Influence of *Pseudomonas fluorescens* on Hyphal Growth and Biocontrol Activity of *Trichoderma harzianum* in the Spermosphere and Rhizosphere of Pea

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Published as Idaho Agricultural Experiment Station Paper 92762.
This research was supported in part by USDA-CSRS agreement 91-34239-6532 (Special Project, Cool Season Food Legumes) and by IAES project IDA00941.
We thank D. Eschen, S. Mannai, P. Sudarshana, and E. Thiel for technical assistance.
Accepted for publication 12 November 1992.

ABSTRACT

Dandurand, L. M., and Knudsen, G. R. 1993. Influence of *Pseudomonas fluorescens* on hyphal growth and biocontrol activity of *Trichoderma harzianum* in the spermosphere and rhizosphere of pea. Phytopathology 83:265-270.

Trichoderma harzianum isolate ThzID1 was grown in liquid culture, was formulated with alginate and polyethylene glycol 8000, and was milled into fine granules (average diameter 500 μm). Granules contained chlamydospores, conidia, and hyphal fragments. Viability of the encapsulated fungus remained high for at least 6 mo when stored at 5 C (i.e., >90% of the granules produced hyphal growth when incubated on agar); viability was reduced significantly when granules were stored at 22 C. Application of the granular formulation of *T. harzianum* to pea seeds reduced root rot by *Aphanomyces euteiches* f. sp. *pisi* in growth-chamber experiments and also increased plant top weights compared to noncoated seeds. Seed treatment with slurries of *Pseudomonas fluorescens* strain 2-79RN$_{10}$, which produces a phenazine antibiotic, also reduced Aphanomyces root rot but to a lesser extent than did *T. harzianum* ThzID1. Disease suppression was not significantly different when seeds were treated with a combination of *T. harzianum* and 2-79RN$_{10}$ compared to treatment with *T. harzianum* alone. Root rot was not reduced by the mutant *P. fluorescens* strain 2-79-B46, which lacks phenazine. Treatment with *T. harzianum* plus 2-79-B46 resulted in the same level of disease control achieved by *T. harzianum* alone. These results suggest that the biocontrol mechanism of *P. fluorescens* 2-79RN$_{10}$ neither inhibited nor enhanced the biocontrol activity of *T. harzianum* ThzID1. In other experiments, density of *T. harzianum* hyphae originating from coated pea seeds in soil was not affected by the addition of 2-79RN$_{10}$, but when 2-79-B46 was added, density was greater after 5 days. The colony radius of *T. harzianum* was initially enhanced (at 3 days) by the addition of either strain, but the effect diminished by day 5. The same treatments were then applied to peas and to glass beads of equivalent size, and similar effects of the added bacterial strains were observed on both substrates, suggesting that the growth enhancement of *T. harzianum* in the presence of bacteria was not the direct result of stimulation of seed exudation by the bacteria. Our results provide a potentially improved formulation methodology for coating seeds with biocontrol organisms and methods for evaluating the compatibility of fungal and bacterial biocontrol agents applied to seeds.

Biological control of seedling diseases using nonpathogenic fungi and bacteria has received increasing attention. Antagonistic microorganisms applied to seeds prior to planting colonize the rhizosphere of seedlings and thus are present at or near the pathogens' infection court, where they act by producing antifungal compounds, through hyperparasitism, or by competitively colonizing spermosphere and rhizosphere substrates. Research is needed to optimize the use of biocontrol agents for practical disease control. For example, biocontrol formulations with relatively long-term viability and convenient application need to be developed. Also, because individual biocontrol agents may not control a sufficiently wide spectrum of pathogens, development of compatible combinations of control agents is a promising research direction. However, there has been relatively little investigation of the extent to which biocontrol bacteria that inhibit plant pathogens also may inhibit biocontrol fungi (2,12). Fravel (7) discussed the possibility of deleterious effects of antibiotic and antibioticlike compounds, produced by biocontrol agents, on beneficial microorganisms. Production of the antibiotic phenazine-1-carboxylic acid by the biocontrol agent *Pseudomonas fluorescens* strain 2-79RN$_{10}$ had this effect on *Trichoderma harzianum* Rifai isolate ThzID1 in sterile soil, although the effect was diminished in nonsterile field soil (2). In the spermosphere or rhizosphere, where microbes are in closer proximity in a relatively nutrient-rich environment, interactions may be more likely. Hubbard et al (12) stated that seed-colonizing pseudomonads were largely responsible for the failure of *Trichoderma hamatum* as a seed protectant in New York soils. A major research focus in our laboratory is the development of methods for compatible pairing of bacterial and fungal biocontrol agents, in appropriate formulations, to

increase their spectrum of useful biocontrol activity in the rhizosphere.

Several biocontrol agents, including bacterial strains of *Pseudomonas fluorescens* and *P. cepacia* and the fungus *T. harzianum*, have been shown to antagonize seedling and root pathogens of pea (*Pisum sativum* L.); experimental biocontrol of *Pythium* spp. and *Aphanomyces euteiches* f. sp. *pisi* usually has been obtained by treating seeds with liquid suspensions of living biocontrol agents before planting (9,12,19,25,29,30). However, this method may not be practical for large-scale applications because of the need to formulate bacterial or fungal spore suspensions immediately before application. A promising development in formulation of fungal biocontrol agents for soil application is the incorporation of hyphal biomass or spores into alginate pellets (8,16,17,20,21,35). Knudsen et al (16) described a method of treating pelletized biocontrol fungi with the osmoregulant polyethylene glycol (PEG), which enhanced the rapidity of hyphal growth and sporulation. Methods of quantifying hyphal growth of *T. harzianum* from alginate pellets have been described (2,14,18). Knudsen et al (15) described the colonization of sclerotia of *Sclerotinia sclerotiorum* by *T. harzianum* formulated in alginate pellets under laboratory and field conditions. Results suggested that biocontrol efficacy is likely to increase with enhanced growth of the biocontrol agent (2,15).

In this report, we first describe a method of incorporating biocontrol fungi and PEG into a fine, granular alginate-based formulation suitable for convenient storage and subsequent treatment of seeds. Second, we describe experiments to evaluate the compatibility of formulated biocontrol fungus *T. harzianum* isolate ThzID1 with known biocontrol bacteria applied to pea seeds. The ability of the agents to supress root rot of pea by *Aphanomyces euteiches* Drechs f. sp. *pisi* W. F. Pfender & D. J. Hagedorn was evaluated. Finally, we describe experiments to quantify the influence of the same bacterial strains on the hyphal growth of *T. harzianum* when applied to pea seeds.

MATERIALS AND METHODS

Experimental organisms. *T. harzianum* isolate ThzID1 was recovered from field soil at Moscow, ID, and was maintained on Difco potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) at 4 C.

P. fluorescens strains 2-79RN$_{10}$ and 2-79-B46 were provided by D. M. Weller and L. S. Thomashow (USDA-ARS, Pullman, WA). Strain 2-79RN$_{10}$ is a spontaneous mutant of strain 2-79 (NRRL B-15132) and is resistant to rifampicin and nalidixic acid antibiotics (36). Strain 2-79-B46 is a Tn5 mutant of 2-79RN$_{10}$ and is deficient in production of phenazine antibiotic (34). The strains were maintained at −80 C in 15% glycerol and were grown on King's medium B (13) agar with rifampicin and nalidixic acid, each at 100 μg/ml (KMB-RN). Cell concentrations for experiments were estimated using spectrophotometer readings at 640 nm compared to a standard curve. *A. e. pisi* was obtained from W. J. Kaiser (USDA-ARS, Pullman, WA) and was maintained on PDA at 4 C. Oospores were produced in V8-broth culture (5).

Sand and soil. Either commercially obtained silica sand or Palouse-Latahco silt loam soil was used in these experiments. Silt loam soil was obtained from the University of Idaho Parker Farm at Moscow. Soil analysis (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 per gram. Soil pH in the soil/water (2:1) solution was approximately 5.9; the pH of the sand was approximately 6.0. Soil was sieved (5-mm mesh) prior to use.

Formulation of *T. harzianum*. *T. harzianum* was grown for 1 wk in 1-L Erlenmeyer flasks containing 500 ml of potato dextrose broth plus streptomycin sulfate (25 μg/ml) on a rotary shaker (120 rpm) at 24–26 C. The fermenter biomass consisted of hyphal segments, chlamydospores, and conidia. PEG plus alginate solutions were prepared by dissolving 240 g of PEG 8000 (Sigma Chemical Company, St. Louis, MO) in 1 L of fermenter liquid plus biomass and then adding 1.2 g of medium-viscosity sodium alginate (Sigma Chemical Company) dissolved in 200 ml of distilled water. The water potential of the aqueous PEG solution was −526 kPa at 22 C, according to the equation of Michel (24). Drops of the mixture were added to 0.25 M aqueous $CaCl_2$. Pellets formed in the $CaCl_2$ solution were removed by screening and were placed on waxed paper and allowed to fully air-dry for 48 h at 25 ±3 C with 20–50% relative humidity. Dried pellets were ground into fine granules in a Wiley mill. Granules were sieved through a pair of mesh screens (0.75-mm and 0.375-mm mesh size). Only those granules that were retained on the lower screen were used for the experiments. Mean particle diameter (obtained from microscopic measurements of 200 granules) was 500 μm. Granules were stored in covered glass beakers at either 22 ±2 C or 5 ±1 C with ambient relative humidity at 20–50%. Viability of the fungus in individual granules was determined after 0, 1, 2, and 6 mo of storage. At each sample time, approximately 0.1 g of granules was removed from each beaker, and the granules were scattered onto the surface of PDA plates. After 48 h of incubation at 25 C, the plates were examined under a dissecting microscope, and the presence or absence of hyphal growth from a random sample of 200 granules was noted. Viability was recorded as the percentage of granules on which hyphal growth was observed. For the following experiments, the granules used were stored at 5 C and were less than 1 mo old.

Compatibility of *T. harzianum* ThzID1 and 2-79RN$_{10}$ or 2-79-B46. Pea seeds (cv. Columbia) were surface-sterilized in 5% sodium hypochlorite for 5 min, followed by several rinses in sterile distilled water. To inoculate seeds with either *P. fluorescens* 2-79RN$_{10}$ or 2-79-B46, bacteria were first grown on KMB agar at 28 C for 48 h. Cells were scraped from plates and were suspended (10^{10}–10^{11} cells/ml) in sterile phosphate buffer. Individual seeds were placed in 1 ml of either bacterial suspension or sterile distilled water for approximately 15 min. For treatments receiving *T. harzianum*, seeds were removed from water or bacterial suspension and were rolled in the *T. harzianum* formulation until coated (10% by weight). Seeds were allowed to air-dry at 22 C for 24 h prior to planting.

Spermosphere population counts of 2-79RN$_{10}$ and 2-79-B46 were obtained after air-drying by suspending three peas per treatment, each in 1 ml of sterile phosphate buffer, and vortexing for 1 min. Rhizosphere counts of the two *P. fluorescens* strains were made at the end of the experiment by suspending the root system of 10 replicates in 5 ml of sterile phosphate buffer and vortexing for 1 min. Serial dilutions were made, and 0.1-ml aliquots were spread on duplicate plates of KMB-RN. Plates were incubated at 25 C, and colonies were counted after 3 days.

Sand (100 g per tube) was added to plastic planting tubes (4 × 20.5 cm, Ray Leach Cone-Tainer, Ray Leach, Canby, OR), and 1 ml of an *A. e. pisi* oospore suspension (corresponding to 125 oospores/g of sand) was applied to the surface of the sand and was covered with 15 g (approximately 3 mm in depth) of sand. The control seeds had no oospores added. Peas (one pea per Cone-Tainer) were then placed on the sand surface and were covered with an additional 35 g of sand. Treatments were arranged in a completely randomized design in racks (98 Cone-Tainers per rack) that were placed in a growth chamber at 20 C, with a photoperiod consisting of 16 h of light per 8 h of dark. There were 20 single-plant replicates per treatment, and the experiment was repeated. After 3 wk, plants were removed from the sand, and the roots were washed and rated on a 0–4 rating scale for Aphanomyces root rot (0 = plant healthy, 1 = root slightly discolored, 2 = root extensively discolored but not shrunken, 3 = root extensively discolored and shrunken, and 4 = root partially to completely rotted through or plant dead) (30). Top and root wet weights were obtained for each plant. Data were analyzed using the general linear models procedure of the SAS statistical package (32).

Effect of 2-79RN$_{10}$ and 2-79-B46 on radial growth and hyphal density of ThzID1 in the spermosphere. Soil was autoclaved for 90 min on two consecutive days and was adjusted to a soil matric potential of −100 kPa as described previously (14). Pea seeds were surface-sterilized and were treated with bacteria and/or *T. harzianum* as described above. Spermosphere counts of 2-79RN$_{10}$

and 2-79-B46 were obtained by washing and plating seeds before planting and when hyphal growth was measured at 3 and 5 days. Methods for measuring the density of hyphae originating from alginate pellets in soil, described by Bin et al (2), were used to measure hyphal growth of *T. harzianum* from pea surfaces. First, 9-cm diameter glass petri dishes were filled approximately half full with soil. A single pea seed treated either with *T. harzianum* alone, *T. harzianum* plus 2-79RN$_{10}$, or *T. harzianum* plus 2-79-B46 was placed on the soil surface in the center of a dish. The pea and the soil surface were overlaid with two layers of 1-mm^2 nylon mesh, and additional soil was added to fill the dishes. Soil was gently compressed to an average bulk density of 1.2 g/cm^3. Dishes were placed in plastic bags with moist paper towels and were incubated at 20 C. There were four replicates per treatment per sample time, and the experiment was repeated.

Radial growth and density of *T. harzianum* hyphae were measured after 1, 3, and 5 days. At each sample time, the upper layer of nylon mesh (with the soil above it) was removed from sampled petri dishes. The lower layer of mesh served as a reference scale for measuring radial growth and density of hyphae in the observed plane, to a depth of 1 mm (2). The radial-growth pattern of the colony originating from each pellet was observed at 40× with a stereomicroscope and was mapped on graph paper. Maps were digitized (Jandel Scientific, Corte Madera, CA) to estimate the area of each map, and the mean colony radii were estimated from area values. Hyphal density was estimated at 1-mm increments in each of four directions from the pellet, using a visual assessment key generated by a computer simulation (14). Hyphal densities in each direction were averaged for each successive 1-mm distance from the pellet; the total visible density of hyphae (in millimeters) 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 around the pellet center (2).

The possibility that the effects of bacteria on the growth of *T. harzianum* in the spermosphere of pea might be mediated by the stimulation of pea exudates was explored by comparing hyphal growth from pea surfaces with growth from surfaces of chemically inert spheres of approximately equivalent size. Glass beads (4-mm diameter) were washed and autoclaved and then were coated with the experimental organisms in the manner described for pea seeds. Pea seeds were coated at the same time, for comparison, and hyphal-growth measurements were made as described above. The experiment was replicated.

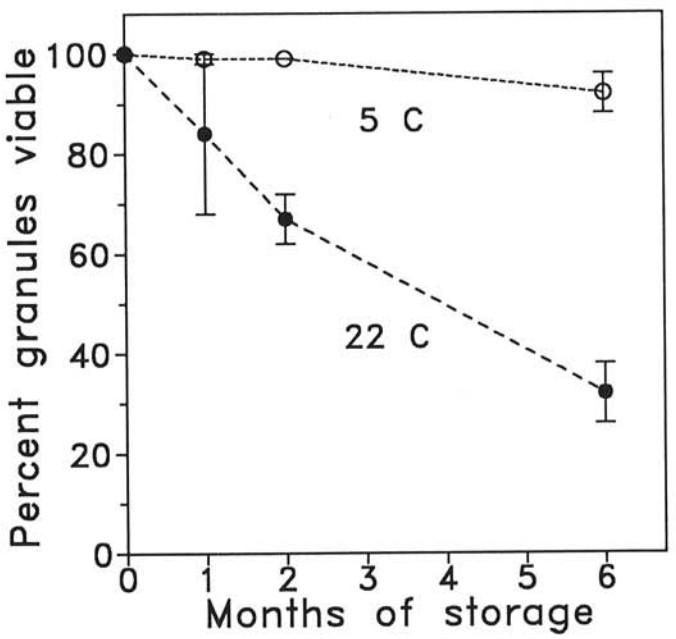

Fig. 1. Viability of *Trichoderma harzianum* isolate ThzID1 encapsulated in alginate plus PEG granules, after 0, 1, 2, and 6 mo of storage at 5 or 22 C. Percent viability was determined from the presence or absence of hyphal growth in a random sample of 200 granules incubated 48 h at 25 C on water agar. Vertical bars represent ±1 SE.

RESULTS

Formulation and viability of *T. harzianum* ThzID1. Initially, virtually all granules (>99%) of the formulation contained viable propagules of *T. harzianum* isolate ThzID1, representing a mixture of conidia, chlamydospores, and hyphal fragments. Over 6 mo of storage, viability of granules stored at 22 C (room temperature) dropped significantly, while viability of granules stored at 5 C (refrigerated) decreased only slightly (Fig. 1). Also, variability among samples was generally greater for granules stored at room temperature than for those that were refrigerated (Fig. 1). Regardless of storage conditions, the formulation adhered readily to seed surfaces moistened with water prior to planting.

Compatibility of *T. harzianum* ThzID1 and *P. fluorescens* 2-79RN$_{10}$ or 2-79-B46. Treatment by experiment interactions were not significant ($P > 0.05$), and data from both experiments were pooled. Rhizosphere population counts of 2-79RN$_{10}$ and 2-79-B46, either in the presence of absence of *T. harzianum*, were approximately 10-fold lower (approximately 10^7 colony-forming units (cfu)/g of root) at the end of the experiment compared to initial levels on pea seeds (10^8 cfu/seed). Seed treatment with *T. harzianum* significantly ($P < 0.05$) reduced Aphanomyces root rot of pea compared to the infested control and the two *P. fluorescens* strains (Table 1). Root rot was reduced by *P. fluorescens* 2-79RN$_{10}$ compared to the infested control, but suppression was significantly less than with *T. harzianum* ThzID1. The level of disease suppression with *T. harzianum* and either 2-79RN$_{10}$ or 2-79-B46 was intermediate, although not significantly different from either *T. harzianum* alone or 2-79RN$_{10}$ alone. The mutant strain of *P. fluorescens*, 2-79-B46, did not reduce root rot symptoms compared to the infested soil (Table 1).

Seed treated with *T. harzianum*, either alone or in combination with 2-79RN$_{10}$ or 2-79-B46, resulted in a significant increase in top weight compared to the noninfested control (Table 1). Top weight of peas treated with 2-79RN$_{10}$ alone was significantly greater than the weight of nontreated peas in the presence of *A. e. pisi* but was not significantly greater than the weight of the noninfested control. Treatment with 2-79-B46 alone did not increase top weight over that of nontreated peas. Similarly, root weights of plants in all *Trichoderma* treatments were significantly higher when *A. e. pisi* was present than were the root weights of nontreated peas; in most cases *Trichoderma* treatments were

TABLE 1. Influence of *Trichoderma harzianum* isolate ThzID1, *Pseudomonas fluorescens* strain 2-79RN$_{10}$, and *P. fluorescens* strain 2-79-B46, alone and in combination, on Aphanomyces root rot of pea, top and root weight

Treatment[x]	Aphanomyces rating[y]	Top weight (g)	Root weight (g)
Noninfested	0.45 a[z]	0.35 b	0.51 b
ThzID1	0.68 ab	0.51 a	0.58 ab
ThzID1 + 2-79-B46	1.03 bc	0.49 a	0.62 a
ThzID1 + 2-79RN$_{10}$	1.10 bc	0.54 a	0.61 a
2-79RN$_{10}$	1.28 c	0.36 b	0.45 b
2-79-B46	2.89 d	0.24 c	0.32 c
Infested	3.08 d	0.19 c	0.23 c

[x] Pea seeds were nontreated or treated with formulated granular ThzID1 and/or bacterial suspensions and were planted in noninfested sand or sand infested with *Aphanomyces euteiches* f. sp. *pisi* oospores (125 oospores/g of sand).

[y] Means of 40 observations per treatment (20 replicates per experiment; two experiments were pooled for analysis). Aphanomyces root rot rating scale: 0 = plant healthy, 1 = root slightly discolored, 2 = root extensively discolored but not thrunken, 3 = root extensively discolored and shrunken, and 4 = root partially to completely rotted through or plant dead (30).

[z] Means within each column followed by the same letter are not significantly different ($P > 0.05$) according to least significant difference (LSD) analysis.

also higher than root weights of nonifested controls (Table 1). As with top weights, treatment with 2-79RN$_{10}$ alone also resulted in increased root weights in the presence of *A. e. pisi*; treatment with 2-79-B46 alone had no significant effect (Table 1).

Radial growth and hyphal density. On either peas or glass beads, bacterial populations varied but generally decreased over the course of the experiment from initial levels of approximately 10^6–10^8 cfu per pea (or bead) to approximately 10^6–10^7 cfu per pea (or bead). For radial-growth and hyphal-density measurements, treatment by experiment interactions were not significant ($P > 0.05$), and data from both experiments were pooled. After 1 day, no hyphal growth was observed in any of the treatments. Treatment with *T. harzianum* plus 2-79RN$_{10}$ resulted in no significant differences ($P > 0.05$) in the hyphal density of *T. harzianum* grown out from pea surfaces after 3 or 5 days compared to treatments with *T. harzianum* alone (control) (Fig. 2). Although addition of 2-79-B46 to *T. harzianum* had no effect on hyphal density at day 3, it did significantly increase hyphal density at 5 days compared either to *T. harzianum* alone (control) or to *T. harzianum* plus 2-79RN$_{10}$ (Fig. 2). The colony radius after 3 days was greater in the presence of either *T. harzianum* plus 2-79RN$_{10}$ or *T. harzianum* plus 2-79-B46, compared to *T. harzianum* alone (control), but any enhancement of radial growth by either strain was no longer evident by day 5 (Fig. 3).

The possibility that faster initial radial growth of ThzID1 in the spermosphere of peas treated with bacteria was the result of pea exudate stimulation by bacteria was tested indirectly by comparing treatments on peas and glass beads. Enhanced radial growth was observed in the presence of either bacterial strain on either substrate after 3 days but not after 5 days. Radial growth in the presence of either bacterial strain was significantly ($P > 0.05$) greater from beads than from peas after both 3 days (3.3 vs. 0.8 mm) and 5 days (19.5 vs. 5.8 mm). Addition of 2-79-B46 again significantly stimulated visible hyphal density of ThzID1 (averaged over pea and bead), whereas 2-79RN$_{10}$ did not. As with radius, hyphal density with either strain present was significantly greater from beads than from peas, both at 3 days (1,292 vs. 908 mm, averaged over both strains) and 5 days (6,999 vs. 4,963 mm). Substrate by strain interactions were not significant ($P > 0.05$) for either radial growth or hyphal density.

DISCUSSION

The effectiveness of biocontrol agents may depend partially on their ability to proliferate during a short period of favorable environmental conditions before they encounter plant pathogens. More rapid growth and sporulation of fungi from biocontrol formulations may significantly enhance efficacy in the field. Knudsen et al (16) showed that PEG treatment of *T. harzianum* in alginate pellets enhanced hyphal extension of the fungus in soil, although it did not enhance production of conidia on pellet surfaces. In this study, we described how similar methods may be used to produce granular formulations of biocontrol fungi suitable for coating seeds. Unlike conventional alginate pellets, pellets with PEG incorporated could be milled easily into fine, relatively uniform granules. The exact mechanisms by which PEG affects storage and growth characteristics of formulated fungi are not known, but the effects may be similar to those observed with osmotically primed seeds, in which water availability is controlled by osmotic agents such as PEG, limiting the rate and total amount of imbibition and reducing the membrane disruption and intracellular solute leakage associated with rapid embryo swelling (11,26,33). Seed-priming treatments also allow presprouting metabolism to occur while preventing seeds from sprouting (11,26). Although similar effects may occur when pelletized fungi are treated with PEG solutions, the effects may vary with the fungi used or with the types of fungal propagules encapsulated (e.g., chlamydospores versus conidia or hyphal fragments). Further investigation of the mechanisms involved may identify common physiological effects of "priming" fungi and may allow further improvement of desirable attributes of biocontrol formulations. In preliminary experiments (L. M. Dandurand and G. R. Knudsen, *unpublished data*), we produced similar formulations with different bacterial strains used for plant-disease biocontrol or chemical-pollutant bioremediation. These methods are promising for customized mixing of microbial strains prior to application.

Seed treatment with *T. harzianum* isolate ThzID1 in the granular alginate plus PEG formulation resulted in lower amounts of Aphanomyces root rot, as well as increased top and root growth

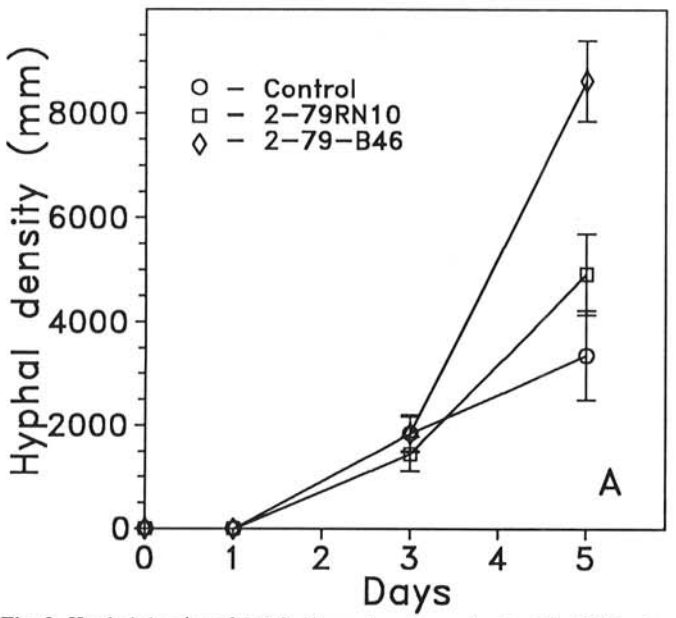

Fig. 2. Hyphal density of *Trichoderma harzianum* isolate ThzID1 in the spermosphere of pea after 1, 3, and 5 days of incubation at 20 C. Hyphal density was determined as the total visible length of hyphae in a cross-sectional view, to a depth of 1 mm. Seeds were coated with a granular formulation of *T. harzianum*, with or without *Pseudomonas fluorescens* strains 2-79RN$_{10}$ or 2-79-B46. Density was measured after 1, 3, and 5 days of incubation at 20 C. Pooled means (vertical bars represent ±1 SE) from two experiments are shown.

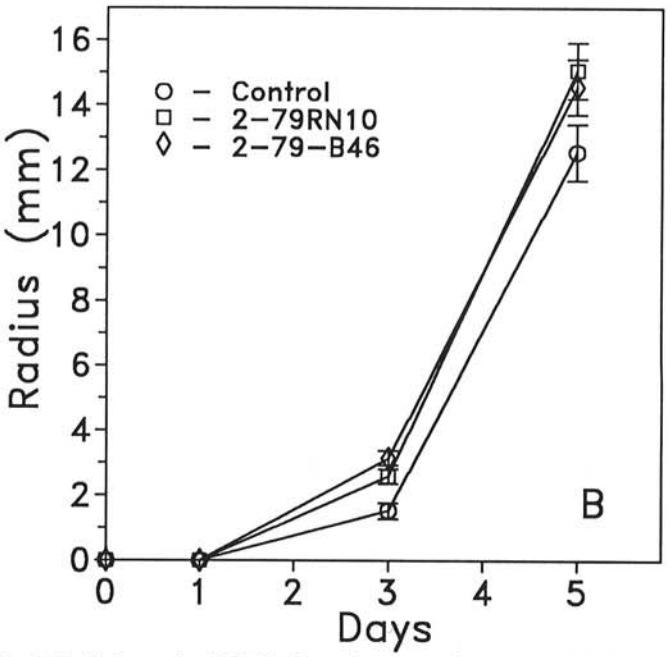

Fig. 3. Radial growth of *Trichoderma harzianum* isolate ThzID1 in the spermosphere of pea after 1, 3, and 5 days at 20 C. Seeds were coated with a granular formulation of *T. harzianum*, with or without the addition of *Pseudomonas fluorescens* strains 2-79RN$_{10}$ or 2-79-B46. Pooled means (vertical bars represent ±1 SE) from two experiments are shown.

of pea seedlings. Different mechanisms have been described for biocontrol of various plant-pathogenic fungi by *Trichoderma* species; these include mycoparasitism and production of antifungal metabolites (6,7,12,15,22,28). Plant-growth promotion by isolates of *T. harzianum* also was reported and was attributed to production of one or more growth-promoting substances by the fungus (37), although plant-growth reduction by *T. harzianum* also has been reported (23). Despite the fact that the specific mechanism or mechanisms operating in our system are undetermined, we believe that *T. harzianum* shows potential as a biocontrol agent for Aphanomyces root rot of pea. Further studies, including trials in *Aphanomyces*-infested fields, will be needed to confirm this potential.

The phenazine antibiotic-producing *P. fluorescens* strain 2-79RN$_{10}$ also reduced Aphanomyces root rot, although the level of suppression was less than that observed with *T. harzianum* isolate ThzID1. Plant-growth enhancement was not observed. *P. fluorescens* strain 2-79-B46, which does not produce the phenazine antibiotic, did not suppress Aphanomyces root rot; as a result, a role for strain 2-79RN$_{10}$'s phenazine antibiotic is implicated as a biocontrol mechanism. This role has been demonstrated in other biocontrol systems (3,27,34,36).

Our results indicate that 2-79RN$_{10}$ did not have a significant detrimental effect on the biocontrol activity of *T. harzianum* in the spermosphere and rhizosphere of pea. Although suppression of Aphanomyces root rot was less with 2-79RN$_{10}$ alone than with *T. harzianum* alone, the presence of 2-79RN$_{10}$ in combination with ThzID1 did not alter the biocontrol activity of *T. harzianum*. Similarly, addition of the phenazine-minus mutant strain, 2-79-B46, had no effect on the biocontrol efficacy of *T. harzianum*. These results are similar to those of Hubbard et al (12), who observed that pseudomonads did not reduce the biocontrol ability of *T. hamatum* in sand, although efficacy was reduced in steamed soil containing little (approximately 1 μg/g) plant-available iron. In a previous study in our laboratory (2), Bin et al observed that *P. fluorescens* 2-79RN$_{10}$ suppressed radial growth and hyphal density of *T. harzianum* on agar and in sterile bulk soil. However, in nonsterile soil, biocontrol efficacy (measured as colonization by *T. harzianum* of sclerotia of *S. sclerotiorum*) was not significantly affected by the addition of bacteria. Growth of *T. harzianum* from alginate pellets in sterile soil was reduced when bacterial populations were 10^4–10^7 cfu/g of soil, and because numbers of 2-79RN$_{10}$ declined below those levels in field soil, Bin et al speculated that the reduced bacterial populations may have resulted in reduced inhibitory activity (2). However, in the current study, the numbers of 2-79RN$_{10}$ appeared to be within the range reported by Bin et al (2) as having a negative impact on growth and activity of *T. harzianum* in sterile bulk soil.

When we specifically measured the growth of *T. harzianum* from pea surfaces, the growth was only slightly, but positively, affected by the presence of 2-79RN$_{10}$. Radial growth was initially stimulated in the presence of 2-79RN$_{10}$, but this enhancement was not observed after 5 days. The presence of 2-79-B46 enhanced hyphal density of *T. harzianum* by approximately 2.5-fold. Hyphal radius also was stimulated by 2-79-B46, but, as with 2-79RN$_{10}$, the effect was ephemeral and by the end of the experiment was no longer observed. Because bacteria have been reported to stimulate seed and root exudates (1,31), we hypothesized that such stimulation may have resulted in increased amounts of nutrients available to *T. harzianum*, perhaps counteracting any inhibitory effects of antibiotic production by 2-79RN$_{10}$. However, when the experiment was repeated with the additional treatment of an inert substrate (glass beads), the presence of strain 2-79RN$_{10}$ again slightly stimulated hyphal growth of *T. harzianum*, and strain 2-79-B46 significantly enhanced hyphal growth as well. Indeed, hyphal-growth stimulation from beads was greater, overall, than stimulation from peas. Thus, the evidence is against our hypothesis, and the bacteria apparently did not affect hyphal growth primarily by causing increased seed exudates from peas. It is possible to speculate about other possible factors, however. For instance, the general decrease in bacterial numbers in all treatments suggests that dead bacterial cells may have provided nutrients for fungal growth. This area is worthy of further investigation because it suggests the possibility that dying biocontrol agents may provide nutrients for plant pathogens, indicating that the application of overly large numbers of biocontrol agents may be counterproductive. Other possible factors may include localized changes in CO_2 concentration or pH by bacteria, both of which have been shown to affect growth of *Trichoderma* spp. (4,10,28). However, Ownley et al (27) reported that 2-79RN$_{10}$ controlled the take-all fungus, via phenazine production, over a wide pH range (4.9–8.0).

Lumsden et al (23) pointed out that the importance of biomass in ecological interactions is difficult to assess and proposed that the biocontrol activity of *T. harzianum* is linked primarily to a transient increase in biomass, so that high-propagule numbers may not be needed to achieve control. Our results agree with this statement: although biomass (estimated by hyphal density) of *T. harzianum* was greater in the presence of strain 2-79-B46 and was increased slightly by the addition of strain 2-79RN$_{10}$, biological control of Aphanomyces root rot was not improved by addition of either bacterial strain. Finally, although there was no apparent advantage to combining 2-79RN$_{10}$ with *T. harzianum* to Aphanomyces root rot suppression, the two biocontrol agents did not appear to be incompatible for combination as a seed treatment. Because commercial biocontrol formulations ideally will be effective against a wide spectrum of plant pathogens, continued investigation of the compatibility and efficacy of combinations of biocontrol agents is necessary.

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