Biological Control of Rhizoctonia solani Damping-Off with Wheat Bran Culture of Trichoderma harzianum

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ABSTRACT

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An isolate of *Trichoderma harzianum* directly attacked the mycelium of *Rhizoctonia solani* when the two fungi were grown together on a glucose plus minerals medium. No antibiotic activity of *T. harzianum* towards the pathogen could be detected. When grown on a mineral medium containing laminarin or chitin as carbon sources, *T. harzianum* produced the enzymes β-(1-3) glucanase and chitinase. In the greenhouse, *T. harzianum,* applied in the form of wheat bran culture to *R. solani*-infested soil, effectively controlled damping-off of bean, tomato, and eggplant seedlings. Low concentrations of PCNB, ineffective alone, improved disease control when applied together with *T. harzianum.*

Additional key words: biocontrol, integrated control, fungal antagonism.

In preliminary experiments, 70 different cultures of bacteria and fungi were isolated from loamy sand infested with *Rhizoctonia solani* Kühn. When tested on a glucose mineral synthetic medium, the most potent antagonist was an isolate of *Trichoderma harzianum* Rifai. This finding was not surprising because members of the genus *Trichoderma* are active both as hyperparasites and as antibiotic producers (2, 11).

Weindling and Emerson (26) isolated substances toxic to *R. solani* from a culture of *T. lignorum*. This antagonist, later identified as a species of *Gliocladium* rather than *Trichoderma* (25), controlled damping-off caused by *Rhizoctonia* sp. in citrus seedlings (27).

The antagonistic activity of *T. harzianum* against several fungi, including *R. solani*, was reported by Dennis and Webster (8). Wells et al (28) were the first to report field control of *Sclerotium rolfsii* Sacc. by the infestation of soil with *T. harzianum* grown on an autoclaved mixture of ryegrass seeds and soil. Backman and Rodriguez-Kabana (1) used molasses-enriched clay granules both as a food base for growing the same antagonist, and as a carrier to facilitate dispersal in the field. They observed a significant decrease in *S. rolfsii* damage to peanuts and an increase in yield during a 3-yr test. These granules however, were ineffective when used by Kelley (13) for the control of *Phytophthora cinnamomi* causing damping-off in pine seedlings.

In this study, we determined the mode of antagonism of *T. harzianum* against *R. solani* and studied the potential use of the antagonist, grown in wheat bran culture, for control of damping-off in greenhouse-grown vegetable seedlings.

MATERIALS AND METHODS

Antagonism in culture.—The antagonistic ability of fungi, isolated from *R. solani*-infested soil, against soilborne pathogens was tested as described by Dennis and Webster (8) using synthetic medium (SM) agar containing (g/liter): $MgSO_4 \cdot 7H_2O$, 0.2; K_2HPO_4, 0.9; KCl, 0.2; NH_4NO_3, 1.0; glucose, 15; Fe^{2+}, 0.002; Mn^{2+}, 0.002; Zn^{2+}, 0.002; thiamine hydrochloride, 0.0001; agar, 20; according to Okon et al (20).

The most efficient antagonist against *R. solani* was isolated, grown on malt extract agar (Difco), and identified as *Trichoderma harzianum* (23). Samples for scanning electron microscopy, using a Cambridge Stereoscan Model F-180 electron microscope, were prepared as described by Lisker et al (14).

Production of lytic enzymes by Trichoderma harzianum.—Erlenmeyer flasks (250-ml) each containing 50 ml of SM, were seeded with 0.1 ml of a suspension containing 0.1 ml of 10^6 conidia/ml) and incubated in a rotary shaker (New Brunswick Scientific Co., New Brunswick NJ 08903 USA) at 180 rpm for 3 days at 30 C.

Cultures were centrifuged aseptically as 27,000 *g* for 20 min at 4 C. The mycelium was washed with sterile tap water, transferred to flasks containing fresh growth media of various compositions, and incubated for 6 hr as described above. The mycelium was then collected by centrifugation at 27,000 *g* for 20 min at 4 C. The supernatant was filtered through Whatman No. 1 filter paper and lyophilized.

Assay procedures.—Chitinase [E.C.3.2.1.14] activity was determined by following the release of N-acetylglucosamine according to Reissing et al (22). Specific activity was expressed as μmoles N-acetylglucosamine/mg protein/hour. The reaction mixture contained 2.0 ml of 0.1 M phosphate buffer (pH 5.1), 1.6 mg colloidal chitin [prepared according to Collins (5)] or *R. solani* cell walls [prepared according to Chet and Henis (4)], and lyophilized material prepared from 10 ml cell-free culture supernatant. The reaction was carried out for 2 hr in a water-bath at 37 C and stopped by immersing the tubes in boiling water for 15 min.

The activity of β-(1-3) glucanase [E.C.3.2.1.39] was determined by following the release of free glucose, using the glucose oxidase reagent (Sigma Chemical Co., St.

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Louis, MO 63178 USA) according to the directions of the manufacturer. Specific activity was expressed as μmole glucose/mg protein/hour. The reaction mixture contained 2.0 ml of 0.1 M citrate buffer (pH 4.7), 1.6 mg soluble laminarin (Sigma) or cell walls of *R. solani*, and lyophilized material from 10 ml of cell-free culture supernatant. The reaction, carried out for 2 hr at 45 C, was stopped by immersing the tubes in a boiling water-bath for 15 min.

Protein content of the enzyme solution was determined by the Folin phenol reagent, according to Lowry et al (15).

Greenhouse experiments.—noninfested, natural loamy sand (82.3% sand, 2.3% silt, 15.4% clay, and 0.45% organic matter; pH 7.4; moisture-holding capacity, 12.2%) was used in all greenhouse experiments. This soil was artificially infested with *R. solani*. The fungus, grown as a thin layer of mycelium on yeast extract dextrose broth (9) for 8 days at 28 C, was homogenized in a Waring Blendor for 60 sec and mixed with the soil at a final concentration of 250 mg (wet wt)/kg soil. Medium for growing inocula of *T. harzianum* was prepared by autoclaving a wheat bran and tap water mixture (1:2 v/v) for 1 hr a day at 121 C on two successive days. Flasks containing this medium were inoculated with *T. harzianum*, incubated for 3 days at 30 C, illuminated for 12 hr and then incubated for 5 days. A preparation of *T. harzianum* at various concentrations, was mixed with the soil after the infestation with *R. solani*.

The following plants were used: eggplant (*Solanum melongena* L. 'Black Queen'), tomato (*Lycopersicon esculentum* Mill. 'Rehovot 13') and bean (*Phaseolus vulgaris* L. 'Brittle Wax').

Beans were sown in plastic boxes (9×9×10 cm) (nine seeds per box) containing 500 g of infested soil. Disease incidence was recorded 21 days after inoculation. Eggplant and tomato seedlings (1 day after emergence) were planted in infested soil (nine seedlings per box). Disease incidence was recorded until damping-off stopped. Plants were grown under greenhouse conditions at 24-30 C. Disease incidence was expressed either as percentage of diseased seedlings, or determined according to the disease index of Sneh et al (24). Isolation from randomly selected diseased seedlings, yielded the same pathogen that had been used for inoculation. All treatments were carried out in six replicates. Experiments were repeated at least twice.

RESULTS

Antagonism and lytic activity.—The spectrum and nature of the antagonism of *T. harzianum* towards the following soilborne plant pathogens were tested: *Sclerotium rolfsii* Sacc., *Fusarium oxysporum* f. sp.

TABLE 1. The effect of various carbon sources on the activity of extracellular β-(1-3) glucanase and chitinase produced by *Trichoderma harzianum* grown on a mineral medium

Carbon source in the culture medium	Substrate in the reaction mixture	β-(1-3) glucanase activity (μmole glucose/mg protein/hr)	Chitinase activity (μmole N-acetylglucosamine/mg protein/hr)
Laminarin[a]	Laminarin	62.0[b]	
Chitin	Chitin		2.50
R. solani cell wall	Laminarin	43.1	
R. solani cell wall	Chitin		1.03
R. solani cell wall	*R. solani* cell wall	15.6	0.27

[a]Laminarin, chitin, and *Rhizoctonia solani* cell wall concentration: 2 mg/ml.
[b]Each number represents the mean of at least three replicates. The experiment was repeated twice.

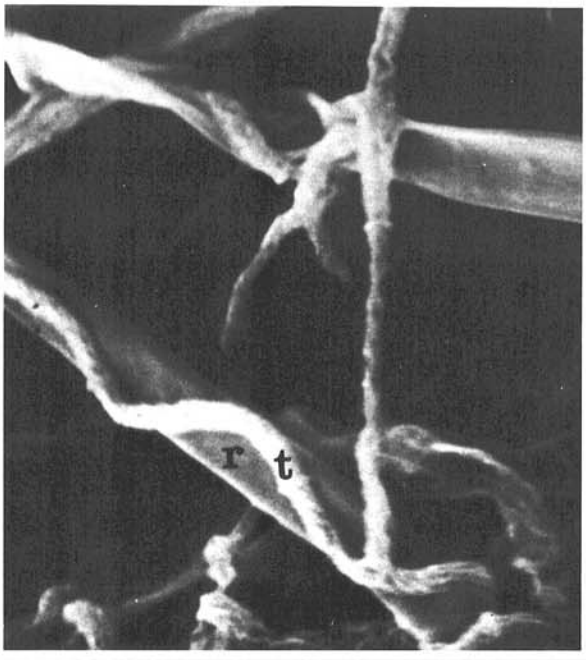

Fig. 1. Scanning electron micrograph of hyphal interaction between *Trichoderma harzianum* (t) and *Rhizoctonia solani* (r). (×1,300).

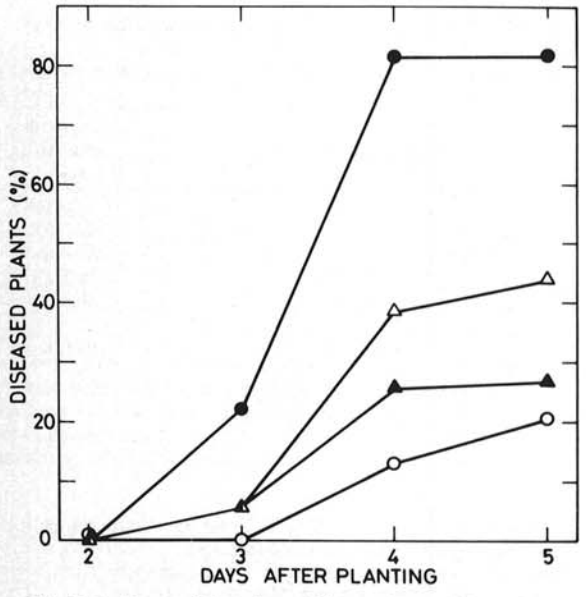

Fig. 2. Incidence of damping-off in eggplant seedlings planted in soil infested with *Rhizoctonia solani* (•——•) compared with seedlings planted in infested soil amended with *Trichoderma harzianum* preparation: 1.5 g/kg (Δ——Δ); 2.5 g/kg (▲——▲); 3.5 g/kg (o——o). Treatments were significantly different ($P = 0.05$) from the control from the third day, using Duncan's multiple range test.

lycopersici Sacc. Snyd. and Hans., *Sclerotinia sclerotiorum* de Bary, *Rhizoctonia solani* Kühn, and *Pythium aphanidermatum* Edson (Fitz.). *Trichoderma harzianum* attacked only *R. solani* and *P. aphanidermatum*. Using the methods of Hsu and Lockwood (11) and Mughogho (19), no antibiotic activity could be detected towards any of the tested fungi.

Trichoderma harzianum and *R. solani* were grown towards each other on a 74-μm (200 mesh) nylon net, placed on SM agar until they met after 50 hr. Then the net was transferred either to carbonless mineral medium or to a synthetic one containing glucose as a carbon source. *Trichoderma harzianum* attacked the mycelium of the pathogen when it served as a sole carbon source; this attack, however, was much more rapid and efficient in the presence of glucose in the medium. Phase-contrast microscopy showed degradation of *R. solani* mycelium in regions of interaction with the antagonistic fungus. Such mycoparasitism was observed by scanning electron microscopy (Fig. 1).

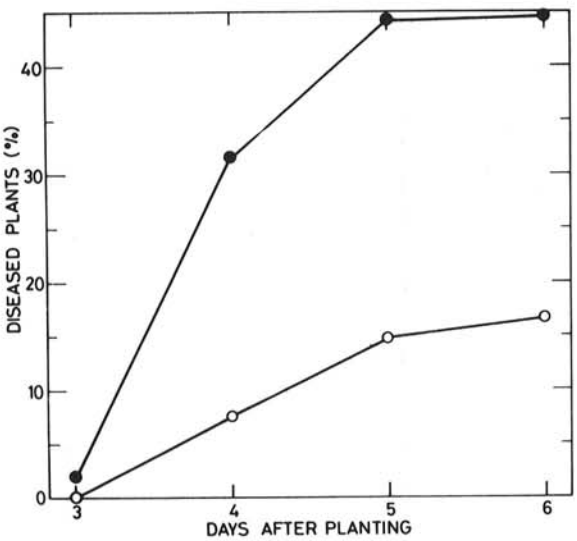

Fig. 3. Incidence of damping-off in tomato seedlings planted in soil infested with *Rhizoctonia solani* (•——•) compared with seedlings planted in infested soil amended with *Trichoderma harzianum* preparation: 1.5 g/kg (o——o). The treatment was significantly different ($P = 0.05$) from the control from the fourth day, using Duncan's multiple range test.

When grown on laminarin- or chitin-supplemented mineral medium, *T. harzianum* produced extracellular β-(1-3) glucanase or chitinase which degraded these polymers, respectively. Chitinase activity (2.5 μmole N-acetylglucosamine/mg protein/hour) was much lower than that of β-(1-3) glucanase (62 μmole glucose/mg protein/hour). Both enzymes were released into the medium when *T. harzianum* was grown on *R. solani* cell walls as a sole carbon source. The enzyme preparation was capable of degrading laminarin or chitin as well as *R. solani* cell walls (Table 1).

The effect of Trichoderma harzianum preparation on damping-off disease under greenhouse conditions.—Of the various agricultural wastes tested, such as manure, sawdust, and wheat bran, wheat bran was the best medium for growth and sporulation of *T. harzianum*. It was chosen therefore as a food base and a carrier for the application of *T. harzianum* to soil. Fungal preparations contained 2.9×10^9 spores/g dry wt. Eighty percent of *T. harzianum* spores in this preparation were viable even after storage for 6 mo at either 4 C or room temperature (24-30 C).

Biological control of damping-off disease, caused by *R. solani*, in beans, tomatoes, and eggplants was achieved by applying *T. harzianum* preparation to infested soil (Fig. 2 and 3 and Table 2). *Trichoderma harzianum*, applied to *R. solani*-infested soil at rates of 1.5-3.5 g/kg soil planted with eggplants, delayed the appearance of symptoms and decreased disease incidence from 80 to 20% (Fig. 2). Similar, though less prominent, control was obtained with bean seedlings (Table 2). In this experiment, two growth cycles (3 wk each) of bean seedlings were carried out in the same soil. *Trichoderma harzianum* had a long-term effect, its efficiency increased during the second growth cycle, which resulted in disease index decline to

TABLE 2. Biological control of *Rhizoctonia solani* by *Trichoderma harzianum* in bean seedlings grown two successive times in the same soil

Trichoderma preparation (g/kg soil)	First growth cycle		Second growth cycle	
	Diseased plants (%)	Disease index[x]	Diseased plants (%)	Disease index[x]
0	52.0 a[y]	1.17 a	69.9 a	1 a
2	48.0 a	1.13 a	50.0 a	0.75 a
6	50.0 a	0.94 a	11.3 b	0.13 b
8	36.5 a	0.46 a	10.0 b	0.12 b
10	30.8 a	0.46 a	5.7 b	0.06 b
Sterile wheat bran (10 g/kg)[z]	94.0 b	2.60 b	No emergence	

[x]Disease index: 0 for healthy plants, and 5 for killed plants.
[y]Column values followed by the same letter are not significantly different ($P = 0.05$), using Duncan's multiple range test. The experiment was carried out in six replicates and repeated twice.
[z]Additional control: killed wheat bran culture of *T. harzianum* enhanced disease caused by *R. solani*.

TABLE 3. The effect of *Trichoderma harzianum* on the incidence of damping-off disease, caused by *Rhizoctonia solani*, in bean seedlings grown in naturally infested soil

Trichoderma preparation (g/kg soil)	Diseased plants (%)	Disease index[x]
0	55 a[y]	1.37 a
3	33 ab	0.64 ab
6	14 b	0.27 b

[x]Disease index: 0 for healthy plants, 5 for killed plants.
[y]Column values followed by the same letter are not significantly different ($P = 0.05$), using Duncan's multiple range test.

0.06. In this cycle only 5.7% diseased plants were observed in the treated soil compared with 69.2% in the control and 30.8% in the treated soil during the first cycle (Table 2). A similar positive effect of *T. harzianum* also was observed with tomato seedlings grown in infested soil (Fig. 3).

Naturally infested terra-rossa soil (20% sand, 15% silt, 65% clay, and 3.04% organic matter; pH 7.45) was taken from a potato field and treated with *T. harzianum* preparation at two concentrations. Reduction in the percentage of both diseased bean plants and disease index was observed with both concentrations of the antagonist (Table 3).

When small noneffective doses (1-2 μg/kg) of pentachloronitrobenzene (PCNB) were applied to soil along with the *Trichoderma* preparation (2 g/kg), disease incidence of eggplants declined from 40 to 13%, while *T. harzianum* alone reduced disease incidence only to 26%. PCNB alone had no effect on disease incidence of eggplant seedlings.

DISCUSSION

When grown on a glucose-mineral medium, our isolate of *T. harzianum* did not excrete any antibiotics. This species is known as a poor antibiotic-producer when compared with other *T.* species groups (7, 19).

In culture *Trichoderma harzianum* attacked *R. solani* and *P. aphanidermatum* but not *S. rolfsii*, *S. sclerotiorum*, and *F. oxysporum*. It is a well-known phenomenon that species or strains of *Trichoderma* may be differentially selective against different fungi (7, 8, 19, 28). Our isolate produced extracellular β-(1-3) glucanase and chitinase which are key enzymes in the lysis of fungal cell walls (4, 18). The antagonist directly attacked *R. solani* in culture, when the pathogen served as a sole carbon source. Of the lytic enzymes produced by *T. harzianum*, β-(1-3) glucanase activity was much higher than that of chitinase. The lytic extracellular enzymes were capable of degrading *Rhizoctonia* cell walls. *Rhizoctonia solani* belongs to the Homobasidiomycetes, the cell walls of which are composed mostly of glucans with only about 6-8% chitin (3). It seems, therefore, that β-(1-3) glucanase is more important in the degradation of cell walls of *R. solani*. Similarly, Jones and Watson (12) showed that β-(1-3) glucanase was the key enzyme in the solubilization of hyphae of *S. sclerotiorum* by *T. viride*.

Whereas Mitchell (16) and Mitchell and Alexander (17) used cell wall components as soil amendments to induce the indigenous lytic soil microflora, in our system a lytic organism was added to the soil along with its food base in order to control *R. solani*. The antagonist was applied to the soil in the form of a wheat bran culture. This nutrient was found to be the best substrate for both the growth of *T. harzianum* and its application to the soil.

In greenhouse experiments damping-off caused by *R. solani* was controlled in eggplants, tomatoes, and beans. The decreasing percentage of diseased plants was positively correlated with the increasing amount of *T. harzianum* preparation added to the soil. This preparation remained active during more than one growth cycle of beans. These results are similar to those published by Wells et al (28) and Backman and Rodriquez-Kabana (1) who used ryegrass and molasses, respectively, as a food base for *T. harzianum*.

Control of damping-off obtained by this preparation was caused specifically by *T. hirzianum*, since wheat bran even alone doubled disease incidence in bean seedlings. A killed culture of *T. harzianum* had no effect on *R. solani*. Similar results, confirming those obtained in the greenhouse, were found with naturally infested soil, too.

Integration of biological and chemical control seems to be a very promising way of controlling pathogens with a minimal interference with biological equilibrium (10, 21). Recently, Curl et al (6) obtained only a slight additive benefit to biological control of the antagonist towards *R. solani* by adding PCNB, at amounts of 2 and 10 μg/g soil, together with *Trichoderma* sp. to sterilized soil. In this study, however, ineffective amounts (1-2 μg/g soil) of PCNB applied together with the *T. harzianum* preparation controlled the disease more effectively than did *T. harzianum* alone. Using a nutrient-rich food base allows *T. harzianum* to continue its growth in the soil and to attack plant pathogens. Moreover this preparation interacts synergistically with sublethal applications of fungicides.

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