

**Influence of an Antagonistic Strain of *Pseudomonas fluorescens* on Growth and Ability of *Trichoderma harzianum* to Colonize Sclerotia of *Sclerotinia sclerotiorum* in Soil**

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This research was supported in part by a research grant from the University of Idaho Research Council, and by the Idaho Agricultural Experiment Station, project IDA00941.

We thank S. Mannai and D. Muck for technical assistance. We also thank L. M. Dandurand, D. M. Weller, M. V. Wiese, and two anonymous reviewers for their helpful comments and suggestions.

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Accepted for publication 29 April (submitted for electronic processing).

**ABSTRACT**

Bin, L., Knudsen, G. R., and Eschen, D. J. 1991. Influence of an antagonistic strain of *Pseudomonas fluorescens* on growth and ability of *Trichoderma harzianum* to colonize sclerotia of *Sclerotinia sclerotiorum* in soil. *Phytopathology* 81:994-1000.

*Pseudomonas fluorescens* strain 2-79RN<sub>10</sub> (nalidixic acid and rifampicin-resistant mutant of wild type strain 2-79) was used to study potential effects of bacterial antagonism in soil on growth and biocontrol efficacy of the biocontrol fungus *Trichoderma harzianum* isolate ThzID1, which was formulated into alginate pellets. In steamed soil (25 C, -100 or -500 kPa matric potential), strain 2-79RN<sub>10</sub> maintained its initial high populations (approximately  $3 \times 10^4$  or  $3 \times 10^7$  cfu/g of soil) over a 14-day period, and significantly reduced hyphal radius, hyphal density, and recoverable numbers of propagules of ThzID1. In raw soil under similar environmental conditions (22-25 C, -10 to -1,000 kPa), populations of 2-79RN<sub>10</sub> decreased by approximately four log<sub>10</sub> units over a 3-wk period, and did not affect the ability of *Trichoderma* spp. to colonize sclerotia of *Sclerotinia sclerotiorum*. In two years of field experiments using raw or steamed soil in microplots, populations of 2-

79RN<sub>10</sub> decreased gradually after 1-2 wk and did not reduce the ability of *Trichoderma* spp. to colonize sclerotia of *S. sclerotiorum*. Colonization of sclerotia by *Trichoderma* spp. after 9 wk was significantly higher in steamed soil (mean = 65%) than in raw soil (mean = 30%) when ThzID1 was added, suggesting possible inhibition of ThzID1 by indigenous soil microbes, or utilization by ThzID1 of nutrients released by steaming of soil. In treatments where ThzID1 was not added, low levels of colonization of sclerotia were observed, apparently due to indigenous *Trichoderma* spp., and these levels were higher in raw soil (mean = 18%) than in steamed soil (mean = 5%). These results suggest that under certain restrictive conditions, high population levels of antagonistic bacteria in bulk soil suppressed a fungal biocontrol agent, but that this suppressive effect was reduced or eliminated when a high bacterial population was not present.

Isolates of *Trichoderma harzianum* Rifai have been reported as antagonists (including mycoparasites) of mycelia or sclerotia of several soilborne plant pathogens (1,2,5,9,16,19,23,27). *T. harzianum* formulated in alginate pellets (16) colonized sclerotia of *Sclerotinia sclerotiorum* (Lib.) de Bary, a widely distributed and destructive plant pathogen, under laboratory and field conditions (14).

The use of mycoparasitic fungi to attack sclerotia, thus reducing pathogen inoculum in soil, is a potential means of biocontrol. Biocontrol efficacy is likely to increase with increasing growth of the biocontrol agent, suggesting that quantitative studies of abiotic and biotic factors affecting growth and proliferation of biocontrol agents in soil are necessary. Abiotic factors affecting growth or efficacy of biocontrol fungi such as *Talaromyces flavus*, *Trichoderma harzianum*, and *Gliocladium* spp. have been studied (3,4,7,18,22). Effects of soil matric potential and temperature on hyphal growth of the biocontrol agent *T. harzianum* isolate ThzID1 were reported by Knudsen and Bin (13); radial growth rate increased with temperature within the range of 15-25 C, but the effects of matric potential (-30 to -500 kPa) and added

bran were not significant. However, addition of bran increased the density of hyphal growth (13).

Significant biotic factors may include soil bacteria or fungi that are antagonistic to biocontrol fungi. Some strains of fluorescent pseudomonads inhibit many soil fungi, including *Trichoderma* spp., due to the production of siderophores or antibiotic compounds (10,17). One strain of *Pseudomonas fluorescens* Migula, designated 2-79, has received considerable attention as a potential biocontrol agent against *Gaeumannomyces graminis* var. *tritici* (24-26). Strain 2-79 produced the pigmented antibiotic phenazine-1-carboxylic acid (PCA), and production of PCA was demonstrated on roots of wheat colonized by the bacterium and grown in steamed and natural soils (8,24). Because of the extensive characterization of strain 2-79, it represents an appropriate choice as a model to investigate possible interactions with a fungal biocontrol agent.

Depending on the method of application of a biocontrol fungus, and its target plant pathogen, effects of antagonistic microbes may be relatively greater in the rhizosphere than in bulk soil. In the case of ThzID1 applied in pellet form to attack sclerotia of *S. sclerotiorum*, biocontrol activity and interactions with other soil microbes will take place primarily outside the rhizosphere.

The purpose of this study was to quantify effects of a known microbial antagonist, *P. fluorescens* strain 2-79RN<sub>10</sub>, on a biocontrol isolate of *T. harzianum*, under a range of conditions in steamed and natural soil.

## MATERIALS AND METHODS

**Experimental organisms.** *Trichoderma harzianum* isolate ThzID1 was recovered from field soil near Moscow, Idaho. ThzID1 was maintained on Difco potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) at 4 C. Alginate granules (pellets) that contained hyphal biomass of ThzID1 plus wheat bran were formulated as described previously (13,14). Pellets were air dried overnight on waxed paper, and stored at room temperature in covered glass beakers. Pellets used in all experiments were 1–3 mo old.

*P. fluorescens* strain 2-79RN<sub>10</sub> was provided by D. M. Weller, USDA-ARS, Pullman, Washington. Strain 2-79RN<sub>10</sub> is a spontaneous mutant of strain 2-79 (NRRL B-15132), resistant to the antibiotics rifampicin and nalidixic acid (25). Strain 2-79RN<sub>10</sub> was maintained at 4 C on King's medium B (11) agar with rifampicin and nalidixic acid each at 100 µg/ml (KMB-RN). Bacterial suspensions for use in the following experiments were prepared as follows. Cells of 2-79RN<sub>10</sub> from agar culture were inoculated into 500 ml of KMB-RN broth and incubated at 22 C for 48 h on a rotary shaker. The culture was centrifuged at 2,500 g for 5 min, the supernatant was discarded, and the precipitate was resuspended in 500 ml of phosphate buffer (1.2 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.2). The suspension was centrifuged again for 5 min, and the precipitate was finally suspended in phosphate buffer. Bacterial concentrations for experiments were estimated by using spectrophotometer readings at A<sub>640 nm</sub> compared to a standard curve. Bacteria were added to soil by hand mixing appropriate dilutions with soil to obtain appropriate population densities and matric potential levels.

*S. sclerotiorum* was isolated from the stem of a diseased lentil (*Lens culinaris* Medik.) plant. The fungus was routinely cultured on PDA. Mycelial disks were transferred to sterilized sliced carrots contained in 1-L Erlenmeyer flasks. After approximately 4 wk incubation at 22 C, sclerotia were harvested, rinsed with water, air dried, and stored at 22 C for 2–8 wk before use.

**Inhibition of ThzID1 by 2-79RN<sub>10</sub> in vitro.** A variation of the methods described by Weller et al (26) was used to test the ability of 2-79RN<sub>10</sub> to inhibit growth of ThzID1 on agar. Log-phase cells of 2-79RN<sub>10</sub> were spotted twice at 5-mm distances from opposite edges of Petri dishes that contained either KMB agar or PDA. The bacteria were allowed to grow for 48 h at 24 C. Control plates had no bacteria added. A 6-mm-diameter plug from the outer margin of a 5-day-old dilute PDA culture of ThzID1 was placed in the center of each agar plate. After 5 days at 20 C, zones of inhibition of fungal growth were measured. The zone of inhibition was defined as the distance between the leading edge of the bacterial colony and the nearest edge of the fungal colony. The experiment was replicated five times, and mean widths of inhibition zones were determined. The experiment was repeated once.

**Soil.** Palouse-Latahco silt loam soil was obtained from the University of Idaho, Parker Farm, at Moscow. Soil analysis results (University of Idaho Analytical Services Laboratory) indicated that the soil contained 20% sand, 20% clay, and 60% silt by weight, with 82.2 µg/g of plant-available iron. Soil pH in soil/water (2:1) solution was approximately 5.9. For all experiments, soil was sieved (5-mm mesh) before use.

**Effect of 2-79RN<sub>10</sub> on radial growth, hyphal density, and populations of ThzID1 in steamed soil.** Soil was steamed in an autoclave at 100–110 C for 1 h as described previously (13). Either sterile phosphate buffer or the bacterial suspension was hand mixed with 120-g amounts of soil to obtain matric potential levels (determined from a standard curve) of –100 or –500 kPa, and bacterial population levels of 0, or approximately 3 × 10<sup>4</sup> or 3 × 10<sup>7</sup> cfu/g. Glass Petri dishes (15 cm diameter) were half-filled with approximately 60 g of the soil preparation. A single

pellet was placed on the soil surface in the center of the dish. The pellet was overlaid with two layers of nylon mesh (1-mm<sup>2</sup> mesh), and then covered by the remainder of the soil preparation. Soil in the Petri dish was compressed to a bulk density of approximately 1.2 g/cm<sup>3</sup>. The Petri dishes were placed in a plastic bag with a wet paper towel to maintain high humidity, and were incubated at 25 C in the dark for either 7 or 14 days. The experimental variables were: soil matric potential (–100 or –500 kPa), initial population density of 2-79RN<sub>10</sub> (0, 3 × 10<sup>4</sup>, or 3 × 10<sup>7</sup> cfu/g of soil), and incubation time (7 or 14 days). The experiment was performed initially with three replicates per treatment, and the experiment was repeated once.

Radial growth and density of hyphae of ThzID1, and populations of ThzID1 and 2-79RN<sub>10</sub> in soil, were measured initially (within 2 h of preparation) and after 7 and 14 days. At each sample time, the upper layer of nylon mesh (with the soil above it) was removed from sampled Petri dishes and saved. The lower layer of mesh served as a reference scale for measuring radial growth and density of hyphae in the observed plane and to a depth of 1 mm (13). The radial growth pattern of the colony that originated from each pellet was observed at ×40 with a stereomicroscope and mapped on graph paper. Maps were then digitized (Jandel Scientific, Corte Madera, CA) to estimate the area of each map, and mean colony radii were estimated from area values. Hyphal density was estimated at 1-mm increments in each of four directions from the pellet by using a visual assessment key generated by a computer simulation (13). Hyphal densities in each direction were averaged for each successive 1-mm distance from the pellet, then the total visible density of hyphae (mm) was estimated by calculating the volume under the surface that would be generated by rotating, in the visible plane, mean density values at each distance about the pellet center.

At each sample time, recoverable populations of 2-79RN<sub>10</sub> and ThzID1 were determined as follows. The soil from both upper and lower layers in each sampled Petri dish was thoroughly mixed in a plastic bag, then a 1-g sample was randomly taken and suspended in 9 ml of sterile phosphate buffer. Serial dilutions were made, and aliquots (100 µl) were spread on duplicate plates of KMB-RN for 2-79RN<sub>10</sub>, and modified TME agar (18) (per liter: 1.0 g of glucose, 20 g of agar, 200 ml of V8 juice, 0.1 g of neomycin, 0.1 g of bacitracin, 0.1 g of penicillin, 0.025 g of chlortetracycline, 0.02 g of nystatin, and 0.5 g of propionic acid, 800 ml of water) to isolate ThzID1. Plates were incubated at 25 C, and colonies of 2-79RN<sub>10</sub> and ThzID1 were counted after 3 days and 7–10 days, respectively.

The experiment was analyzed as a split-plot design with sample time as a main plot effect (20,21) and radial growth and density of hyphae and populations of 2-79RN<sub>10</sub> and ThzID1 as dependent variables.

**Effect of 2-79RN<sub>10</sub> on colonization of sclerotia by ThzID1 in raw soil under controlled environmental conditions.** Raw field soil (360-g amounts), as described, was mixed with phosphate buffer or a suspension of 2-79RN<sub>10</sub> to give matric potential levels of –50 or –100 kPa and bacterial population levels of 0, or approximately 3 × 10<sup>4</sup> or 3 × 10<sup>7</sup> cfu/g. Ten-centimeter-diameter plastic pots were filled with soil to within 5 cm of their tops. One pellet of ThzID1 was placed in the center and 10 sclerotia of *S. sclerotiorum* and three pea (*Pisum sativum* L.) seeds were randomly scattered on the soil surface, and the appropriate soil mixture was added to a depth of 3 cm. Pots were placed on a laboratory bench under fluorescent light with a photoperiod of 12 h/12 h (light/dark). Soil temperature and matric potential were estimated daily using soil temperature probes and gypsum soil moisture blocks, respectively, placed in three unsampled pots. Sensors were connected to a datalogger (Campbell Scientific Co., Logan, Utah), and soil moisture in all pots was maintained in the approximate range of either –10 to –100 kPa, or –100 to –1,000 kPa, by adding small amounts of water according to values indicated by the datalogger, allowing approximately 2 h for equilibration each time. Soil temperature remained between 22 and 25 C over the course of the experiment.

Initial population levels of 2-79RN<sub>10</sub> were estimated through

soil dilution plating as described. Subsequent population estimates and estimates of the proportion of sclerotia colonized by *Trichoderma* spp. were obtained after 7, 14, and 28 days. At each sampling time, all of the soil in each sampled pot was placed in a plastic bag and mixed thoroughly. Then, a 1-g sample was randomly taken and suspended in 9 ml of phosphate buffer. Serial dilutions were plated on KMB-RN agar. Colonies were counted after 3 days incubation at 25 C.

After removing soil samples for population counts, the remaining soil was washed through a 2-mm screen to recover the sclerotia. Sclerotia were surface disinfested (10 min in a solution of 10% ethanol, 0.05% sodium hypochlorite, 80% distilled water), rinsed in sterile distilled water, and dried on paper towels. Sclerotia were placed on PDA plates and incubated at 25 C for 1 wk. Colonized sclerotia were identified by the colonies of *Trichoderma* spp. growing from them. *Trichoderma* spp. were identified by a combination of colony color and morphology of conidia and conidiophores, however colonies were not identified to species.

Experimental variables were population of 2-79RN<sub>10</sub> (three levels), and soil matric potential (two levels). Treatments were arranged in a balanced split-plot design, with time as a main effect and with five replicates. The experiment was repeated once.

**Effect of 2-79RN<sub>10</sub> on colonization of sclerotia by *Trichoderma* spp. in field microplots.** Field trials were initiated on 1 June 1989 and 20 May 1990, at the University of Idaho Parker Farm. Each microplot was made by burying a plastic bucket (25 cm diameter × 30 cm deep) to its rim in a hole dug by a tractor-mounted posthole digger. Buckets each had four 3-cm<sup>2</sup> drainage holes on the sides near the bottom. Microplots were separated from one another by approximately 0.75 m of turf. Each bucket was filled to within 5 cm of the top with sieved (5-mm mesh) Palouse silt loam, and 12 pea seeds were placed on the soil surface in each pot.

Sclerotia and ThzID1 (as appropriate) were mixed with raw or steamed soil (500 g) and added to the pots. Depth of the added soil was approximately 4 cm. Finally, to each bucket was added either 80 ml of phosphate buffer or 80 ml of bacterial suspension (10<sup>8</sup> cfu/ml in 1989, 10<sup>10</sup> cfu/ml in 1990), as appropriate, by pouring the liquid over the soil surface. Initial concentration of 2-79RN<sub>10</sub> in the upper 4 cm of soil, in treatments where 2-79RN<sub>10</sub> was added, was approximately 3 × 10<sup>4</sup> cfu/g of soil in 1989, and between 10<sup>5</sup> and 10<sup>7</sup> cfu/g of soil in 1990.

The experimental design consisted of the following eight treatments in a randomized block design with four replicates per treatment: 1) sclerotia of *S. sclerotiorum* (Ss) in raw soil; 2) Ss + ThzID1 (pellets with bran incorporated, 1 × 10<sup>4</sup> pellets per square meter [= 500 per microplot or 500 kg/ha]) in raw soil; 3) Ss + 2-79RN<sub>10</sub> in raw soil; 4) Ss + ThzID1 + 2-79RN<sub>10</sub> in raw soil; 5) Ss in steamed soil; 6) Ss + ThzID1 in steamed soil; 7) Ss + 2-79RN<sub>10</sub> in steamed soil; and 8) Ss + ThzID1 + 2-79RN<sub>10</sub> in steamed soil.

Gypsum blocks and temperature sensors connected to a datalogger were buried 4 cm deep in the centers of three microplots to monitor soil moisture and temperature. Over the first 2 wk of the experiment, plots were watered to maintain soil matric potential at approximately -50 kPa. Relatively uniform soil moisture was obtained by timing the duration of watering for each microplot. After 2 wk, plots were no longer watered.

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. Microplots were sampled after 1, 2, 3, 5, 7, and 9 wk, by removing soil to a depth of 4 cm and placing it in a plastic bag. Subsamples (approximately 5 g) were taken, diluted serially in sterile water, and plated on TME and KMB agar to estimate soil population levels of *Trichoderma* spp. and 2-79RN<sub>10</sub> as described. The remaining soil in each sample was washed over a 1.4-mm mesh screen, and 20 sclerotia per microplot were selected from the remaining debris. Sclerotia were surface-disinfested and placed on PDA, and the number colonized by *Trichoderma* spp. was determined after a 2-wk incubation period.

Proportion values were arc-sine transformed (21), and the experiment was analyzed as a split-plot design with sample time as a main plot effect (20,21). Results from treatments 1 and 2 (raw soil, without 2-79RN<sub>10</sub> added), from 1989 only, have previously been reported (14).

Although apothecial production from sclerotia was observed in both years, weather conditions were not conducive to disease (*Sclerotinia* white mold) development, thus disease was not rated in either 1989 or 1990.

## RESULTS

**In vitro assay for inhibition of ThzID1 by 2-79RN<sub>10</sub>.** Strain 2-79RN<sub>10</sub> inhibited the growth of ThzID1 on both PDA and KMB agar. On control plates of either medium, ThzID1 colonies grew to the edge of the plates. On PDA with 2-79RN<sub>10</sub>, the mean zone of inhibition was 3 mm. On KMB agar with 2-79RN<sub>10</sub>, the mean zone of inhibition was 6 mm.

**Effect of 2-79RN<sub>10</sub> on hyphal growth and density of ThzID1 in steamed soil.** Populations of 2-79RN<sub>10</sub> remained relatively unchanged from initial levels (either approximately 10<sup>4</sup> or 10<sup>7</sup>

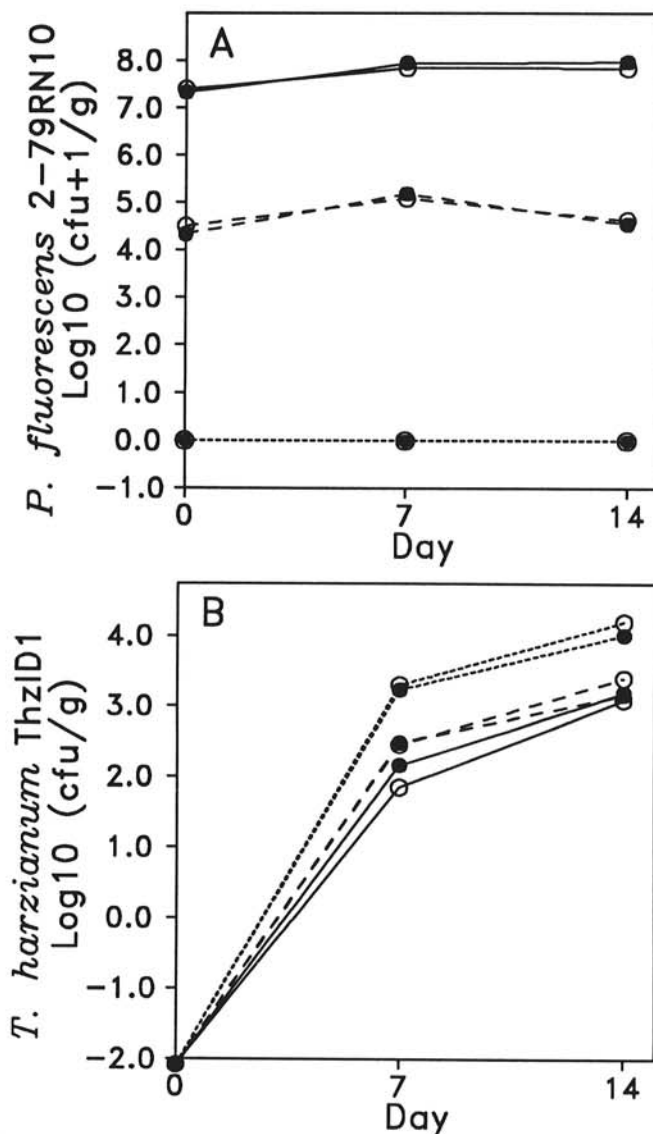


Fig. 1. Populations of (A) *Pseudomonas fluorescens* strain 2-79RN<sub>10</sub> and (B) *Trichoderma harzianum* isolate ThzID1 in steamed soil, in Petri dishes, over 14 days. Strain 2-79RN<sub>10</sub> was applied to soil at initial levels of 0 (dotted line), 3 × 10<sup>4</sup> (dashed line), or 3 × 10<sup>7</sup> (line) cfu/g of soil. Isolate ThzID1 was applied in a single alginate + bran pellet. Soil matric potential was either -100 kPa (○) or -500 kPa (●). Means of five replicates per treatment are shown.



cfu/g) over the 14-day period (Fig. 1). Recoverable numbers of ThzID1 increased over the same period in all treatments (Fig. 1). Treatments with 2-79RN<sub>10</sub> resulted in significantly lower numbers of ThzID1 compared to controls ( $P < 0.01$ ), but differences between bacterial concentrations of  $10^5$  and  $10^8$  cfu/g were not significant ( $P > 0.05$ ). Mean log<sub>10</sub> colony-forming units of ThzID1 per gram of soil, averaged between 7 and 14, was 3.7 for treatments without 2-79RN<sub>10</sub> compared to 2.8 for treatments with 2-79RN<sub>10</sub>. Neither the effect of matric potential nor the interaction between 2-79RN<sub>10</sub> and matric potential was significant ( $P > 0.10$ ) for ThzID1 numbers, colony radius, or hyphal density. Time  $\times$  treatment interactions also were not significant ( $P > 0.05$ ).

Addition of 2-79RN<sub>10</sub> at either  $3 \times 10^4$  or  $3 \times 10^7$  cfu/g significantly reduced radial growth of hyphae ( $P < 0.01$ ) and

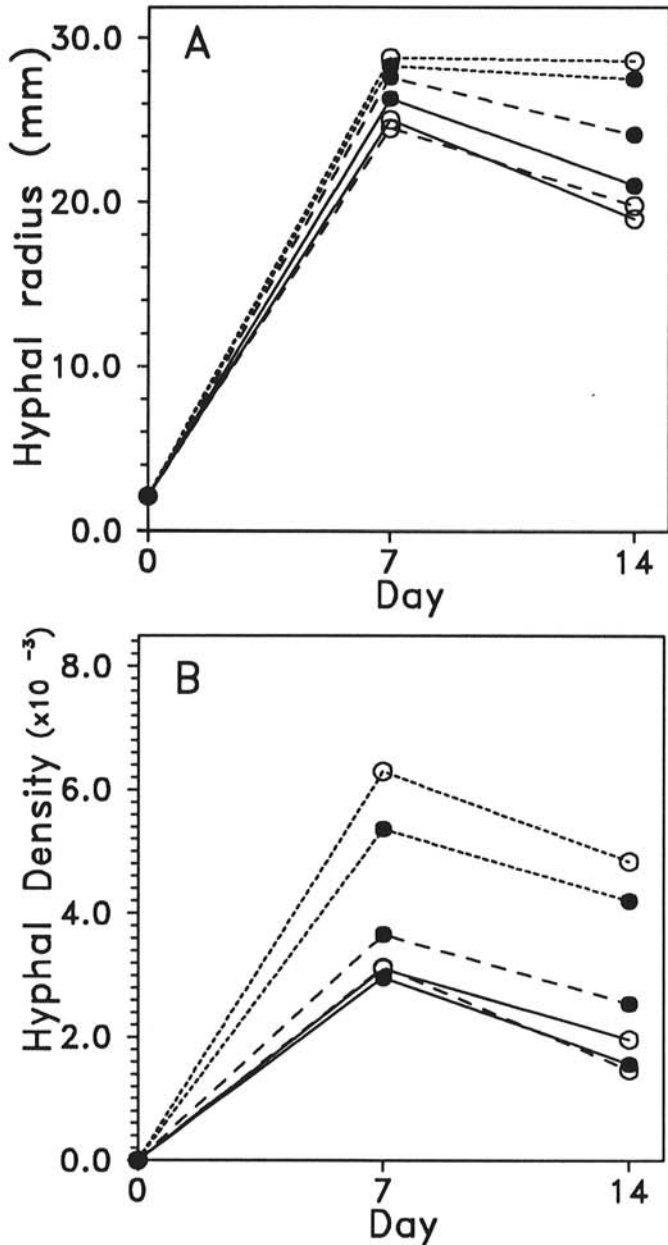


Fig. 2. Radial growth (A) and density of hyphae (B) of *Trichoderma harzianum* isolate ThzID1 in steamed soil, in Petri dishes, over 14 days. Hyphal density was estimated as total visible length (mm) of hyphae in a cross-sectional field of view, to a depth of 1 mm (see text). *Pseudomonas fluorescens* strain 2-79RN<sub>10</sub> was applied to soil at initial levels of 0 (dotted line),  $3 \times 10^4$  (dashed line), or  $3 \times 10^7$  (line) cfu/g of soil. Isolate ThzID1 was applied in a single alginate + bran pellet. Soil matric potential was either  $-100$  kPa (○) or  $-500$  kPa (●). Means of five replicates per treatment are shown.

hyphal density ( $P < 0.01$ ). Differences between the bacterial concentrations of  $3 \times 10^4$  and  $3 \times 10^7$  cfu/g were not significant ( $P > 0.05$ ). Mean colony radius averaged between days 7 and 14 was 28.3 mm in the absence, compared to 23.4 mm in the presence of 2-79RN<sub>10</sub> (Fig. 2), and mean hyphal density was  $5.6 \times 10^3$  mm in the visible plane without 2-79RN<sub>10</sub> compared to  $3.0 \times 10^3$  mm with 2-79RN<sub>10</sub> present (Fig. 2).

**Effect of 2-79RN<sub>10</sub> on colonization of sclerotia by ThzID1 in raw soil under controlled environmental conditions.** In contrast to results observed from steamed soil, populations of 2-79RN<sub>10</sub> in raw soil declined by approximately four log<sub>10</sub> units over the 4-wk course of the experiment (Fig. 3). Also, in contrast to results from steamed soil, recoverable numbers of *Trichoderma* spp. in all treatments remained near the initial, background level of approximately 100 cfu/g of soil, over 4 wk (Fig. 3). We were unable to distinguish between introduced and indigenous *Trichoderma* spp. Neither matric potential nor 2-79RN<sub>10</sub> had a significant effect on numbers of *Trichoderma* spp. recovered ( $P > 0.10$ ).

Analysis of variance indicated a significant ( $P < 0.05$ ) main plot (sample time) effect on colonization of sclerotia by

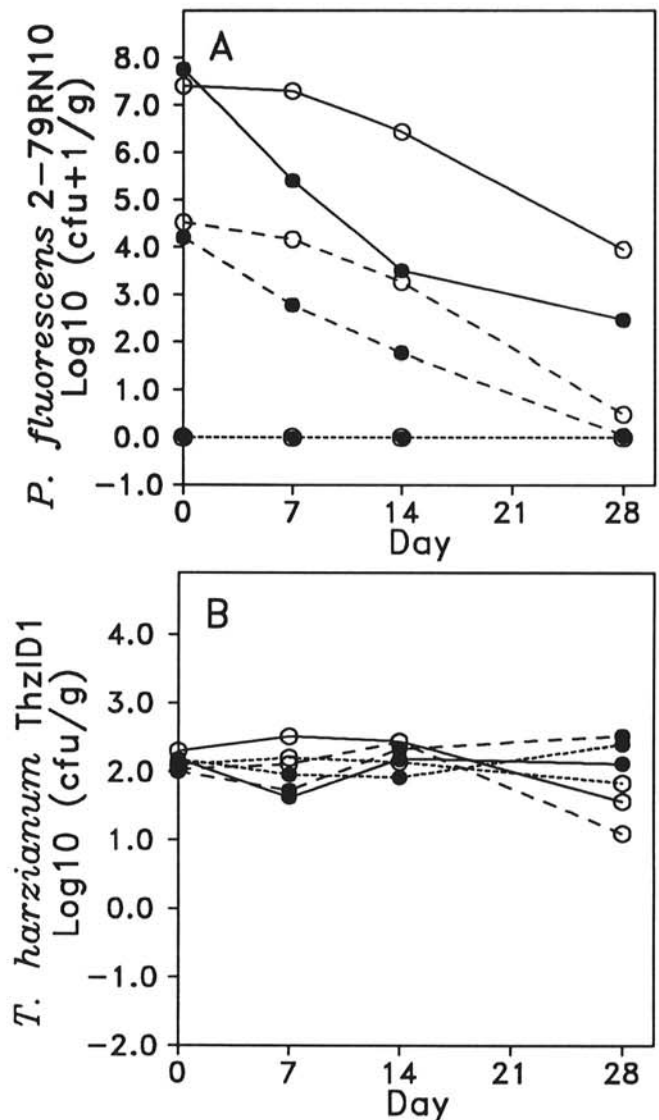


Fig. 3. Populations of (A) *Pseudomonas fluorescens* strain 2-79RN<sub>10</sub> and (B) *Trichoderma harzianum* isolate ThzID1 in raw soil in 10-cm pots, after 7, 14, and 28 days. Strain 2-79RN<sub>10</sub> was applied to soil at initial levels of 0 (dotted line),  $3 \times 10^4$  (dashed line), or  $3 \times 10^7$  (line) cfu/g of soil. Isolate ThzID1 was applied in a single alginate + bran pellet. Soil matric potential was either  $-100$  kPa (○) or  $-500$  kPa (●). Means of eight replicates per treatment are shown.

*Trichoderma* spp., but no treatment  $\times$  sample time interaction ( $P > 0.05$ ). Repetitions of the experiment did not differ significantly, and there was no significant treatment by experiment interaction, so data for the two repetitions were combined. The percentage of sclerotia colonized by *Trichoderma* spp. increased over the 4-wk period in all treatments (Fig. 4) and was not significantly affected by the presence of 2-79RN<sub>10</sub> ( $P > 0.10$ ). Mean percentage of colonization was significantly greater in the wetter soil (38%) than in the drier soil (30%) ( $P < 0.05$ ).

**Effect of 2-79RN<sub>10</sub> on colonization of sclerotia by *Trichoderma* spp. in field microplots.** In 1989, soil moisture levels fell below -1,500 kPa matric potential after approximately 2 wk, and remained there for the remainder of the season. In 1990, soil moisture remained above 100 kPa for almost five weeks before falling below 1,500 kPa. One subsequent rain event in 1990 decreased soil matric potential to about -100 kPa for a 4-day period during the sixth week of the experiment. Populations of 2-79RN<sub>10</sub> declined over the season in both years of the experiment. Although for the first week or two each year, populations remained approximately stable or even increased slightly (Fig. 5). Average rates of decline over the season were similar for both years (Fig. 5).

Numbers of *Trichoderma* spp. recovered were variable and generally low (mean =  $1.5 \times 10^2$  cfu/g) in all treatments at all sample times. None of the experimental variables (1989 vs. 1990, sample time, steamed vs. raw soil, 2-79NR, or addition of ThzIDI pellets) had a significant effect on numbers of *Trichoderma* spp. ( $P > 0.10$ ).

The percentage of sclerotia colonized by *Trichoderma* spp. generally increased over time in both years (Fig. 6). Even in treatments to which ThzIDI was not added, some colonization of sclerotia by *Trichoderma* spp. was observed. Analysis of variance indicated a significant ( $P < 0.05$ ) main plot (sample time) effect on colonization of sclerotia each year, but no treatment  $\times$  sample time interaction ( $P > 0.05$ ). Although mean percentage of colonization was significantly higher in 1990 than in 1989 ( $P < 0.05$ ), there was no treatment  $\times$  year interaction ( $P < 0.05$ ). Addition of pellets of ThzIDI significantly increased colonization of sclerotia (mean = 38% vs. 10%, over all treatments and sample

times) ( $P < 0.05$ ). Over both years, the effect of 2-79RN<sub>10</sub> on colonization of sclerotia by *Trichoderma* was not significant ( $P > 0.05$ ). However, when the two years were analyzed independently, addition of 2-79RN<sub>10</sub> resulted in a slight but significant ( $P < 0.05$ ) increase in the percentage of sclerotia colonized in 1989.

The effect of soil treatment (steamed vs. raw soil) was also significant ( $P < 0.05$ ), with average colonization of sclerotia, over all treatments and sample times, higher in steamed soil (16%) than in raw soil (12%). The interaction between soil treatment and 2-79RN<sub>10</sub> was not significant ( $P > 0.05$ ), but the interaction between soil treatment and ThzIDI was significant ( $P < 0.05$ ). When ThzIDI was added, the percentage of colonization was higher in steamed soil than in raw soil (65% vs. 30% after 9 wk), but when ThzIDI was not added, the percentage of colonization was higher in raw soil than in steamed soil (18% vs. 5% after 9 wk) (Fig. 6).

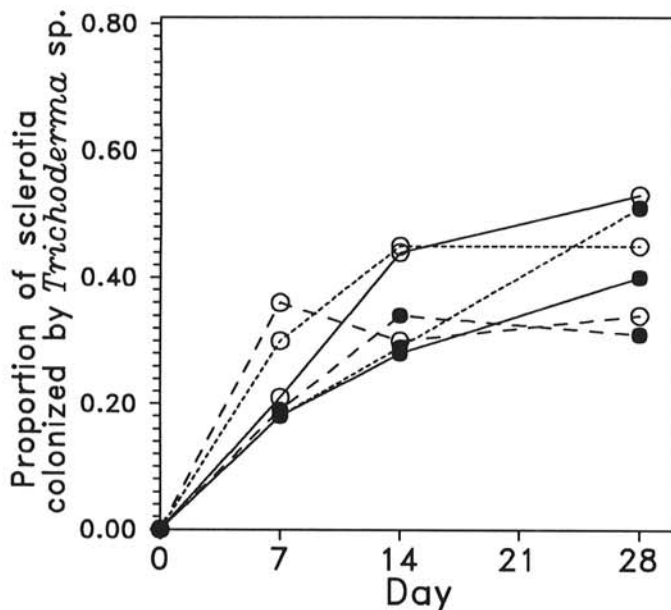


Fig. 4. Proportion of sclerotia of *Sclerotinia sclerotiorum* colonized by *Trichoderma* spp. in raw soil in 10-cm pots, after 7, 14, and 28 days. *Pseudomonas fluorescens* strain 2-79RN<sub>10</sub> was applied to soil at initial levels of 0 (dotted line),  $10^4$  (dashed line), or  $10^7$  (line) cfu/g of soil. *T. harzianum* isolate ThzIDI was applied in a single alginate + bran pellet. Soil matric potential was maintained within a range of either -10 to -100 kPa (○), or -100 to -1,000 kPa (●). Means of eight replicates per treatment are shown.

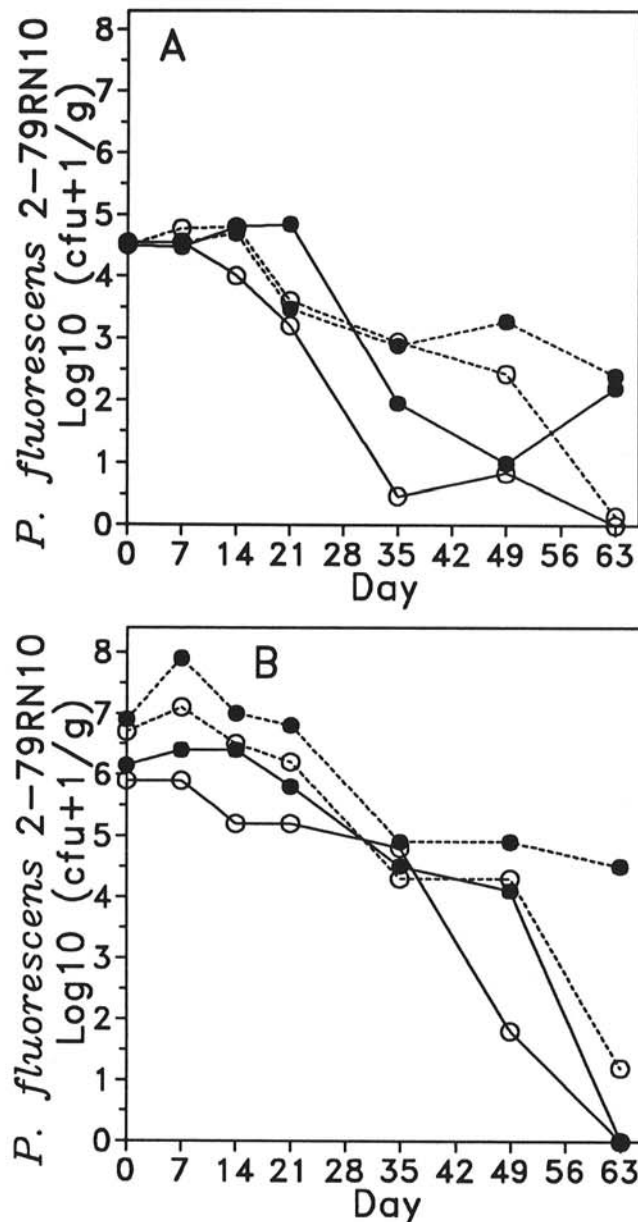


Fig. 5. Populations of *Pseudomonas fluorescens* strain 2-79RN<sub>10</sub> recovered from field microplots in (A) 1989 and (B) 1990. Strain 2-79RN<sub>10</sub> was applied to soil at initial levels of approximately  $5 \times 10^4$  (1989) or  $5 \times 10^6$  (1990) cfu/g of soil. Soil was initially steamed (dotted line) or raw (line). *Trichoderma harzianum* isolate ThzIDI was applied in alginate + bran pellets at densities of either 0 (○) or approximately 1 pellet per gram (●) of soil. Means of four replicates per treatment are shown for each year.

## DISCUSSION

Fravel (6) discussed the possibility of deleterious effects of antibiotic and antibioticlike compounds, produced by biocontrol agents, on beneficial microorganisms. Our results suggest minimal effects on the beneficial ThzID1 from the presence of the known antagonist 2-79RN<sub>10</sub> in bulk soil. In vitro, *P. fluorescens* strain 2-79RN<sub>10</sub> inhibited radial growth of ThzID1, on both PDA and KMB agar. Inhibition on PDA, a relatively high iron medium, suggests that production of PCA may have been a mechanism of inhibition (26), although zones of inhibition were narrower than observed on KMB. Antibiosis has been shown to be an important mechanism of antagonism by 2-79RN<sub>10</sub> against other fungal species (8,24).

By using steamed soil in the first laboratory experiment, we attempted to eliminate the possibility of significant direct effects of microbes other than the added 2-79RN<sub>10</sub>. The lowest level

of added 2-79RN<sub>10</sub>,  $3 \times 10^4$  cfu/g of soil, was sufficient to reduce growth and proliferation of ThzID1. Strain 2-79RN<sub>10</sub> was able to maintain high populations in the soil over the 14-day period of the experiment, perhaps due in part to nutrients made available when the soil was steamed. Although the reduction in growth and proliferation of ThzID1 in the presence of relatively high populations of 2-79RN<sub>10</sub> was significant, it is questionable whether this reduction would significantly reduce potential biocontrol efficacy of the fungus. Large numbers of hyphae originate from pellets in soil, and it is probable that radial growth is the most important factor determining contact with "target" sclerotia (13-15). Relatively high numbers of 2-79RN<sub>10</sub> caused only a modest reduction (about 17%) in average radial growth after 7-14 days.

Using raw soil in the laboratory, we tested for possible interactions between ThzID1 and Pf2-79 in a relatively controlled physical environment, but with other soil microbes present. In this system, the level of precision in estimating fungal growth parameters was necessarily reduced. Because we cannot differentiate hyphae from different sources in raw soil, monitoring colonization of target sclerotia was an indirect assay of the biocontrol agent's growth ability in raw soil (15). In this and a previous study (14), both in the laboratory and the field, we have observed low levels of colonization of sclerotia by indigenous *Trichoderma* spp., which are not easily distinguished from the added ThzID1. However, this "background" level of colonization was consistently small compared to that of added ThzID1 (14).

Compared with the results from steamed soil, the antagonistic effect of 2-79RN<sub>10</sub> on ThzID1 was not apparent in raw soil under controlled environmental conditions. Populations of 2-79RN<sub>10</sub> in bulk soil samples decreased over time in all treatments, which may partially explain the apparent lack of inhibition of sclerotial colonization. Whether the decline in populations of 2-79RN<sub>10</sub> was due to physical environment (somewhat drier soil than in the previous experiment) or nutritional inadequacy is not known, but poor conditions for bacterial growth are fundamental constraints to antibiotic production in soil (24). Even when high populations ( $>10^6$  cfu/g) of 2-79RN<sub>10</sub> were present for at least 14 days, inhibition of ThzID1 was not apparent (Fig. 4). However, it is quite possible that localized high populations of bacteria would be present around germinating seeds, plant roots, or exudate-producing sclerotia. Also, the physiological status of antagonistic bacteria may be equally important as their actual numbers (12).

As in the controlled environment experiment, numbers of *Trichoderma* spp. recovered from field samples did not increase over time, despite the general increase in colonization of sclerotia. It is likely that colonization of sclerotia in the field resulted mainly from initial hyphal growth from pellets (14). Again, an overall decline in recoverable numbers of 2-79RN<sub>10</sub> over the season was observed, and 2-79RN<sub>10</sub> did not reduce colonization of sclerotia. The higher level of sclerotial colonization in steamed soil suggests that steaming may have reduced populations of other competitive or antagonistic microflora. However, it is also possible that ThzID1 was able to utilize nutrients released during steaming of soil.

Although we observed little or no effect of 2-79RN<sub>10</sub> in bulk soil on colonization of sclerotia by ThzID1, it is quite possible that the situation would be different if both organisms were present in the rhizosphere, where conditions may be favorable for growth and antibiotic production by 2-79RN<sub>10</sub> (10,24,25). This is a logical area for future investigation.

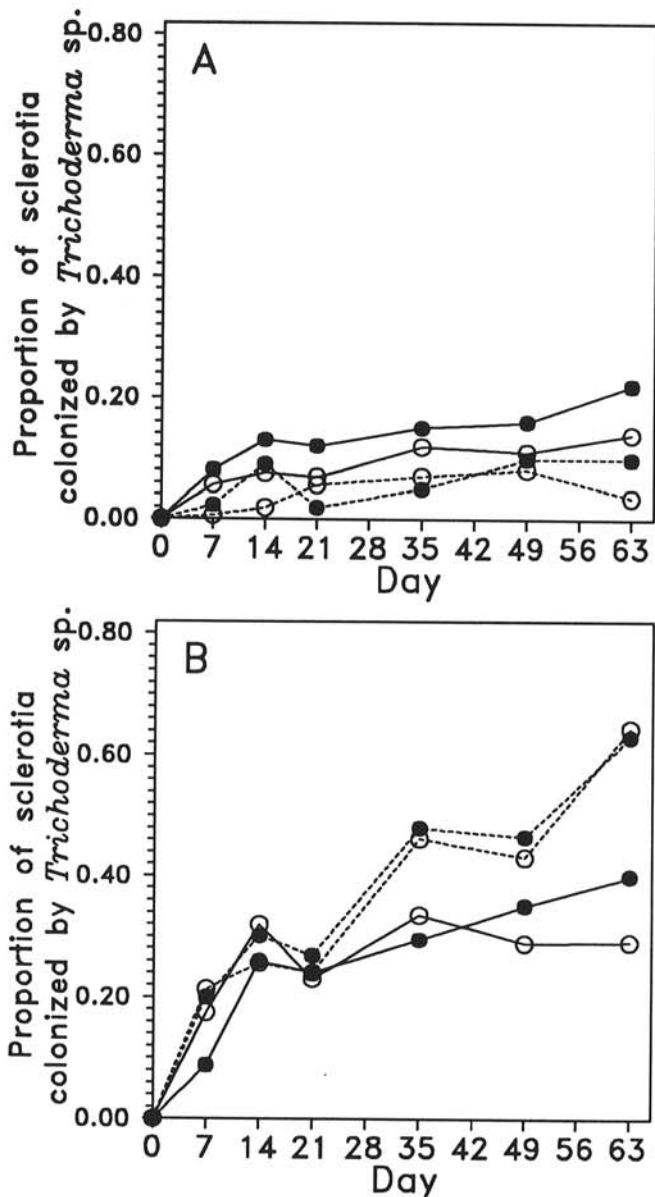


Fig. 6. Proportion of sclerotia of *Sclerotinia sclerotiorum* colonized by *Trichoderma* in field microplots, 1989 and 1990 combined. Soil was initially steamed (dotted line) or raw (line), and *T. harzianum* isolate ThzID1 was applied in alginate + bran pellets at densities of (A) 0 pellets per gram of soil or (B) approximately 1 pellet per gram of soil. *Pseudomonas fluorescens* strain 2-79RN<sub>10</sub> was applied to soil at initial levels of either 0 cfu/g of soil (○), or between  $5 \times 10^4$  and  $5 \times 10^6$  cfu/g of soil (●). Means of eight replicates (two years combined) per treatment are shown.

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