Isolation and Biocontrol Potential of *Trichoderma hamatum* from Soil Naturally Suppressive to *Rhizoctonia solani*

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ABSTRACT

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A soil that appeared to be naturally suppressive to *Rhizoctonia solani* was collected from Columbia, South America. Suppressiveness was confirmed in the laboratory by comparing its conducive index (0.2) to that of a conducive Fort Collins clay loam (0.85). *R. solani* propagules repeatedly were added to both soils and the resultant disease incidence on radishes grown in the suppressive soil was lower ($P = 0.01$) than in comparable inoculations in the conducive Fort Collins clay loam. The Colombian soil contained 10^8 propagules of fungi per gram of which 8×10^5 propagules per gram was *Trichoderma hamatum*. When conidia of this fungus were placed in Fort Collins clay loam at 10^6 propagules per gram, the soil became suppressive to *R. solani*. *T. hamatum* attacked the mycelium of *R. solani* when the two microorganisms were grown in two-membered culture. It also produced the cell wall degrading enzymes β-(1-3) glucanase and chitinase, but there was no detectable antibiotic activity in vitro when *T. hamatum* was added to conducive Fort Collins clay loam at 10^6 conidia per gram soil. It became suppressive to *R. solani* not only on radishes, but on beans as well. Such treatment also induced suppressiveness to *Pythium* spp. attacking peas, and *Sclerotium rolfsii* in beans.

Additional key words: antagonism, mycoparasitism, lysis, soilborne plant pathogens.

Only a few soils with experimentally demonstrated natural suppressiveness to soilborne plant pathogenic fungi were found in nature. As early as 1892, Atkinson (1) reported soils suppressive to *Fusarium*. Louvet et al (15) confirmed the suppressiveness of some soils to a *Fusarium* sp. causing wilt of muskmelon in France. In the Salinas Valley in California, despite long cultivation of a variety of susceptible crops, Fusarium wilt diseases do not occur (21,22). Recently, Scher and Baker (19) obtained control of Fusarium wilt pathogens with bacteria isolated from this *Fusarium*-suppressive soil. Shipton (20) reported that the incidence of take-all disease (induced by *Gaeumannomyces graminis* var. *tritici*) decreases after 3–4 yr of continuous wheat monoculture. Weinhold et al (23) and Menzies (16) reported a decline of common scab of potatoes caused by *Streptomyces scabies* after monoculturing. Henis et al (12) succeeded in generating soil suppressive to *Rhizoctonia solani* Kühn by successive monoculture of radishes. A significant decrease in disease incidence resulting from an increase in soil suppressiveness was observed after five successive plantings at weekly intervals. Recently, Liu and Baker (14) reported that soil suppressiveness was accompanied by a significant increase in propagule density of *Trichoderma harzianum*. Inoculum density of *R. solani* was inversely proportional to the density of the *Trichoderma* propagules following radish monoculture. A modification of Koch's postulates was utilized to demonstrate that *T. harzianum* was the causal agent responsible for suppressiveness. In the present work, a Colombian soil that appeared to be suppressive to *R. solani* in nature was characterized, and the microorganism responsible for this phenomenon was isolated, identified, and utilized successfully as a biocontrol agent.

MATERIALS AND METHODS

In all experiments, two soils were used: Fort Collins clay loam (4,12), and a soil planted with carnations collected from the area around Bogotá, Colombia, South America. The soils were analyzed by the Soil Testing Laboratory at Colorado State University and their characteristics are shown in Table 1. Microbial analyses of soils were done with the dilution plate method using tap water containing 0.08% agar for soil dilution. Bacteria and actinomycetes were counted on soil-extract agar, and fungi on chloramphenicol-supplemented potato-dextrose agar (PDA-Difco Laboratories, Detroit, MI 48233). The number of propagules of *Trichoderma* was estimated on a selective medium prepared according to Elad et al (8). The inoculum density of *R. solani* was determined on the medium of Ko and Hora (13) with the aid of the multiple pellet soil sampler (12). Soil moisture was adjusted to and maintained at 15% (−0.7 bars) based upon the weight of oven-dry soil for experiments with *R. solani* and *S. rolfsii*, and at 18% (−0.5 bars) for experiments with *Pythium* spp.

Plastic pots each containing 100 g of soil were seeded with 32 seeds per pot of radish (*Raphanus sativus* L. 'Early Scarlet Globe'), or five seeds of pea (*Pisum sativum* L. 'Thomas Laxton'), or bean (*Phaseolus vulgaris* L. 'Bush Blue Lake 47').

Inoculum of *R. solani* isolate R-3 (3) was produced on chopped potato soil (CPS). Large propagules (>589 μm) were separated and used as described by Henis et al (12). Sclerotia of *S. rolfsii*, grown on potato-dextrose agar (PDA) were used for soil infestation. Fort Collins clay loam soil naturally infested with *P. aphanidermatum*, *P. ultimum*, and *P. oligandrum* (11) was used for testing for control of these pathogens by antagonists isolated from *Rhizoctonia*-suppressive soils.

Pots were covered with transparent Mylar® (E. I. du Pont de Nemours Co., Wilmington, DE 19898) and incubated at 25 ± 1 C under continuous illumination (approx. 5,000 lx). Disease incidence (DI) or conducive index (CI) was assessed by calculation (12,14).

Trichoderma spp. were grown on PDA in the dark and conidia were collected and added to soil. In some cases, the *Trichoderma* was grown in 1-L Erlenmeyer flasks, each containing a solid medium composed of soil:peat:cellulose (1:1:1, v/v) supplemented with (w/v) 0.2% glucose (as a "starter"), 0.1% NH_4NO_3, and 0.1% KH_2PO_4.

Each experiment contained at least five replicates and was performed twice. Cell walls of *R. solani* were prepared according to

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the method of Chet et al (5). The activities of lytic enzymes, β-(1-3) glucanase and chitinase, were determined as was previously described (10).

RESULTS

When soils in the vicinity of Bogotá, Colombia, were steamed, losses induced by *R. solani* occurred, yet diseases induced by this pathogen were rarely or never observed in nontreated areas. Thus, the raw soil evidently contained propagules of *R. solani* that had greater inoculum potential after reinvasion from adjacent nonsteamed areas, but was apparently suppressive to this pathogen. The soil was collected and analyzed for its physical properties and compared with Fort Collins clay loam. The physical properties were different as seen in Table 1. The nature of the microbial populations of the two soils also was quite different (Table 2). The most pronounced difference was in the number of fungi. Whereas in Fort Collins soil the total number was 10^3 propagules per gram of soil, an extremely high propagule density (10^8 propagules per gram) was found in the soil from Colombia. No *Rhizoctonia* was detected by our methods (12) in either soil; however, a significant difference in the density of the *Trichoderma* population was observed. Only 10^2 propagules per gram were found in the Fort Collins clay loam, whereas an unusually high number of *Trichoderma* propagules (8×10^5 per gram) was detected in the Colombian organic soil. A totally different picture appeared when bacteria and actinomycetes were counted. Both groups were present in significantly higher quantities in the Fort Collins soil than in the Colombian soil.

Experiments were done to determine whether the Colombian organic soil was suppressive to *R. solani*. The conducive index (CI) (12) of this soil was 0.2, whereas in all soils tested thus far (4,12,14) the virginal CI has been 0.86 or more. *R. solani* was added to the Colombian soil at 900 large propagules per 100 g (12) at weekly intervals, and at each date of inoculation radishes were planted. The amount of damping-off during this monoculture did not exceed 10% (Fig. 1). At the fifth replanting, the inoculum density added to the soil was doubled to 1,800 large propagules per 100 g soil. The amount of damping-off increased to 40% in the subsequent weekly planting of radish; disease incidence again decreased to 10% after the second week. The high density of propagules of *Trichoderma* together with information from the previous studies, suggested that this organism might be the antagonist responsible for suppressiveness. All isolates had similar cultural morphology and 30 were tested for antagonistic ability against *R. solani* on agar medium by using methods described previously (10). All but one had similar characteristics. When growing in the presence of *R. solani*, the *Trichoderma* mycelium covered the plate, some hyphae coiled around the host fungus, and in several cases degradation of *R. solani* mycelium in the region of interaction with the antagonist was observed. The isolates with these attributes were grown on malt agar and were identified as *Trichoderma hamatum* (Bon.) Bain (18). The fungus grew on cell walls of *R. solani* or cellulose as a sole carbon source in liquid salt medium. When grown on cell walls, it released extracellular β-(1-3) glucanase and chitinase. No inhibition zone, indicative of antibiotic excretion, was observed when the isolate was grown in the presence of *R. solani, R. rolfsii,* and *Pythium* spp.

Conidia of *T. hamatum* were added to Fort Collins clay loam at different concentrations. Concentrations higher than 10^2 conidia per gram of soil significantly reduced the CI (Fig. 2).

Fort Collins clay loam was infested with large propagules of *R. solani* (900 propagules per 100 g of soil). A portion of the soil was infested with 10^6 propagules per gram of *T. hamatum*. The pH of a portion of this infested soil (virginal pH 8.1) was adjusted to pH 6. Radishes were planted at weekly intervals. There was significantly more damping-off in the inoculated control than in either of the other treatments, and disease incidence increased to 100% by the

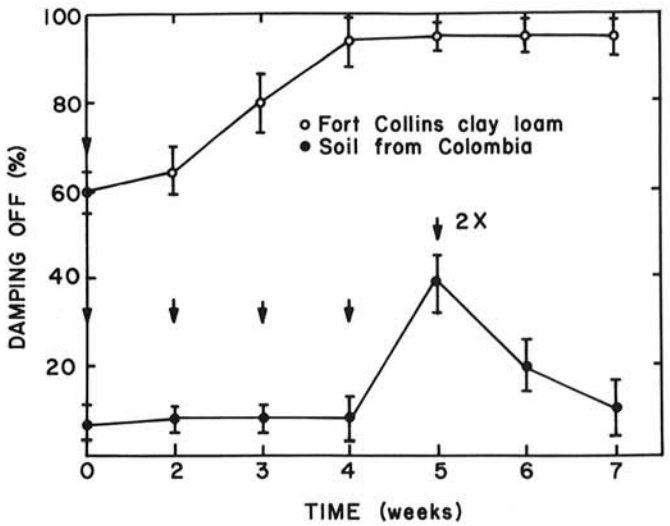

Fig. 1. Percent damping-off of radishes planted (32 seeds per treatment) at weekly intervals in a Columbian soil compared with a Fort Collins clay loam when radishes (32 seeds per treatment) were planted at weekly intervals. Inoculum of *Rhizoctonia solani* was added to the Colombian soils before each planting for the first 5 wk as indicated by arrows. During the first 4 wk, 900 propagules per gram and the fifth wk 1,800 propagules per gram of soil was added. Fort Collins soil was infested only in the first week. There were five replications. The standard deviation (SD), shown as vertical bars, was computed from original data.

TABLE 1. Analysis of Fort Collins soil compared with a Colombia, South America, soil suppressive to *Rhizoctonia solani*

Location	pH	Conductivity (mmhos/cm)	Lime[b] (%)	Organic matter (%)	DPTA[a]-extractable mineral nutrients					Texture
					NO_3-N (μg/g)	P (μg/g)	K (μg/g)	Zn (μg/g)	Fe (μg/g)	
Fort Collins[c]	8.1	0.4	>2	1.9	4	10	139	16.5	2.5	Clay loam
Colombia[d]	5.1	4.7	<1	35.6	92	23	288	35.0	162	Organic

[a] Acronym DPTA stands for diethylenetriamine penta-acetic acid extraction.
[b] >2% is considered high and <1% is considered low.
[c] Soil series name is Fort Collins clay loam.
[d] Soil series name of soil from Colombia is not available.

TABLE 2. Microbial population[a] of Fort Collins clay loam and a Colombia, South America, organic soil

Soil	Bacteria	Actinomycetes	Fungi	*Trichoderma*	*Rhizoctonia*
Fort Collins clay loam	$2 \times 10^8 \pm 7\%$	$3 \times 10^6 \pm 8\%$	$2 \times 10^3 \pm 10\%$	$1 \times 10^2 \pm 11\%$	Not detected
Colombian organic soil	$3 \times 10^7 \pm 6\%$	$1 \times 10^3 \pm 14\%$	$1 \times 10^8 \pm 12\%$	$8 \times 10^5 \pm 9\%$	Not detected

[a] Microbial populations were determined by the dilution plate method using soil extract agar for bacteria and actinomycetes, PDA + chloramphenicol for fungi, and selective media of Elad et al (8) and Ko and Hora (13) for *Trichoderma* and *Rhizoctonia solani*, respectively.

third replanting (Fig. 3). Where *T. hamatum* was added to the soil, damping-off was reduced significantly. There also was significantly less disease incidence during the first three plantings when the soil was acidified as compared with the nonacidified soil.

The use of *T. hamatum* for controlling disease induced by *R. solani* in beans also was tested (Fig. 4). A significant reduction in disease incidence in Fort Collins clay loam from 70 to 22% was achieved by the addition of the antagonist to soil at 10^6 propagules per gram. Similar suppressiveness also was induced when *T. hamatum* was added to soil naturally infested with *Pythium* spp. and planted with peas (Fig. 5). Damping-off occurred only during the first 6 days, but was significantly reduced in soil containing *T. hamatum*.

No antagonism was apparent between *T. hamatum* and *S. rolfsii* growing on PDA. The two fungal colonies made contact and ceased growing toward each other. However, when conidia of the antagonist (10^6 per gram soil) were added to soil infested with sclerotia of *S. rolfsii*, a significant reduction in disease incidence in beans was observed. When beans were replanted in the treated soil, an even lower disease incidence (22%) occurred (Fig. 6). Similar results also were achieved when *T. hamatum* was added as propagules grown on and mixed with cellulose-soil mixture were used.

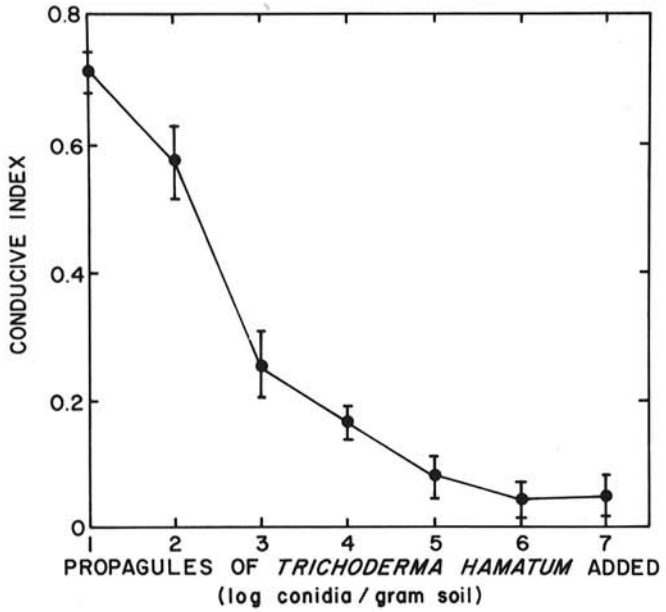

Fig. 2. The decreases in conducive indices (CI) of Fort Collins clay loam originally conducive to *Rhizoctonia solani* after infestation with condia of *Trichoderma hamatum*. There were five replications, each containing 32 radish seeds. The standard deviation (SD), shown as vertical bars, was computed from original data.

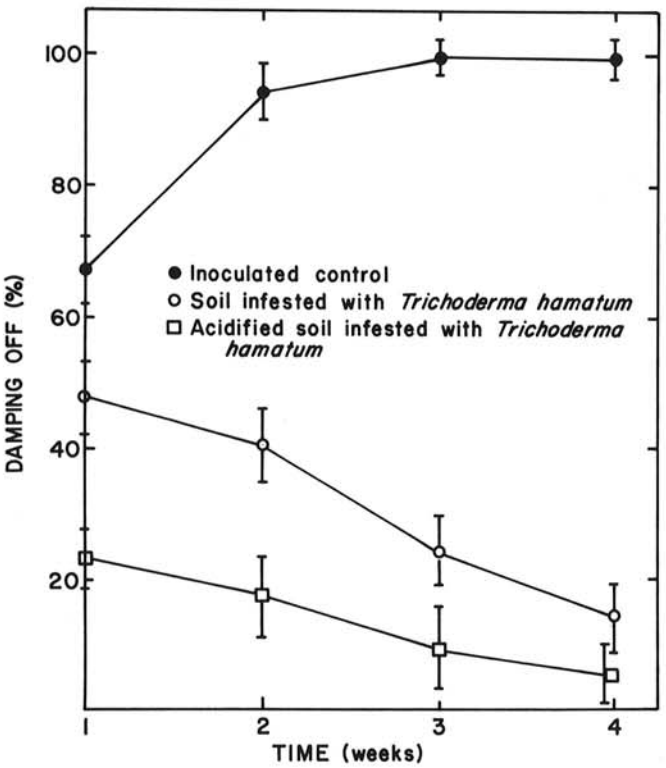

Fig. 3. Damping-off in radish seedlings (32 seeds per treatment) grown in monoculture at weekly intervals in Fort Collins clay loam infested with *Rhizoctonia solani* compared with seeds replanted in infested soil amended with *Trichoderma hamatum* at 10^6 propagules per gram, or in acidified soil (pH 6.0) amended with *T. hamatum*. There were five replications. The standard deviation (SD), shown as vertical bars, was computed from original data.

DISCUSSION

Soils suppressive to soilborne plant pathogenic fungi are found in a number of agricultural areas (1,4,12,14,15,16,19,21,22); however, no naturally occurring soil suppressive to *R. solani* has

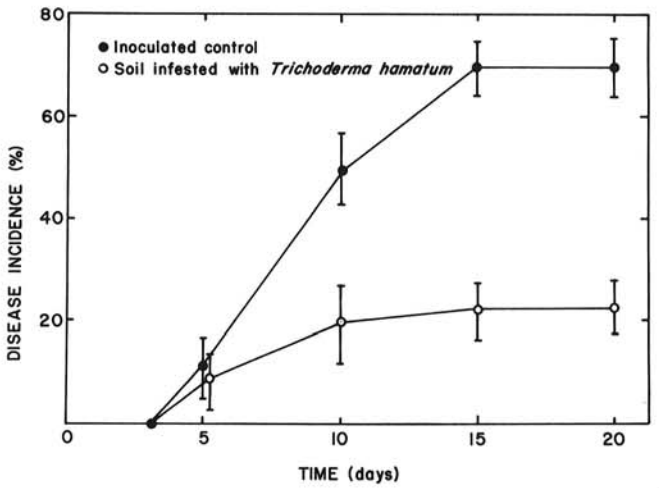

Fig. 4. The effect of *Trichoderma hamatum* added to conducive soil at 10^6 conidia per gram on the incidence of stem rot of beans induced by *Rhizoctonia solani*. There were five seeds in each treatment in five replications. The standard deviation (SD), shown as vertical bars, was computed from original data.

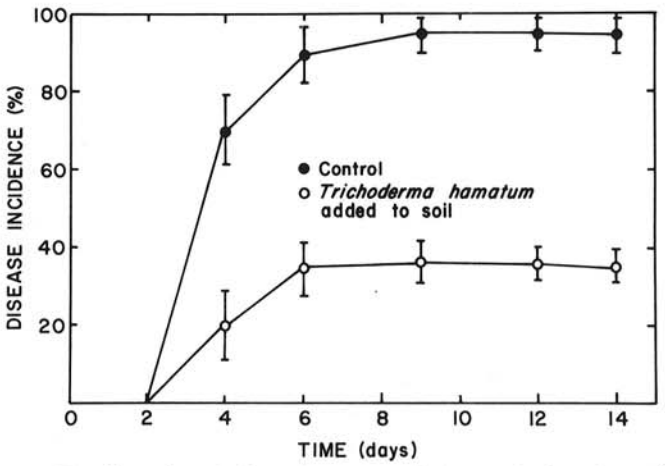

Fig. 5. The effect of *Trichoderma hamatum* added to conducive soil at 10^6 propagules per gram on reduction of damping-off in peas over time in soil naturally infested with *Pythium* spp. pathogenic to peas. There were five seeds in each treatment in five replications. The standard deviation (SD), shown as vertical bars, was computed from original data.

been reported until now. The organic Colombian soil was suppressive to this pathogen (Fig. 1). Both soil and microbiological analyses showed a pronounced difference between this soil and the conducive Fort Collins clay loam. The Columbian soil had a low pH (5.1) which apparently enhanced the activity of fungi. As a consequence, it contained a much higher proportion of fungi in the microbial population than did the Fort Collins soil, whereas the latter contained considerably more bacteria and actinomycetes. Soil of low pH was favorable for development of suppressiveness and the increase of antagonistic *Trichoderma* (4). The Colombian soil contained 8×10^5 propagules per gram of *Trichoderma,* whereas Fort Collins clay loam (pH 8.1) contained only 10^2 propagules per gram of soil. The high density of *Trichoderma* propagules indicated that this organism might be responsible for soil suppressiveness. The fungus was isolated and the predominant species was identified as *T. hamatum* (18). *T. viride* (25), and especially *T. harzianum* (2,9 10,24), are known to be biocontrol agents; however, little has been done with *T. hamatum*. Dennis and Webster (6,7) found that some isolates produced antibiotics, especially at low pH. *T. hamatum*, in contrast to other *Trichoderma* species, was ineffective against *Armillariella mellea* (17). Our isolate differed from those described previously; it produced no antibiotic substances. However, it produced β-(1-3) glucanase and chitinase, and grew on isolated *R. solani* cell walls. This isolate also grew on cellulose as a sole carbon source. Production of cellulase, in addition to other cell wall degrading enzymes, may explain its ability to control diseases induced by *Pythium* spp. (in addition to those caused by *R. solani* and *S. rolfsii*) since *Pythium* contains cellulose in its cell walls (7). These findings differ, therefore, from those reported for *T. harzianum* which attacked *R. solani* or *S. rolfsii* (2,9,10), but not *Pythium*. Application of conidia of *T. hamatum* conferred suppressiveness to the conducive Fort Collins clay loam and, therefore, was found to be effective for controlling *R. solani* in radish seedlings. The reduction in the percentage of damping-off was even higher when the antagonist was added to acidified soil (pH 6) (Fig. 3). Thus, these findings corroborate former research results showing that low pH favors suppressiveness induced by *T. harzianum* (4,14). *T. hamatum* also significantly reduced diseases induced by *R. solani* and *Pythium* spp. in beans and peas, respectively (Figs. 4 and 5). It should be noted that peas became resistant to the pathogens after 6 days (Fig. 5), as was also observed by Harman et al (11). Similar results were obtained with *S. rolfsii*, which is favored by warm climate. A second replant of beans in the treated soil showed even a better control of disease (Fig. 6), apparently due to the establishment of *T. hamatum* in soil.

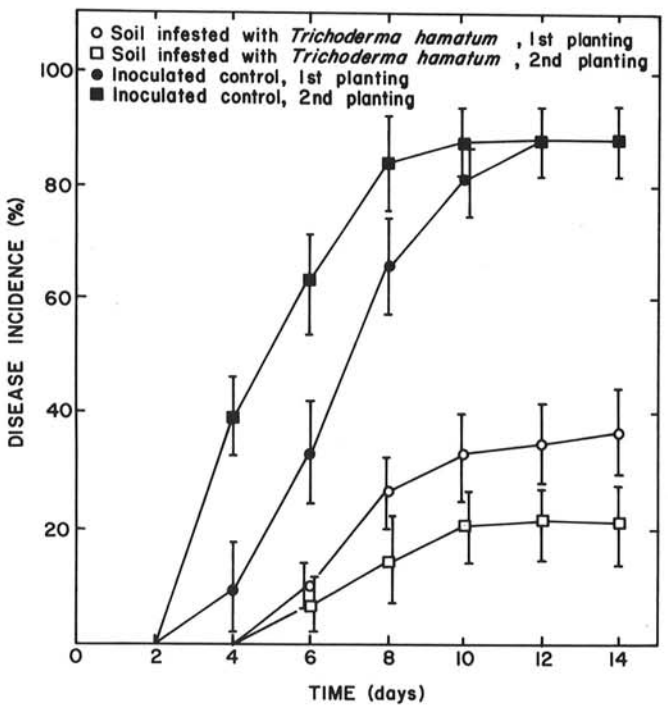

Fig. 6. Effect of *Trichoderma hamatum* on disease induced in beans by *Sclerotium rolfsii*. Conidia of *T. hamatum* were added at 10^6 propagules per gram of soil. Each treatment contained five plants and there were five replications. Fourteen days after the first seeding, plants were removed and seeds were placed in the soil for the second time. The standard deviation (SD), shown as vertical bars, was computed from original data.

Our data indicate that *T. hamatum* has potential as a biocontrol agent. It attacks both *Pythium* spp. and *R. solani* and thus controls most of the causal agents of damping-off. Unlike other *Trichoderma* spp. which attack only *R. solani* (10,14), the hazard of increase in density of *Pythium* spp. in soil as a result of the control of *R. solani* does not seem to be a potential problem when *T. hamatum* is applied.

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