Survival and Proliferation of Propagules of *Trichoderma* spp. and *Gliocladium virens* in Soil and in Plant Rhizospheres

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ABSTRACT

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Laboratory-grown conidia of *Trichoderma* spp. and *Gliocladium virens* were sensitive to soil fungistasis. Germination of laboratory-grown conidia in natural soil varied from 1 to 22%, depending on the species used. In contrast, chlamydospores from a liquid fermentation system or from potato-dextrose broth germinated readily in soil (39–99%). *Trichoderma* spp. and *G. virens* grew vertically downward through soil (<2.0 cm) and the numbers of propagules increased 100-fold (from ~ 10^4 to 10^6) when either of the two genera was added in fermentor biomass containing traces of a food base and consisting mostly of chlamydospores. No significant increases occurred when conidia without a food base were added to soil. *Trichoderma* spp. were isolated from the rhizosphere of potato plants 4 and 8 wk following seed piece treatment with fermentor biomass of both fungi. Populations of both *Trichoderma* spp. and *G. virens* increased in soil planted with cotton to which fermentor biomass was added. However, the rhizosphere and nonrhizosphere populations were not significantly different which indicates that the food base was one of the factors responsible for the increase.

Species of the genera *Trichoderma* and *Gliocladium* have been evaluated by many workers for efficacy in the biocontrol of fungal plant pathogens (13). These potential biocontrol agents produce both chlamydospores and conidia. Chlamydospores of *Trichoderma* and *Gliocladium* form in liquid and solid media (6) and on organic substrates buried in soil (7). Chlamydospores may be more important than conidia in the long-term survival of these genera in soil (4,7,13).

Although there is considerable published information on soil fungistasis and its effects on conidia of *Trichoderma* spp. (9,16,17), nothing is known about its effects on chlamydospores. Since 1958, when Caldwell (4) published the results of studies on the fate of spores of *T. viride* in soil, and until recently (6,7), chlamydospores were not considered in ecological studies of the two genera. Conidia of *Trichoderma* spp. were found to be either very sensitive to fungistasis (16) or relatively insensitive (10,11). Various organic amendments added to soils partially or completely annulled fungistasis to conidia of *Trichoderma* spp. with the effect being more pronounced in acid than in alkaline soil (17). Also, a relationship seemed to exist between the spore type or size of propagules of a fungus sensitive to fungistasis, with smaller spores being more sensitive to fungistasis than larger ones (16) and hyphae being less sensitive than conidia (5). The role of fungistasis in the survival of chlamydospores of *Trichoderma* and *Gliocladium* has not been studied.

An inexpensive liquid medium (molasses and brewer's yeast) has been used in a fermentation system to produce biomass of *Trichoderma* spp. and *Gliocladium* spp. (14). The biomass, consisting largely of mycelial fragments and chlamydospores plus residual nutrients, will be hereafter referred to as fermentor biomass (FB). Dry, ground FB that passed a 425-μm pore size sieve contained aggregates of chlamydospores held together by mycelial fragments and occasional conidia. Ground FB mixed with Pyrax® ABB and added in small amounts (0.1%, w/w) to nonsterile soil proliferated greatly in a few weeks from 5×10^3 colony-forming units (cfu) per gram of dry soil at zero time to as high as 7×10^7 cfu per gram of soil (14). In contrast, the numbers of conidia added to soil remained constant for 10–20 days and then declined.

The present study was undertaken to determine the differential effects of soil fungistasis on propagules of three *Trichoderma* spp. and *G. virens*, elucidate the reasons for increased cfus when the fungi are added to soil as FB, and determine whether the two antagonists colonize the rhizosphere of crop plants when added to soil or seed.

MATERIALS AND METHODS

The following isolates of *Trichoderma* and *Gliocladium* were used: *T. hamatum* (Bon.) Bain. (Tri-4), *T. harzianum* Rifai (WT-6), *T. viride* Pers. ex Gray (benomyl resistant mutant T-1-R9), and *G. virens* Miller et al (Gl-21). Stock cultures were maintained on V-8 juice agar (200 ml of V-8 juice, 800 ml of water, 15 g of agar, and 6.0 ml of 1.0 N NaOH) slants.

Production of propagules. For production of conidia, all isolates were grown on V-8 juice agar in petri plates under continuous cool white fluorescent light (700 μEin/m^2/sec) at room temperature for 7 days. Conidial suspensions were prepared by gently washing the agar surface with sterile distilled water with the use of a cotton-tipped applicator. Chlamydospores were produced by growing the isolates for 2 days in 0.1% Difco potato-dextrose broth (PDB) (6). Conidia and chlamydospore suspensions were washed three times by alternate centrifugation (1,085 RCF, 10 min each) and resuspensed in sterile distilled water. FB was produced in 20-L autoclavable polypropylene carboys in a molasses brewer's yeast medium as described previously (14). The mycelium was harvested by filtration through a cotton muslin filter on an 18-cm Büchner funnel, ground in a blender for 5 min, and passed through a 10-μm pore size nylon mesh (Nitex® Swiss nylon monofilament fabric,

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Tetko, Inc., Elmsford, NY) to remove conidia and lose hyphal fragments. The residue was washed into a Ten Broeck® tissue grinder and ground for several minutes. The suspensions were washed through a 15-μm pore size nylon mesh to obtain chlamydospores in the filtrate.

Germination of propagules in vitro and in soil. One-half-milliliter suspensions containing 5.0×10^4 washed conidia or chlamydospores were added to 25-mm diameter type HA Millipore® membrane filters (0.45 μm) and the liquid was removed by filtration. Membranes with conidia or chlamydospores were either placed on sterile moistened filter paper in petri plates or buried in a Rumsford sandy loam (pH 6.3, −0.1 bars moisture) in Coors crucibles (1) at room temperature. Each treatment was replicated four times. After 24 hr, membranes were removed from dishes or soil, placed on glass slides, stained with 0.02% trypan blue in lactophenol (lactic acid:phenol:glycerine:distilled water [1:1:1:1]) and examined with a bright-field microscope for conidium and chlamydospore germination. One hundred spores were counted per membrane.

Analysis of variance was conducted using a factorial treatment arrangement including the following factors: isolate (T-1-R9, WT-6, Tri-4, Gl-21), spore type (conidia, chlamydospores), and treatment (soil, no soil). Planned orthogonal comparisons were run by partitioning treatment sums of squares. All experiments were repeated a second time with four replications.

Soil colonization by *Trichoderma* spp. and *G. virens*. Conidia and FB were evaluated for their ability to grow and colonize soil placed in segmented plastic cylinders (3.1 cm ID). Cylinder segments each 2 cm in height were taped together in groups of four (four replications). The lower three segments of the cylinders were filled with uninfested Rumsford sandy loam (−0.1 bars moisture). The top segment of each cylinder was filled with soil infested with 10^4 cfu per gram of soil of either washed conidia or FB of *T. viride* (T-1-R9), *T. harzianum* (WT-6), *T. hamatum* (Tri-4), or *G. virens* (Gl-21). Close contact between infested and uninfested soil was made and care was taken to avoid compacting the soil in the cylinders. The bottom and top of each cylinder were plugged with a rubber stopper covered with aluminum foil. Each treatment was replicated eight times and the experiment was done five times. The 2-cm soil cylinder segments were separated after 0, 3, 6, 12, and 24 days and aqueous dilutions were made by suspending 1-g soil samples from each segment into distilled water. Precautions were taken to avoid cross contaminations when sampling from soil and cylinders. One milliliter of suspensions were placed on Trichoderma medium E (TME) (15). Plates were incubated under continuous light for 6 days and colonies were counted. Analysis of variance was conducted and means were separated by using an LSD test, $P = 0.05$.

Colonization of the rhizosphere. Dried FB of *T. viride* (T-1-R9) or *T. harzianum* (WT-6) was added to Pyrax® ABB (anhydrous aluminum silicate, pH 7.0, R. T. Vanderbilt Co., Inc., Norwalk, CT) to provide a mixture containing 10^5 cfu per gram. Bare conidia were not added to potato seed pieces or cotton soil since previous tests indicated that such propagules do not colonize the rhizospheres (12). Whole potato tubers (*Solanum tuberosum* L. 'Superior') were dusted with 0.5 g each of FB containing mycelial fragments and chlamydospores of the antagonists and planted singly in 25.4-cm-diameter plastic pots containing 6 kg of sieved Rumsford sandy loam. Twenty potatoes were planted per pot (treatment) and each treatment had six replications. Potatoes dusted with Pyrax® ABB alone served as a control. The plants were harvested after 4 and 8 wk, and soil closely adhering to the tuber surface, the root surface of as many roots as could be recovered from soil, and nonrhizosphere soil was collected. One-gram samples were diluted with distilled water to appropriate dilutions and 1-ml quantities were placed on TME. Plates were incubated for 6 days under continuous fluorescent light and colonies were counted. All experiments were performed twice. Analysis of variance was done and means were separated using an LSD test. Data are presented as the means from one experiment.

Dried FB of *T. viride* (T-1-R9), *T. harzianum* (WT-6), and *G. virens* (Gl-21) was added to 4-kg batches of sieved Rumsford sandy loam to provide 10^3–10^4 cfu per gram of soil. A control treatment consisted of noninfested soil. Soil was dispersed into 12-cm-diameter pots. Cotton (*Gossypium hirsutum* L. 'Acala 4-42') seed dusted with metalaxyl (Ciba-Geigy Corp., Greensboro, NC) were planted in infested and noninfested soil, and after 0, 2, and 5 wk, cotton plants were removed from pots and allowed to dry at room temperature. Nonrhizosphere soil was collected from areas in the soil where roots had not grown. Rhizosphere soil samples from soil clinging to the roots and nonrhizosphere soil were assayed for the test fungi with the dilution-plate method on the TME medium as before. All experiments were done twice with six replications. Analysis of variance was done and means were separated by using an LSD test. Data are presented as the mean of two experiments.

TABLE 1. Germination of conidia and chlamydospores of *Trichoderma* spp. and *Gliocladium virens* in vitro and in soil

Species and isolate no.	Germination (%)			
	In vitro[y]		In soil[z]	
	Conidia	Chlamydospores	Conidia	Chlamydospores
T. viride (T-1-R9)	56	67	1	39
T. harzianum (WT-6)	15	89	22	94
T. hamatum (Tri-4)	57	92	6	54
G. virens (Gl-21)	81	93	13	99

[y]Spores were placed on Millipore membranes (5×10^4 spores per 0.5-ml drop) on moist filter paper in petri dishes. After 24 hr spores were stained and germination counts were made with a light microscope.

[z]Spores were placed on millipore membranes (5×10^4 spores per 0.5-ml drop) and buried in Rumsford sandy loam in Coors crucibles. After 24 hr, membranes were removed from soil, stained with 0.02% trypan blue in lactophenol, and examined with a bright-field microscope. One hundred spores were counted for germination.

RESULTS

Germination of propagules of *Trichoderma* spp. and *G. virens* in vitro and in soil. Fungal isolate, spore type, and treatment each had a significant effect ($P < 0.05$) on germination of *Trichoderma* and *Gliocladium* conidia and chlamydospores. All main effects and interactions were significant. Planned orthogonal contrasts of conidia versus chlamydospores in soil for each fungal isolate revealed a significant effect of soil on germination of *Trichoderma* and *Gliocladium* spores (Table 1). Washed conidia of *Trichoderma* and *Gliocladium* were sensitive to soil fungistasis. No more than 22% germination of conidia had occurred within 24 hr in the soil treatments, regardless of isolate. In contrast, washed chlamydospores germinated readily and in large numbers (39–99%) within 24 hr after placement in soil. This was true of all isolates. Chlamydospores of *T. viride* germinated in soil less frequently than those of *T. harzianum*, *T. hamatum*, and *G. virens*. Washed chlamydospores grown in 0.1% PDB also germinated readily (63–93%) within 24 hr when placed on membranes in soil.

Soil colonization. FB (consisting mostly of chlamydospores) added to the soil in the first cylinder segment (0–2 cm) at initial concentrations of 10^4 propagules per gram increased an average of 100-fold within 6 days (Table 2). All four isolates were consistently isolated from soils in the second segment (2–4 cm) in which numbers of propagules were not significantly different from numbers present in the top segment in most treatments. Since care was taken to avoid contamination during the sampling of soil segments, results indicate that *Trichoderma* spp. and *G. virens* added as FB grew through soil and increased over time. Total growth of the fungi evaluated did not exceed 2 cm in any treatment since *Trichoderma* spp. and *G. virens* were not isolated from soils in the lower third and fourth segments. Data from segments 3 and 4 are not shown in the table.

Population densities of *Trichoderma* spp. and *G. virens* did not significantly increase in numbers when added to soil as conidia (data not in Table 2). Washed conidia added initially to segment 1 at concentrations of 10^4 propagules per gram remained at this concentration in segment 1 (10^4 propagules per gram) throughout

the 24-day experiment. Propagules of *Trichoderma* spp. and *G. virens* were isolated from soils in the second segment. However, numbers of propagules were significantly less than those added initially to the top segment.

Colonization of the rhizosphere. Populations of *T. viride* (T-1-R9) and *T. harzianum* (WT-6) were consistently isolated from the rhizosphere of potato plants 4 and 8 wk following treatment of the seed piece with FB (Table 3). By 4 wk. *T. viride* (T-1-R9) was recovered at 10^3 cfu per gram of rhizosphere soil. *T. viride* (T-1-R9) survived better than *T. harzianum* (WT-6) in both the rhizosphere and on the tuber surface after 8 wk. A higher population density of both the antagonists was observed in soil adhering to the potato tuber surface than in rhizosphere soil. The fungi either were not recovered from nonrhizosphere soil or were present in low numbers. *T. viride* or *T. harzianum* were not recovered from rhizosphere, nonrhizosphere, or tuber surface soil in the control treatments.

Propagules of all the isolates added as FB increased by 2 wk in soil planted to cotton (Table 4). The 100-fold increase in the population densities of *Trichoderma* spp. and *G. virens* occurred whether soil was planted to cotton or left unplanted (Table 2). *Trichoderma* spp. and *G. virens* were present in cotton rhizosphere soil at high population densities (10^6 cfu per gram of soil). However, no rhizosphere effect was demonstrated since numbers of propagules in rhizosphere soil were not significantly different from numbers in nonrhizosphere soil. By 5 wk, lower population densities of the antagonists were present in both rhizosphere and nonrhizosphere soils than population densities at 2 wk. *T. viride*, *T. harzianum*, and *G. virens* were not isolated from rhizosphere or nonrhizosphere soil in the uninfested control treatment.

DISCUSSION

Conidia of all three *Trichoderma* spp. and *G. virens* germinated poorly in soil and were sensitive to soil fungistasis whereas chlamydospores germinated readily and in great numbers in the soil used in these experiments. The differential effect of soil fungistasis on the two kinds of propagules was evident regardless of the initial medium used to grow the fungi or the fungal species evaluated. Significant differences within the genus *Trichoderma* in the total percent germination of chlamydospores were evident (Table 1). Chlamydospores of *T. viride* germinated less often in soil than those of the other two species and of *G. virens*. This study supports the findings of Steiner and Lockwood (16) who concluded that large fungal spores tend to germinate rapidly and exhibit lower sensitivity to soil fungistasis than small spores. This study also supports the initial conclusions of others that chlamydospores of *Trichoderma* and *Gliocladium* are more important than conidia in the survival and proliferation of the two genera in soil (4,7).

The addition of FB containing chlamydospores of *Trichoderma* spp. and *G. virens* to soil resulted in a burst of activity of the antagonist. The residual nutrients (molasses and brewer's yeast) present in close association with the chlamydospores in the dried FB supplied an adequate food base to support growth and sporulation of the fungi in soil. Significant increases in antagonist propagules were not obtained when washed conidia were added to soil without a food base. Dried FB consists of many chlamydospores held together in mycelial mats (14). The breakdown of chlamydospore aggregates by lytic soil microbes, however, is not an adequate explanation for the great increase in the numbers of propagules of *Trichoderma* spp. and *G. virens* in the soil. Several 100-fold increases in population densities were found in soil adjacent to antagonist-infested soil in the cylinders. Since proliferation occurred away from the infested soil segment (Table

TABLE 2. Growth and colonization of soil in plastic cylinders by fermentor biomass of *Trichoderma* spp. and *Gliocladium virens*[y]

Species and isolate	Soil segment	Colony-forming units ($\times 10^3$) per gram of soil at day: 0	3	6	12	24
T. viride						
T-1-R9	1	18 b[z]	350 a	1,100 a	2,000 a	560 a
T-1-R9	2		18 b	1,200 a	230 b	160 a
T. hamatum						
Tri-4	1	9 b	11 a	5,000 a	960 a	460 a
Tri-4	2		1 a	2,700 a	310 a	400 a
T. harzianum						
WT-6	1	11 b	1,000 a	3,700 a	3,100 a	480 a
WT-6	2		1,200 a	3,700 a	1,300 a	390 a
G. virens						
Gl-21	1	11 b	280 a	8,600 a	6,100 a	2,400 a
Gl-21	2		37 a	1,400 a	220 b	430 a

[y] Plastic soil cylinders 2 cm long were taped together in groups of four and filled with soil. In segment 1, soil was infested with 10^4 colony-forming units of fermentor biomass of *Trichoderma* and *Gliocladium* per gram of soil while segments 2 through 4 were filled with uninfested soil. The ends of each cylinder were plugged with aluminum foil-covered rubber stoppers. Segment 1, 0–2 cm depth; segment 2, 2–4 cm depth. Colony-forming units were determined by the dilution plate method on Trichoderma medium E (15). No *Trichoderma* or *Gliocladium* was recovered from segments 3 and 4 in any treatment.

[z] Means in a column for each species followed by the same letter are not significantly different according to LSD ($P = 0.05$) test. Each mean was based on eight replications.

TABLE 3. Proliferation of propagules from fermentor biomass of *Trichoderma viride* and *T. harzianum* in rhizosphere, nonrhizosphere, and potato tuber-surface soils[y]

Species and isolate no.	Treatment	Colony-forming units per gram of soil: 4 wk	8 wk
T. viride			
T-1-R9	Nonrhizosphere	0[z]	0
T-1-R9	Rhizosphere	1,520 b	730 b
T-1-R9	Tuber surface	81,000 a	21,000 a
T. harzianum			
WT-6	Nonrhizosphere	43 b	0
WT-6	Rhizosphere	1,690 ab	290 a
WT-6	Tuber surface	5,300 a	330 a
Pyrax control			
None	Nonrhizosphere	0	0
None	Rhizosphere	0	0
None	Tuber surface	0	0

[y] Fermentor biomass was applied to potato tubers at 1.3×10^5 (T-1-R9) and 6.0×10^4 (WT-6) colony-forming units per seed piece. Colony-forming units were determined with the dilution plate method on Trichoderma medium E (15).

[z] Means in a column for each species followed by the same letter are not significantly different according to the LSD ($P = 0.05$) test.

TABLE 4. Survival of fermentor biomass of *Trichoderma viride*, *T. harzianum*, and *Gliocladium virens* in cotton rhizospheres and in nonrhizosphere soil[y]

Species and isolate	Treatment	Colony-forming units per gram of soil (10^4): 0 wk	2 wk	5 wk
T. viride				
T-1-R9	Nonrhizosphere	0.5	50.0 b[z]	13.5 a
T-1-R9	Rhizosphere		53.0 b	7.0 b
T. harzianum				
WT-6	Nonrhizosphere	6.0	320.0 a	5.7 a
WT-6	Rhizosphere		220.0 a	3.6 b
G. virens				
Gl-21	Nonrhizosphere	0	270.0 a	14.8 a
Gl-21	Rhizosphere		300.0 a	11.7 a
Noninfested				
None	Nonrhizosphere	0	0	0
None	Rhizosphere		0	0

[y] Fermentor biomass was added to soil at 10^4 colony-forming units per gram of soil. Rhizosphere and nonrhizosphere soil was sampled and dilutions were made on Trichoderma medium E (15).

[z] Means in a column for each species followed by the same letter are not significantly different according to the LSD ($P = 0.05$) test.

2), it is believed that the chlamydospores germinated and the resulting hyphae grew through soil and produced conidia and chlamydospores. The green color of the soil visible in the plastic segmented cylinders at points below the initial antagonist-infested soil also supports this observation.

Trichoderma spp. were isolated from the rhizosphere of potato plants both 4 and 8 wk following treatment of the seed pieces with FB containing chlamydospores of the antagonists. It is not clear whether the presence of *Trichoderma* spp. in the rhizosphere was due to an active increase via germination and growth of hyphae from the antagonist-infested seed piece down the roots. Potato roots develop from the underground stem between the seed piece and the soil surface. Roots growing near the tuber surface may have carried *Trichoderma* with them as they grew through soil. The downward movement of water in the pots also may have been responsible for movement of the antagonist to the roots.

Propagules of *Trichoderma* spp. and *G. virens* added as FB proliferate in soil and on roots of cotton. Although the antagonists were isolated from the rhizosphere of cotton, no rhizosphere effect was demonstrated since high levels of the antagonists were found in nonrhizosphere soil. However, the presence of the antagonists at high population densities on the roots of cotton may be sufficient to suppress root pathogens. The increase was not observed in other rhizosphere studies when conidia were added to soil (12).

A potential method for introducing species of *Trichoderma* and *Gliocladium* into soils or on seed is via the addition of FB of the two fungi. The establishment of viable populations of antagonists in soil and on roots is necessary for disease control. Disease incidence, severity, sclerotial viability, and soilborne inoculum levels of *Rhizoctonia solani* on potato were reduced by FB containing chlamydospores of *T. viride* and *G. virens* (2,3). Sodium alginate pellets containing FB of *Trichoderma* spp. incorporated into soil also suppressed soilborne populations of *R. solani* (8). The active proliferation of the antagonists concommitant with disease reduction provided an effective means of biocontrol in these studies. Similar effects were not observed when conidia were used in such studies.

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