Colonization of the Rhizosphere by Biological Control Agents Applied to Seeds

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ABSTRACT

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The ability of various fungi and bacteria to move from inoculated seeds to developing roots was studied. Plant roots growing into soil-free moist chambers were not colonized by *Trichoderma* spp., but were colonized by *Enterobacter cloacae*. When roots were grown in sterile soil, *Trichoderma harzianum* was detected in the rhizosphere of the upper half of the roots, while *E. cloacae* colonized the entire rhizosphere. In untreated soil, none of the organisms studied (*T. harzianum, T. koningii, Gliocladium virens, Penicillium funiculosum, E. cloacae, Pseudomonas fluorescens, P. putida*, or another fluorescent *Pseudomonas* sp.) could be detected in the rhizosphere more than 3 cm below the planted seed. In autoclaved soil to which fungi were added, *E. cloacae* colonized roots well, while *T. harzianum* was inhibited. Conversely, in autoclaved soil to which soil bacteria were added, *E. cloacae* was inhibited, and *T. harzianum* grew well. Percolating water enhanced the downward movement of both bacterial and fungal propagules.

Additional key words: pea, *Pisum*.

Soil-inhabiting fungi cause numerous root and seed rots. Currently approved fungicides control most seed rots; however, at economically feasible rates, they frequently fail to control root rots. Many investigators have shown that biological control agents (e.g., *Trichoderma* spp.) can sometimes protect seeds as effectively as chemical fungicides (5,7,8,13). Antagonists that become established around the crowns and roots of tomato seedlings (17), peach (3), and cherry (9) can effectively reduce crown and root disease.

Seed treatment is an attractive method for introducing biological control agents into the soil-plant environment. Other effective means of antagonist delivery directly to soil (e.g., colonized grain or bran) may require such large amounts of material that extensive use of these delivery systems could be economically and logistically impractical. Antagonists applied to seeds may have the opportunity to be the first colonizers of roots. Unfortunately, seed inoculants may not be effective in preventing root disease (14,15,19,23), perhaps because these organisms are either poor root surface colonizers (20,26,28) or they are incapable of being transported by the root through the soil profile (16). However, exceptions have been observed. Fluorescent *Pseudomonas* spp., for example, used to treat wheat seeds or potato seed-pieces were shown to colonize the entire rhizosphere (12,25,27).

Little information is available regarding movement of microorganisms from the seed to the rhizosphere. Hence, studies were conducted to determine the extent to which biological control agents move from the seed into rhizospheres exhibiting different levels of microbial competition.

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MATERIALS AND METHODS

Organisms and media. Fungal and bacterial cultures used in this study are listed in Table 1. Most organisms were chosen because of

their reputed antagonistic activity toward seed- and root-rotting pathogens (e.g., *Pythium ultimum* Trow or *Rhizoctonia solani* Kühn). *Trichoderma harzianum* Rifai, *T. koningii* Oud, and *Enterobacter cloacae* (Jordan) Hormalche and Edwards have been shown to protect pea and cucumber seeds against rots caused by *Pythium* spp. in field soils (5,6). *T. harzianum* T95GA (a benomyl-resistant mutant of *T. harzianum* T-Co) (10), *Gliocladium virens* Miller, Giddens and Foster G1, *Penicillium funiculosum* Thom, and fluorescent *Pseudomonas* sp. 3-2 are known to antagonize *Pythium ultimum* in vivo (*unpublished*). *Pseudomonas fluorescens* Migula TL and *P. putida* (Trev.) Migula BK-1 were chosen for their ability to colonize developing roots and subsequently to become predominant in the rhizosphere (2). *E. cloacae, Pseudomonas* spp., and fungal cultures were maintained on slants of yeast-dextrose-carbonate agar (YDCA), King's B agar (11), and potato-dextrose agar (PDA) (Difco Laboratories, Detroit, MI), respectively. All cultures were stored at 4 C.

To monitor antagonists in soil and on root surfaces, all organisms were labeled with antibiotic resistance markers. A rifampicin-resistant strain of *E. cloacae* (ATCC 39979) and *Pseudomonas* spp. were derived from parent cultures by plating a cell suspension (10^8 cells per milliliter) on trypticase soy agar (TSA) (BBL Microbiology Systems, Cockeysville, MD) or King's B agar containing 100 μg of rifampicin (Sigma Chemical Co., St. Louis, MO) per milliliter, respectively. Developing colonies were selected after 48 hr at 30 C. The stability of these mutants was tested by culturing them through media without rifampicin five times and then growing them on media with and without rifampicin. Counts between media were compared. There was no evidence for reversion of resistant strains back to the parent type. Strains of *T. harzianum* (ATCC 20737), *T. koningii* (ATCC 20738), *P. funiculosum*, and *G. virens* G1 resistant to 100 μg of cycloheximide per milliliter of medium were obtained similarly.

Seed treatments. Fungi were grown on PDA at 20–25 C until sporulation occurred. Conidia were harvested by scraping the culture surface with a spatula and suspending the spores in 2% (w/v) Methocel A4C (Dow Chemical Co., Midland, MI) at a final concentration of about 10^8 conidia per milliliter. *E. cloacae* and *Pseudomonas* spp. were grown in 250-ml Erlenmeyer flasks containing 50 ml of either trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD) or King's B broth, respectively, and incubated for 12 hr at 30 C on a rotary shaker. Cells were collected by centrifugation at 10,400 g for 10 min at 4 C and resuspended in 3 ml of a 2% (w/v) Methocel solution.

Pea seeds (*Pisum sativum* L. 'Venus') were uniformly coated with the conidia or cell suspensions (2 ml/25 seeds), allowed to air dry for 12 hr, and stored at 4 C until use. Seeds coated with *Pseudomonas* were planted immediately after treatment.

Enumeration of microorganisms. At each sampling date, spermosphere (i.e., seed and adhering soil after gentle shaking), rhizosphere (i.e., root and adhering soil after gentle shaking), and soil samples were suspended and dilution series were prepared in 0.85% (w/v) NaCl solution. Populations were determined by using the dilution plates with four replicate samples enumerated at each sampling on various selective media. Colonies of *E. cloacae* were counted on a medium of the following composition: $MgSO_4$, 0.1 g; D-(+) trehalose, 2.0 g; 2,6-diaminopurine, 0.2 g; NaCl, 15.0 g; K_2HPO_4, 0.8 g; KH_2PO_4, 0.8 g; yeast extract, 0.01 g; methylene blue, 65 mg; and agar, 15.0 g per liter. The medium was autoclaved, allowed to cool to 50–60 C, and supplemented with 100 μg per milliliter of both rifampicin and cycloheximide (S. V. Beer and M. Sasser, *unpublished*). *Pseudomonas* spp. were enumerated on King's B agar containing 100 μg of rifampicin, 20 μg of nalidixic acid (Sigma Chemical Co., St. Louis, MO), and 100 μg of cycloheximide per milliliter. Most fungi were enumerated on PDA amended with 75 μg of cycloheximide, 20 μg of nystatin, 100 μg of streptomycin sulfate, and 100 μg of chlortetracycline per milliliter. *T. harzianum* T95GA was counted on PDA amended with benomyl (E. I. duPont de Nemours Inc., Wilmington, DE), 50 μg a.i. per milliliter; streptomycin sulfate, 100 μg per milliliter; and chlortetracycline, 100 μg per milliliter. Unless otherwise mentioned, all antibiotics were obtained from Sigma Chemical Co.

In all experiments, each plant was considered a replicate, there were four replicates per experiment, and each experiment was repeated twice.

Soil and soil column preparation. An Arkport fine sandy loam was used throughout this study. Its pH was 7.3 and its nutrient status was reported elsewhere (10). Before use, the soil moisture content was adjusted to approximately −72 mbar. Portions (32 g) of soil were added to a column (1.8 cm in diameter and 12 cm in height) made of aluminum foil and sealed at the bottom with a cotton plug. The column of soil was compacted to a bulk density of 1.0–1.1 g/cm^3.

Sterile soil was prepared by autoclaving moist soil for 1 hr on 3 consecutive days. To ascertain whether sterility had been achieved, suspensions of soil were plated on 30%-strength TSA containing sufficient agar to make the final concentration 1.5% (w/v) and incubated for 3 days at 30 C.

Infestion of sterile soil with either fungi or bacteria. The fungi used were selected from soil dilution plates containing PDA amended with 100 μg of chlortetracycline and 100 μg of streptomycin sulfate per milliliter. Care was exercised to maintain similar numbers of different colony types in the selected strains as were present on original dilution plates. All fungi selected were spore-forming, and approximately 50% were *Aspergillus* spp. and *Penicillium* spp. Fifty isolates were grown on unamended PDA for 7 days and a spore suspension containing a loopful of spores from each isolate was used to infest sterile soil. The bacteria used were collected from a 1,000-fold soil dilution plate containing 0.3% TSA amended with 100 μg of cycloheximide per milliliter. The plate was washed with sterile distilled water after 48-hr of incubation and the resulting bacterial suspension was added to sterile soil. Infested soils were readjusted to −72 mbar moisture content and incubated at 30 C. After addition of either bacteria or fungi, soils were allowed to equilibrate for 2 wk before use.

Movement of biocontrol agents from the seed into the rhizosphere. Seeds treated with various biological control agents

TABLE 1. Beneficial organisms used in seed treatments

Organisms	Isolated from	Sources
Trichoderma harzianum T95GA	Soil	R. Baker, Colorado State University
T. harzianum ATCC 20737	Soil	G. E. Harman
T. koningii ATCC 20736	Soil	G. E. Harman
Gliocladium virens G1	Soil	G. E. Harman
Penicillium funiculosum	Sporangia of *Pythium ultimum*	G. E. Harman
Enterobacter cloacae ATCC 39979	Germinating cucumber seed (Slicemaster)	G. E. Harman
Fluorescent *Pseudomonas* sp. 3-2	Pea root (Venus)	W. L. Chao
Pseudomonas fluorescens TL-3	Potato (White Rose)	T. J. Burr, Dept. of Plant Pathology New York State Agricultural Experiment Station
Pseudomonas putida BK-1	Potato (White Rose)	T. J. Burr, Dept. of Plant Pathology New York State Agricultural Experiment Station

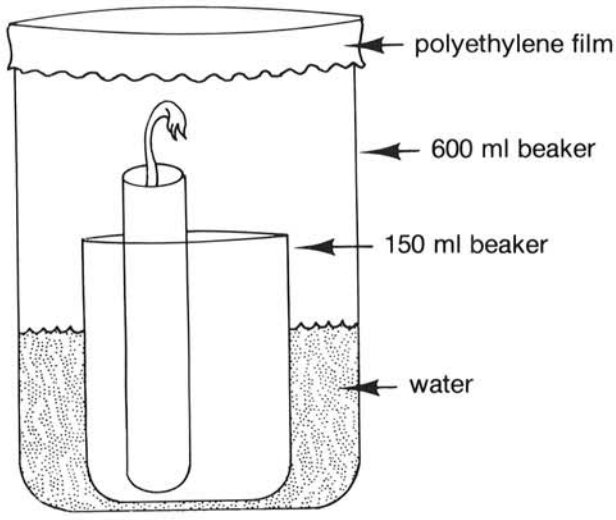

Fig. 1. The apparatus used for determining distribution of biological control agents from treated seeds in the rhizosphere. An aluminum foil tube (center) with the lower 1-cm stoppered with cotton (dashed portion) was filled with moist soil (−72 mbar) and compacted to a density of 1.1–1.2 g/cm^3. A treated or nontreated seed was planted in the upper centimeter. The tubes were incubated for 6 days at 22–25 C and 1-cm root and soil sections were obtained by cutting the cylinders into 1-cm sections.

TABLE 2. Distribution of biological control agents[a] along soil-free pea roots

Distance along root from seed	CFU × 10^3/cm of root		
	Trichoderma harzianum	*Trichoderma koningii*	*Enterobacter cloacae*
1st cm	<0.1 (1/4)[b]	<0.1 (2/4)	10 ± 4.0
2nd cm	<0.1 (1/4)	<0.1 (2/4)	6 ± 3.6
3rd cm	<0.1 (0/4)	<0.1 (0/4)	18 ± 7.1
4th cm	<0.1 (0/4)	<0.1 (0/4)	16 ± 3.3
5th cm	ND[c]	ND	38 ± 2.1
6th cm	ND	ND	440 ± 20
7–9th cm	ND	ND	87 ± 6.7

[a] Seeds were coated with 4.3×10^6, 4.9×10^5, and 6.0×10^7 of *T. harzianum* ATCC 20737, *T. koningii* ATCC 20736, and *E. cloacae* ATCC 39979, respectively. Population were measured 4 days after planting.
[b] Numbers in the parentheses represent the frequency of root sections that have *Trichoderma* which can grow on medium amended with cycloheximide. Numbers following ± represent the standard deviations.
[c] Not determined.

were planted in soil columns and incubated in a moist chamber sealed with polyethylene film (Fig. 1) for 6 days at 22–25 C.

To study the effect of water percolation through soil on downward movement of biocontrol agents, treated seeds were planted in larger aluminum foil cylinders (5.7 cm in diameter and 11 cm in height) filled with 320 g of soil at a bulk density of 1.1–1.2 g/cm^3. After incubation for 6 days, moist chambers were unsealed for 3 days to allow evaporation. Subsequently, 26 ml of water were added to each cylinder over a 45-min period unless stated otherwise. Care was taken to add water only to the center of the cylinders to minimize the flow of water along the inner surface of the cylinder. Rhizosphere soil was sampled 2 days later by cutting the cylinders into 1-cm sections and removing the roots and adhering soil with minimal disturbance. Populations of selected microorganisms were then enumerated as described previously.

Distribution of biological control agents on the pea rhizoplane in a moist chamber. A 1.8-cm-diameter hole was cut in the bottom of a plastic petri dish and the opening was covered with cheese cloth. An antagonist-treated seed was placed over the hole and the dish was filled with nontreated soil. The dish was then placed over a 9-cm-diameter glass jar containing water and the resulting chamber was covered with polyethylene film. The jars were wrapped with aluminum foil to exclude light. After incubation at 22–25 C for 3 days, roots were removed aseptically and cut into several 1-cm sections for microbial analysis.

Scanning electron microscopy. Six-day-old root samples were fixed in 3% glutaraldehyde in 0.05 M phosphate buffer (pH 7) overnight. The specimens were washed four times for 30 min with 0.05 M phosphate buffer, postfixed in aqueous 1% OsO_4 solution, rinsed four times with distilled water, and dehydrated in a graded acetone series. Critical-point-dried specimens were coated with gold and palladium in a Balzers Union sputter coater (Balzers Union Ltd., Liechtenstein) and viewed in an Amray-1000A scanning electron microscope (Amray, Bedford, MA).

RESULTS

To ascertain whether the root can serve as a transport agent for seed inoculants, roots developing from treated seeds were allowed to grow out of soil into a moist chamber to avoid any soil interference. After 4-days of incubation, about 10^4 colony-forming units (cfu) of *E. cloacae* per centimeter of root were detected throughout the entire root system (Table 2). No propagules of *Trichoderma* were detected (at 10^2 dilution) on any portion of the root. Moreover, when roots of seedlings grown from seeds treated with *Trichoderma* were cut into 1-cm segments and placed on selective medium, growth of *Trichoderma* was observed only on the first and the second centimeter of the root away from the seed.

TABLE 3. Vertical movement of fungal biological control agents in the pea rhizosphere from treated seeds[a] planted in untreated soil

Location	CFU × 10^4				
	Trichoderma harzianum[b]	*Trichoderma harzianum*	*Trichoderma koningii*	*Penicillium funiculosum*	*Gliocladium virens*
Spermosphere[c]	42 ± 27 (14 ± 7)[d]	580 ± 160 (840 ± 200)	39 ± 23 (76 ± 20)	530 ± 60 (37 ± 18)	171 ± 83 (127 ± 46)
Rhizosphere[e]					
1st cm	0.13 ± 0.22 (<0.01)	8.5 ± 6.4 (6.6 ± 2.9)	0.09 ± 0.13 (0.05 ± 0.04)	0.04 ± 0.06 (0.21 ± 0.13)	0.07 ± 0.05 (0.52 ± 0.13)
2nd cm	<0.01	0.32 ± 0.40[f] (0.28 ± 0.34)	<0.01	<0.01	<0.01
3rd cm	<0.01	<0.01	<0.01	<0.01	<0.01

[a] Initial seed populations were 5.6×10^5, 4.3×10^6, 4.9×10^5, 4.6×10^6, and 5.0×10^5 colony-forming units (CFU) per seed for *T. harzianum* T95GA, *T. harzianum* ATCC 20737, *T. koningii* ATCC 20736, *P. funiculosum*, and *G. virens* G1, respectively.
[b] Values following the ± symbol are standard deviations.
[c] Values represent number of propagules per spermosphere sample, counted 6 days after planting.
[d] Values in parentheses are CFU per gram of nonrhizosphere soil of the corresponding depth.
[e] Values below are CFU per centimeter of root, beginning just below the seed.
[f] Only from one of four replicates; no CFU could be detected in the other three.

When antagonist-treated seeds were planted in untreated soil, none of the five fungi tested (see Table 1) were able to colonize the rhizosphere more than 2 cm from the seed, and only low antagonist densities were found below the first centimeter of 9-cm-long roots (Table 3). Similar results were observed in nonrhizosphere soil. The bacterial agents were found on the upper portion (1–3 cm) of the roots nearest the seed (Table 4). None of the added bacteria were detected below 6 cm. When studied by using scanning electron microscopy, fungal hyphae were observed in rhizospheres that developed from treated seed planted in sterile soil but not in untreated soil (Fig. 2a and b).

Experiments were conducted to determine which factors prevent the vertical movement of microorganisms along the root. In sterile soil, a more or less uniform population (about 10^7 cfu) of *E. cloacae* was detected throughout the entire rhizosphere (Table 5). Conversely, samples taken at increasing distances from the seed contained increasingly lower levels of *T. harzianum* ATCC 20737 below 4 cm in both rhizosphere and nonrhizosphere soil. Few propagules of *T. harzianum* ATCC 20737 were found below the fifth centimeter of a 9-cm-long root. A greater population of propagules of *E. cloacae* was found in rhizosphere soil than in nonrhizosphere soil (Table 5). The number of propagules of *T. harzianum* ATCC 20737 was generally greater in nonrhizosphere soil than in rhizosphere soil (Table 5).

TABLE 4. Movement of bacteria in the pea rhizosphere from treated seeds[a] planted in untreated soil

	CFU × 10^4		
Location	*Enterobacter cloacae*[b]	Fluorescent *Pseudomonas* sp.	*Pseudomonas putida*
Spermosphere[c]	29,000 ± 16,000	5,100 ± 2,000	2,400 ± 1,600
Rhizosphere[d]			
1st cm	18 ± 4	72 ± 54	19 ± 2.4
2nd cm	2.6 ± 3	26 ± 30	2.5 ± 4.1
3rd cm	<0.01	3.0 ± 3.4	<0.002
4th cm	<0.01	<0.002	<0.002

[a] Initial numbers were 6.2×10^8, 4.4×10^8, and 5.0×10^7 colony-forming units (CFU) per seed for *E. cloacae* ATCC 39979, fluorescent *Pseudomonas* sp. 3-2, and *P. putida* BK-1, respectively.
[b] Values following the ± symbol are standard deviations.
[c] Values in this row are number of propagules per spermosphere sample, counted 6 days after planting.
[d] Values are number of CFU per centimeter of root, beginning just below the seed.

TABLE 5. Vertical distribution of *Trichoderma harzianum* and *Enterobacter cloacae* in the pea rhizosphere at various distances from treated seeds[a] planted in autoclaved soil

	CFU/g oven dried soil[b]			
Distance	*T. harzianum* (× 10^4)[c]		*E. cloacae* (× 10^6)	
Spermosphere	175 ± 25	(150 ± 44[d])	455 ± 145	(140 ± 55)
Rhizosphere[e]				
1st cm	16 ± 8.0	(110 ± 77)	67 ± 12	(35 ± 11)
2nd cm	20 ± 12	(135 ± 62)	107 ± 37	(38 ± 8.0)
3rd cm	20 ± 13	(>100)	27 ± 3.0	(40 ± 10)
4th cm	3 ± 1.4	(15 ± 12)	64 ± 22	(36 ± 6.0)
5th cm	2 ± 1.9[f]	(2.2 ± 2.0)	41 ± 12	ND[g]
6th cm	<0.01		55 ± 20	ND
7–9th cm	<0.01		60 ± 21	ND

[a] Initial numbers were 4.3×10^6 and 6.0×10^7 colony-forming units (CFU) per seed for *T. harzianum* ATCC 20737 and *E. cloacae* ATCC 39979, respectively.
[b] Counted 5 days after planting.
[c] Numbers following the ± symbol are standard deviations.
[d] Values in parentheses represent number of propagules per gram of nonrhizosphere soil sampled from the corresponding depth.
[e] Numbers indicate centimeters below the seed.
[f] Two out of four replicates, no colonies were detected at the lowest dilution.
[g] Not determined.

To find which portion of the native microbial population (bacterial or fungal) has a more pronounced influence on the vertical movement of antagonists in the rhizosphere, a study was carried out in which either resident fungi or bacteria were added to sterile soil. In bacteria-free soil containing 5×10^5 cfu of fungi per gram of oven dried soil, 1.7×10^5 cfu of *E. cloacae* were recovered from the rhizosphere up to 3 cm from the seed 6 days after planting. However, no propagules of *T. harzianum* ATCC 20737 could be detected in the rhizosphere (Table 6). Conversely, in fungus-free soil containing 1.5×10^8 cfu of bacteria per gram of oven-dried soil, *E. cloacae* was detected only in the first centimeter of the rhizosphere, whereas *T. harzianum* ATCC 20737 could still be detected 2 cm from the seed.

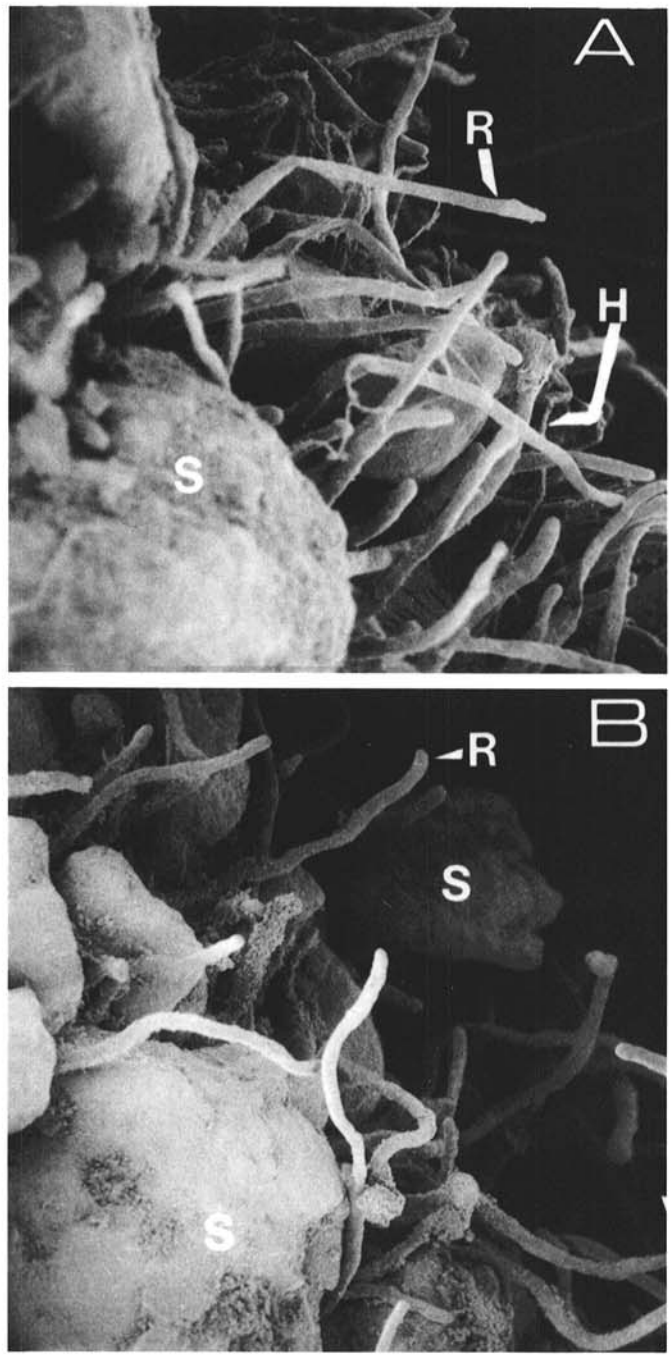

Fig. 2. Scanning electron photomicrographs of the rhizosphere of 6-day-old pea roots approximately 1 cm below seeds treated with *Trichoderma koningii* ATCC 20736. In **A,** seeds and roots are from autoclaved soil, while in **B,** they are from untreated soil. R = root hairs, S = soil particles, and H = fungal hyphae. Items shown are magnified 500-fold. Note absence of hyphae in **B**.

When soils were watered, *T. harzianum* ATCC 20737, *P. fluorescens* TL, and *E. cloacae* were all found in the rhizosphere at greater distances from the seed than if soils were not watered and the depth of their penetration was influenced by the amount of water added (Table 7). For example, when water was added, propagules of *T. harzianum* ATCC 20737 were detected in the rhizosphere 4 cm from the seed; when no water was added, they were detected only in the rhizosphere 2 cm away from the seed.

A method (24) currently used for studying the root-colonizing capacity of various microorganisms was tested. *E. cloacae* was introduced into untreated soil as a seed treatment and a comparison was made among the population levels that developed: in the entire rhizosphere of pea (6.2×10^4 cfu per gram of oven-dried soil), in the upper 2-cm of the pea rhizosphere (2.6×10^5 cfu per gram of oven-dried soil), and in the remainder of the pea rhizosphere (10^2 cfu per gram of oven-dried soil). The density of *E. cloacae* in the upper 2-cm of the rhizosphere was significantly ($P = 0.05$) higher than the density of *E. cloacae* over the entire pea rhizosphere.

DISCUSSION

In the present study, none of the agents tested were able to colonize the rhizosphere more than 2 cm from treated seeds in untreated soil. Although some organisms tested have been reported previously to colonize the root surface of other plant species in other soils (12,25,27), Taylor and Parkinson (26) have shown that the spread of fungi along the length of the root is generally insignificant and the colonization of the root surface occurs mainly from the soil adjacent to the root.

The biotic status of the soil markedly affects the colonization of microorganisms on roots. *E. cloacae* moved from treated seeds onto the entire surface of roots growing in sterile soil or in soil-free moist chambers. However, if seeds treated with *E. cloacae* were planted in nonsterile soil, only the upper one-fourth of the root was colonized by *E. cloacae*. Competition has been suggested to have a profound impact on succession and the composition of microbial communities (1). For example, Rai and Upadhyay (21) show that colonization of pigeon pea substrates by *Fusarium nudum* Butler was highly suppressed if the substrates had already been colonized by other soil fungi. Franco and Vincent (4) demonstrated that related strains of *Rhizobium* and strains with similar growth habits compete with each other in the colonization of the rhizoplane of *Macroptilium atropurpureum* (DC) Urb. In the present study, the results obtained by planting antagonist-treated seeds in soils with part of the microbial population removed indicate that bacteria compete more strongly in the rhizosphere for similar resources (e.g., nutrients and space) with other bacteria than with fungi and vice versa. For example, in soil containing fungi but no bacteria, *T. harzianum* ATCC 20737 did not colonize the pea rhizosphere, while *E. cloacae* colonized a greater portion of the root than it did in untreated soil. These data indicate that failure of the introduced microorganisms to move vertically in the rhizosphere is caused by their inability to compete with the indigenous soil microflora.

When antagonist-treated seeds were planted in sterile soil, more fungal propagules were found in the surrounding soil than in the rhizosphere, suggesting that under favorable conditions (e.g., when nutrients are available) antagonistic fungi are more likely to colonize soil than the root surface. Conversely, the bacteria tested were always found in greater numbers in the rhizosphere than in the surrounding soil. These results suggest that in the rhizosphere, populations of bacteria and fungi may avoid competition by colonizing different niches and/or by some degree of spatial separation within the rhizosphere. Consequently, combinations of compatible bacteria and fungi may provide better control of seed and root-rotting pathogens than either used alone.

Our study demonstrates that, in untreated soil, biological control agents introduced as seed treatments do not move onto roots. Therefore, for either bacterial or fungal seed treatments to be able to protect subterranean plant parts reliably, ways must be found to overcome the deleterious effects of soil microflora. Water used for irrigation will aid to some extent in the vertical distribution of biological control agents applied as seed treatments. Other beneficial practices may include enhancing the growth of specific

TABLE 6. Numbers[a] of *Trichoderma harzianum* and *Enterobacter cloacae* in the pea rhizosphere at various distances from treated seeds[b] planted in bacteria-free or fungus-free soil

	CFU × 10^4			
	Bacteria-free soil		Fungus-free soil	
Location	*T. harzianum*	*E. cloacae*	*T. harzianum*	*E. cloacae*
Spermosphere[c]	12.3 ± 4.9	89,000 ± 32,000	560 ± 290	44,000 ± 8,000
Rhizosphere[d]				
1st cm	<0.002	590 ± 400	4.7 ± 6.6	0.40 ± 0.36
2nd cm	<0.002	630 ± 160	0.04 ± 0.06[e]	<0.002
3rd cm	<0.002	17 ± 21	<0.002	<0.002
4th cm	<0.002	<0.002	<0.002	<0.002

[a] Numbers of *T. harzianum* ATCC 20737 and *E. cloacae* ATCC 39979 were counted 5 and 6 days, respectively, after planting.
[b] Initial numbers were 2.0×10^6 and 9.4×10^7 colony-forming units (CFU) per seed for *T. harzianum* ATCC 20737 and *E. cloacae* ATCC 39979, respectively.
[c] Values in this row are number of CFU per spermosphere sample + standard deviation.
[d] Values are number of CFU per centimeter of root ± the standard deviation; beginning just below the seed.
[e] Two out of four replicates, no CFU were detected at the lowest dilution.

TABLE 7. Numbers[a] of *Trichoderma harzianum, Enterobacter cloacae*, and *Pseudomonas fluorescens* in pea rhizosphere at various distance from treated seeds[b] planted in untreated soil in the presence ($+H_2O$) or absence ($-H_2O$) of percolating water

	CFU × 10^4					
	T. harzianum		*E. cloacae*		*P. fluorescens*	
Location	$+H_2O$[c]	$-H_2O$	$+H_2O$	$-H_2O$	$+H_2O$	$-H_2O$
Spermosphere[d]	109 ± 63	640 ± 220	1,600 ± 1,540	440 ± 350	138 ± 54	2,050 ± 2,230
Rhizosphere[e]						
1st cm	180 ± 96	25 ± 14	7.0 ± 4.8	9.4 ± 7.1	117 ± 89	53 ± 24
2nd cm	9 ± 7.8	1.1 ± 1.6	1.1 ± 0.9	2.5 ± 1.2	10 ± 13	13 ± 13
3rd cm	1.8 ± 2.2	<0.01	0.3 ± 0.3	<0.002	0.2 ± 0.4[f]	<0.002
4th cm	0.1 ± 0.23	<0.01	0.005 ± 0.004	<0.002	<0.002	<0.002
5th cm	0.02 ± 0.04[e]	<0.01	<0.002	<0.002	<0.002	<0.002

[a] Counted 12 days after planting.
[b] Initial numbers were 2.0×10^6, 9.4×10^7, and 9.3×10^8 colony-forming units (CFU) per seed for *T. harzianum* ATCC 20737, *E. cloacae* ATCC 39979, and *P. fluorescens*, respectively.
[c] Water (10 ml) was added every other day for 6 days.
[d] Values in this row are number of CFU per spermosphere sample ± the standard deviation, beginning just below the seed.
[e] Values are number of CFU per centimeter of root ± the standard deviation; beginning just below the seed.
[f] No CFU were detected in two of the four replicates.

organisms by adding selected C or N sources (7), addition of low levels of pesticides or other materials favoring beneficial microorganisms (8,18), modifications of plants to enhance growth of selected microorganisms (22), and selection of organisms with good root colonizing ability (24). However, care should be taken in assessing the root-colonizing capacity of various microorganisms. A false conclusion can be reached by using the entire rhizosphere as a sample, since portions near inoculum sources are likely to be much more heavily colonized than portions further away.

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