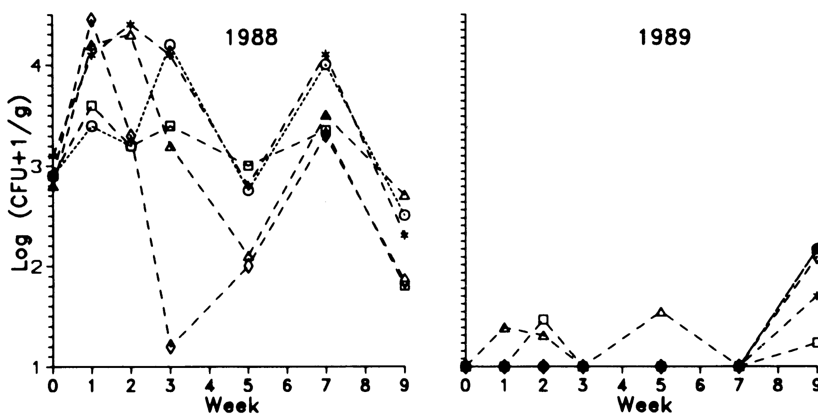

Fig. 2. Numbers of *Trichoderma* spp. recovered during 1988 and 1989 field experiments. (○) = Untreated control; (☆) = 400 (1988) or 1×10^4 (1989) pellets per square meter, without bran; (□) = 1×10^3 (1988) or 2×10^4 (1989) pellets per square meter, without bran; (△) = 400 (1988) or 1×10^4 (1989) pellets per square meter, with bran; (◇) = 1×10^3 (1988) or 2×10^4 (1989) pellets per square meter, with bran.

RESULTS

Colonization of sclerotia under laboratory conditions. Almost invariably, sclerotia from which *Trichoderma* spp. grew did not produce *S. sclerotiorum* or other fungi. Analysis of variance indicated a significant ($P < 0.05$) main plot (week) effect but no treatment × week interaction ($P > 0.05$). Repetitions of the experiment did not differ significantly and there was no significant treatment × experiment interaction, so data for the two repetitions were combined. Significant ($P < 0.05$) single-factor effects were steaming of soil, temperature, matric potential, and ThzID1. The effect of bran was not significant ($P > 0.05$). Over all sample times, mean proportions of sclerotia colonized were higher in steamed soil, at 25 C, at −500 kPa, and with ThzID1 present. Mean percentages of sclerotia colonized by *Trichoderma* spp. over time are shown in Figure 1. Significant ($P < 0.05$) two-factor interactions were steaming × temperature, steaming × matric potential, steaming × ThzID1, temperature × matric potential, temperature × ThzID1, and matric potential × ThzID1.

Field experiments. Recovery of *Trichoderma* spp. in 1988 and 1989 field experiments for different treatments and sampling times is shown in Figure 2. For both 1988 and 1989, the main plot effect of sample time was significant, but there was no sample time × treatment interaction. Populations were higher in 1988 than in 1989 for all treatments and sampling times, averaging approximately 10^4 cfu/g of soil in 1988 compared with approximately 10–100 cfu/g of soil in 1989. There were no differences ($P > 0.05$) in populations of *Trichoderma* spp. among treatments in either year.

Percentages of recovered sclerotia that were colonized by *Trichoderma* spp. in 1988 and 1989 field experiments for different treatments and sampling times are shown in Figure 3. For both 1988 and 1989, the main plot effect of sample time was significant ($P < 0.05$), but there was no sample time × treatment interaction ($P > 0.05$). For 1988, there was no significant treatment effect on proportion of sclerotia colonized ($P > 0.05$). For 1989, both single factor effects of bran and density of ThzID1 were significant ($P < 0.05$). Over all sample times for 1989, mean proportions of sclerotia colonized were higher for pellets with bran than for pellets without bran and higher for treatments with greater pellet

density. The bran × density interaction was not significant ($P > 0.05$).

DISCUSSION

Knudsen and Bin (6) previously described effects of temperature, soil matric potential, and bran on radial growth and density of hyphae originating from alginate pellets containing *T. harzianum* strain ThzID1. Presumably, the proportion of randomly distributed sclerotia that can be colonized by hyphae originating from a pellet is a function of hyphal growth. In our laboratory experiments, after 1 wk of incubation, there was relatively little subsequent increase in proportions of sclerotia colonized. This result is consistent with our previous observations of hyphal growth in steamed soil (6). In those experiments, radial growth from alginate pellets was rapid for about 1 wk, then slowed and almost stopped by 2 wk, with a maximum radius of about 3 cm at 20–25 C and −500 kPa matric potential. Incorporation of wheat bran into pellets did not significantly affect radial growth rates, although it did increase hyphal density and sporulation within the growth radius (6). Radial growth of 3 cm would result in coverage of only 44% of the cross-sectional area in a 9-cm-diameter container, so that, on average, only 44% of randomly distributed sclerotia in such a container would be contacted by hyphae originating from a single pellet. This may help explain why we observed maximum colonization levels of about 40–56% in this study. Although this suggests a potential limitation on biocontrol efficacy in the field, it does not correct for possible continued hyphal growth from newly encountered and colonized substrates in field soil.

Temperature and matric potential both significantly affected colonization of sclerotia in laboratory studies. Colonization was greater at the higher temperature and in the drier soil. The effect of temperature is consistent with the reported growth optimum for *T. harzianum* in culture, which is above 30 C (3). Knudsen and Bin (6) observed significantly faster radial growth of ThzID1 in soil at 20 and 25 C than at 15 C. However, Knudsen and Bin (8) reported that soil moisture within the range of −30–−500 kPa did not affect radial growth rates, although hyphal density was significantly higher in drier soil. Because large numbers of hyphae originate from pellets in soil, it is probable that radial growth is the most important factor determining contact with "target" sclerotia. It is not known whether there is a threshold density of hyphae needed to colonize a sclerotium. Further investigation of temporal and spatial patterns of hyphal growth will be important for predicting the efficacy of biocontrol agents in the field.

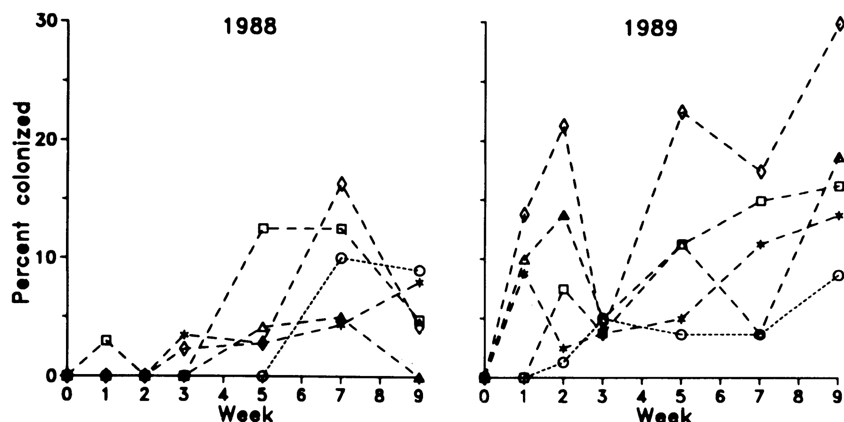

Fig. 3. Mean percentages of sclerotia of *Sclerotinia sclerotiorum* colonized by *Trichoderma* spp. in 1988 and 1989 field experiments. (○) = Untreated control; (☆) = 400 (1988) or 1 × 10^4 (1989) pellets per square meter, without bran; (□) = 1 × 10^3 (1988) or 2 × 10^4 (1989) pellets per square meter, without bran; (△) = 400 (1988) or 1 × 10^4 (1989) pellets per square meter, with bran; (◇) = 1 × 10^3 (1988) or 2 × 10^4 (1989) pellets per square meter, with bran.

In both laboratory and field studies, indigenous populations of *Trichoderma* spp. resulted in colonization of sclerotia. Even in steamed soil, either residual survival of *Trichoderma* spp. (or perhaps recolonization of soil by airborne spores) resulted in low levels of background colonization. The use of a marked strain of a biocontrol agent, such as a benomyl-resistant biotype (10), may be useful in the future to distinguish resident from additional *Trichoderma* spp. However, the stability of such markers in soil is not known.

In our field experiments, the addition of ThzID1 did not significantly increase numbers of propagules of *Trichoderma* spp. recovered over the course of the season in either 1988 or 1989. This suggests that we were mostly recovering the resident, background population of *Trichoderma* spp. However, in some instances, we did observe sporulation of ThzID1 from pellets in the field, especially from pellets with wheat bran incorporated.

The addition of ThzID1 increased colonization of sclerotia in the field in 1989. Although density of the biocontrol agent in the laboratory experiments (about 160 pellets per square meter) was considerably lower than in either field experiment, colonization of sclerotia was greater. Differences in environmental conditions between laboratory and field experiments may have been important. In 1988, soil moisture in the field varied from about −30 to −100 kPa for 2 days after planting and for 1–2 days after the two irrigations, but warm rainless weather resulted in soil matric potential values consistently drier than −1,500 kPa at all other sample times. We speculate that the soil was too dry to support hyphal growth. Relatively higher soil moisture levels during the first 2 wk of the 1989 field experiment may have contributed to the higher levels of colonization observed. The addition of bran to pellets increased colonization of sclerotia in the 1989 field experiment, in contrast with results observed in laboratory experiments. Although the role of bran is unclear, one speculation is that bran helps increase the absorption or retention of water by pellets in drier soils.

Colonization of sclerotia of *S. sclerotiorum* by *T. harzianum* reduces the pathogen's inoculum-producing ability. However, proliferation (increased populations) of ThzID1 in soil was not observed, so it is likely that colonization of sclerotia in the field resulted mainly from initial hyphal growth from pellets. This suggests that, at best, the biocontrol agent would need to be periodically replenished in order to be effective.

ACKNOWLEDGMENTS

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