# Factors Affecting *Trichoderma hamatum* Applied to Seeds as a Biocontrol Agent

G. E. Harman, I. Chet, and R. Baker

Associate professor, Department of Seed and Vegetable Sciences, Cornell University, New York State Agricultural Experiment Station, Geneva 14456; professor, Department of Plant Pathology and Microbiology, the Hebrew University of Jerusalem, Rehovot, Israel; and professor, Department of Botany and Plant Pathology, Colorado State University, Fort Collins 80523. This research was conducted at the Department of Botany and Plant Pathology, Colorado State University.

Published with the approval of the director of the Colorado State University Experiment Station as Scientific Journal Series Paper 2577.
The senior author was supported by funds from the American Seed Research Foundation, Colorado State University, and Cornell University; the second author was supported by funds from Science and Education Project 79004 and Western Regional Project W-147.
Accepted for publication 12 November 1980.

## ABSTRACT

Harman, G. E., Chet, I., and Baker, R. 1981. Factors affecting *Trichoderma hamatum* applied to seeds as a biocontrol agent. Phytopathology 71:569-572.

*Trichoderma hamatum*, applied as a seed treatment, controlled seed rot of pea or radish in soil infested with *Pythium* spp. or *Rhizoctonia solani*, respectively, if soil temperatures were between 17 and 34 C and seeds had been treated with a suspension of conidia with a concentration equal to or greater than $10^6$/ml. Addition of chitin or cell walls of *R. solani* to seed coats increased the ability of the mycoparasite *T. hamatum* to protect seeds against *Pythium* spp. or *R. solani* and resulted in an increase in the population density of *Trichoderma* in the soil. *T. hamatum* with chitin, but not with cell walls of *R. solani*, effectively reduced damping-off resulting from *Pythium* spp. compared with a seed treatment containing only *T. hamatum*. Addition of peat to *T. hamatum* did not increase the protective ability of the agent as a seed treatment, but resulted in an increase in its population density in soil. Addition of a nonpathogenic isolate of *R. solani* had little effect either on protection of seeds or levels of *Trichoderma* in the soil, whereas addition of cellulose tended to decrease both protection and establishment of the biocontrol agent in the soil. Treatment of seeds with both *Rhizobium* and *T. hamatum* had no effect on the nodulating activity of the former or the protective ability of the latter.

We (9) recently reported that seed treatments with the biological control agent *Trichoderma hamatum* (Bon.) Bain protected seeds and seedlings from attack by *Pythium* spp. or *Rhizoctonia solani* Kühn. As a consequence of this seed treatment, numbers of *Trichoderma* propagules increased in the soil, and the soil became suppressive to *Pythium* spp. and *R. solani*. Thus, *T. hamatum* seems to be a promising biocontrol agent. This antagonist was isolated from a highly organic muck soil with low pH (5) and it is a microparasite of *R. solani* and *Pythium* spp. *T. hamatum* produces $\beta$-1,3 glucanase, chitinase, and cellulase (5,6). Therefore, addition of cell wall or organic soil components capable of being degraded by these enzymes to seed treatments with *T. hamatum* may increase the efficacy of this agent. Other biocontrol agents also have shown promising results as seed treatments (3,10,17,18), but their performance tends to be erratic.

To improve the reliability of biocontrol, procedures must be developed for delivery and establishment of biocontrol agents in the potential infection courts of hosts. Seed treatments offer an attractive method of delivery. Addition of appropriate amendments to support the continued activity of biocontrol agents may protect the host after initial application of the antagonist. Fungicidal seed treatments are frequently toxic to *Rhizobium* spp. (14), so effective seed protectants having no deleterious effects on nitrogen-fixing bacteria are needed.

The objectives of the work reported here were to test effects of temperature, conidial concentration, and amendments on the efficacy of seed treatments with *T. hamatum*. We also tested effects of combinations of *T. hamatum* and *Rhizobium* spp. on nodulation and on seedling disease.

## MATERIALS AND METHODS

**Soils and microorganisms.** Unless otherwise noted, all experiments were performed in a clay soil that was obtained near Fort Collins, CO, which had a pH of 7.4 and other characteristics as described elsewhere (9).

*T. hamatum* obtained from a *Rhizoctonia*-suppressive soil near Bogota, Colombia, was used as in earlier investigations (9). The *R. solani* isolate was R3 (9), whereas the *Pythium* spp. were those indigenous to the Fort Collins clay soil, including *P. ultimum* Trow, *P. aphanidermatum* (Edson) Fitzp., and *P. oligandrum* Drechsler (9).

**Seeds and planting conditions.** All experiments except those testing effects of temperature were performed at 25 ± 2 C. Plastic containers 11 cm in diameter and 8 cm deep were used for all plantings. Soil was placed in these containers to a depth of 3 cm for peas (*Pisum sativum* L. 'Laxton's Progress') and 1.5 cm for radishes (*Raphanus sativus* L. 'Early Scarlet Globe'). After seeds were planted, containers were covered with plastic to reduce evaporation.

For tests with *Pythium* spp., four pea seeds were planted 1 cm deep in each container of naturally infested Fort Collins clay soil. Soil moisture was adjusted to 18% (−0.15 bars).

For tests with *R. solani*, 32 radish seeds were planted 1–3 mm deep in each container of soil. Soil moisture was adjusted to 14% (−0.5 bars); at this moisture content and with this host no damage from the resident *Pythium* spp. occurred. Immediately prior to planting, the soil in each container was infested with a pellet of *R. solani* containing approximately 900 large (>589 $\mu$m) propagules (16).

All experiments were conducted with five replicates (containers) of each treatment, and data were analyzed according to the Minitab program (15). All experiments were done at least twice.

**Seed treatments.** Seeds were treated with a conidial suspension of *T. hamatum* in water containing 10% (w/v) Pelgel® (The Nitragin Co., Milwaukee, WI 53209) as a spreader or sticker. We also used 2% (w/v) Methocel A4C Premium® (Dow Chemical Co., Midland, MI 48640) in some experiments. Twenty or 80 ml of spore suspension was used per kilogram of pea or radish seeds, respectively, in all cases.

We added various amendments to conidial suspensions used as seed treatments. These included powdered chitin (technical grade, Sigma Chemical Co., St. Louis, MO 68178), cell walls of *R. solani* prepared by the method of Chet et al (7), which were ground and passed through a screen with 589 $\mu m^2$ openings, carboxymethyl cellulose (Brown Co., Berlin, NH 03570) and cultures of an isolate of a *Rhizoctonia* sp. nonpathogenic to peas and radishes (obtained from C. C. Tu, National Taiwan University, Taipei, Taiwan). For the latter treatment, the nonpathogenic isolate of *Rhizoctonia* was

0031-949X/81/06056904/$3.00/0

grown on Bacto potato-dextrose agar (Difco, Inc., Detroit, MI 48323), and after growth, the culture was allowed to dry thoroughly. The dried culture was ground to a powder in a mortar and pestle and was sieved through a screen with 589 $\mu m^2$ openings. This powder was used as an amendment. All of the above materials were added to conidial suspensions in Pelgel solutions in sufficient quantities to give 3% (w/v) of the amendment. We also ground dried fibrous sphagnum peat moss in a blender and sieved the material through a screen with 150 $\mu m^2$ openings. The resulting powder was added to conidial Pelgel suspensions in quantities sufficient to give a 15% (w/v) peat suspension.

In all experiments with amendments, we used $10^5$ conidia of *T. hamatum* per milliliter of seed treatment suspension. This level was chosen because protection by *T. hamatum* was reduced in comparison to that induced by higher concentrations (Fig. 1); improvements in seed or seedling disease control could not be observed with conidial concentrations of $10^6$–$10^8$/ml. We tested both the radish-*R. solani* and pea-*Pythium* systems in all experiments and assessed both the amount of disease 9 days after planting and the number of *Trichoderma* propagules in soil at the end of the experiments using dilution plate techniques with a *Trichoderma*-selective medium (11).

**Combined treatments with *T. hamatum* and *Rhizobium*.** We determined whether seed treatments with both *Rhizobium* and *T. hamatum* affected performance of either organism. For these tests, seeds of bean (*Phaseolus vulgaris* L. 'Bush Blue Lake 47') were treated with *Rhizobium* inoculum prepared for that species (The Nitragin Co., Milwaukee, WI 53209) according to the manufacturer's instructions. Similar groups of seeds were treated with similar preparations containing $10^8$ *T. hamatum* conidia per milliliter and other groups of seeds were left untreated. Seeds were planted in greenhouse potting soil autoclaved twice for 2 hr on successive days, and plants were grown for 6 wk. Plants were then uprooted and the numbers of nodules were counted.

Conversely, pea seeds were treated with *Rhizobium* suspensions with or without $10^8$ *T. hamatum* conidia and planted in the nontreated Fort Collins clay soil. Nine days later, the amount of disease resulting from the indigenous *Pythium* spp. and the number of propagules of *Trichoderma* in the soil were determined as described above.

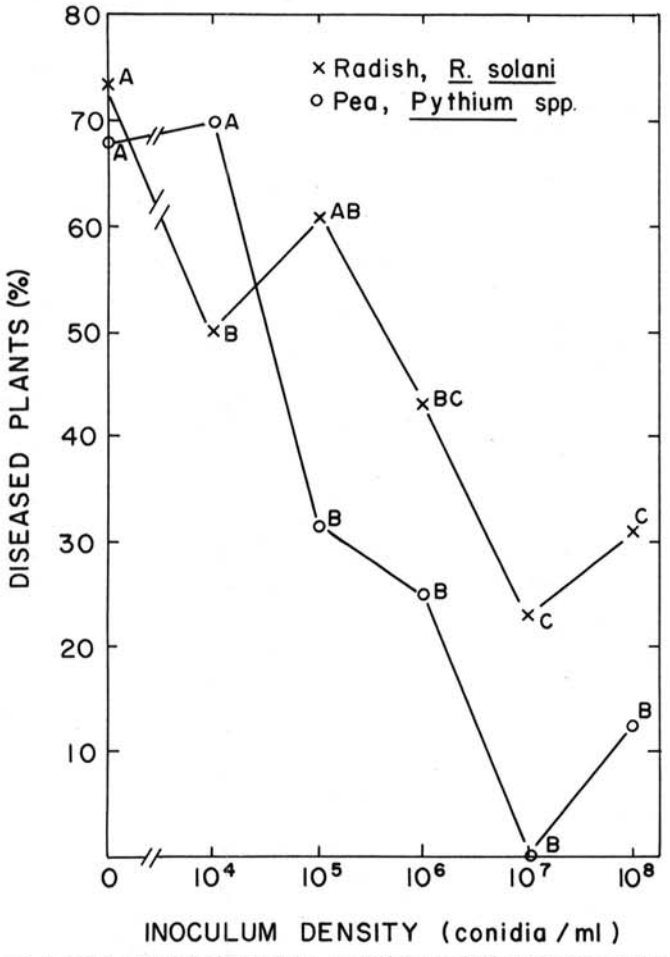


**Fig. 1.** Percentage of diseased pea or radish seeds after treatment with different concentrations of spores of *Trichoderma hamatum*. Points with dissimilar letters are significantly different, $P=0.05$. Pea seeds were planted in soil naturally infested with *Pythium* spp., while radishes were planted in similar soil infested with *Rhizoctonia solani*.

## RESULTS

As a first step in these studies, we compared 2% Methocel with 10% Pelgel as a spreader and sticker. When added alone, neither material had any significant effect on seed or seedling disease of either host. There was also no difference in the efficacy of *T. hamatum* applied in either material. Pelgel had a pH of 5.5 and Methocel a pH of 7.2. Pelgel suspensions were much less viscous than Methocel suspensions and therefore were more convenient to use in these studies.

Conidial concentrations of $10^5$/ml or greater gave protection to peas whereas concentrations of $10^6$/ml or greater gave protection to radish seeds in comparison with nontreated controls (Fig. 1).

We also tested effects of soil temperature on the efficacy of seed treatments with *T. hamatum*. Pea seeds were protected from

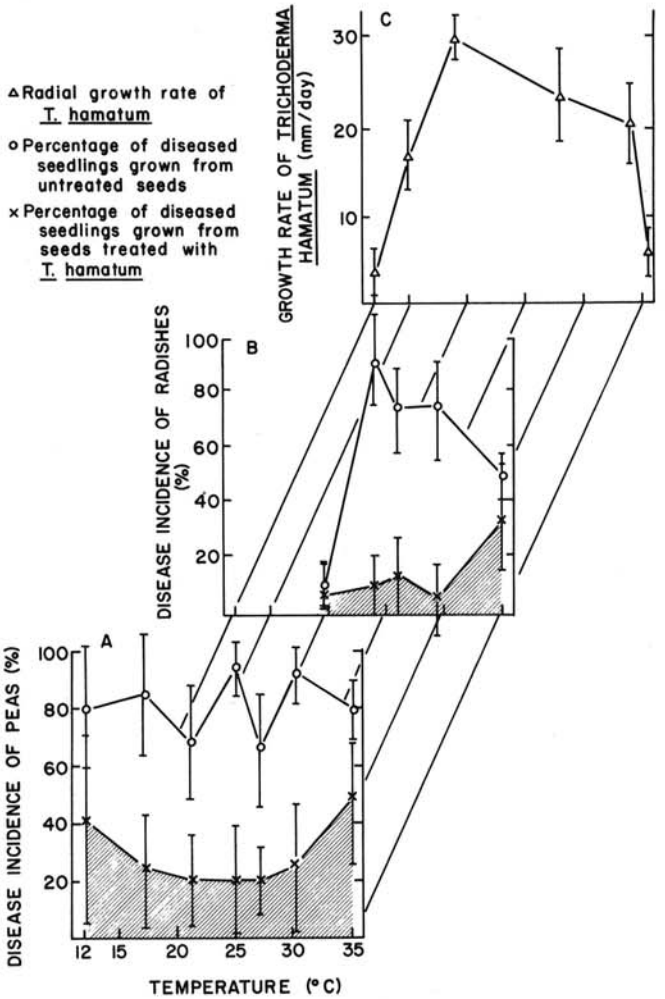


**Fig. 2.** Percentage of diseased **A,** pea or **B,** radish seedlings grown from nontreated or *Trichoderma hamatum*-treated (shaded areas) seeds at different temperatures compared to the growth rate of *T. hamatum* at similar temperatures on potato-dexrose agar (C). The bars represent the standard deviation about each point. Pea seeds were planted in soil naturally infested with *Pythium* spp., while radishes were planted in similar soil infested with *Rhizoctonia solani*.

damping-off induced by *Pythium* spp. at temperatures ranging from 17 to 30 C; whereas at 12 and 35 C, *T. hamatum* gave no significant protection in comparison with nontreated controls (Fig. 2). Similar results were obtained with the radish-*R. solani* system, except that *R. solani* did not induce disease at 20 C or below, and therefore only warmer temperatures were tested. Linear growth rate of *T. hamatum* was inversely related to its protective capacity; at temperatures between 15 and 34 C the fungus grew well, but at higher and lower temperatures growth was much slower (Fig. 2).

Amendments added to conidial suspensions affected the amount of damping-off in seeds treated with *T. hamatum*. Addition of chitin reduced disease incidence in both pea and radish, while addition of *R. solani* cell walls or a nonpathogenic *Rhizoctonia* sp. reduced disease incidence with radish but not with peas. Peat had no effect on either crop species (Table 1). Cellulose increased disease of radish in comparison with the inoculated control but the magnitude was not significantly different.

There was a greater increase in *Trichoderma* propagule numbers in soil planted to seeds with *T. hamatum* and chitin, peat, or cell walls of *R. solani* than in soils planted to seeds with *T. hamatum* alone (Table 1). Conversely, addition of nonpathogenic *R. solani* had no effect on numbers of propagules of *Trichoderma*, while addition of cellulose tended to decrease the number of propagules.

We also mixed conidia of *T. hamatum* at $10^8$/ml, 2% (w/v) chitin, and 1% cell walls of *R. solani*. This mixture (TCC) was applied to seeds and tested as above. Disease incidence was ~20% and similar to that observed when *T. hamatum* was applied alone at $10^8$ conidia per milliliter in either the *Rhizoctonia*-radish or *Pythium*-pea systems. However, populations of *Trichoderma* in soil at the end of 9 days of growth were $3 \times 10^5$ propagules per gram of soil with treated peas and $1 \times 10^3$ with treated radish seeds, as compared with only $8 \times 10^3$ and $5 \times 10^4$ propagules per gram with pea and radish seeds, respectively, after treatment with $10^8$ *T. hamatum* conidia alone.

Addition of *Rhizobium* inoculum to a suspension of *T. hamatum* containing $10^8$ conidia per milliliter for seed treatment of peas resulted in no significant reduction in plant stand relative to seed treatments with *T. hamatum* and without *Rhizobium*. Similarly, addition of *T. hamatum* to *Rhizobium* inoculum of beans resulted in similar numbers of nodules on roots compared to plants grown from seeds treated only with *Rhizobium*.

## DISCUSSION

In this study, we attempted to determine the conditions required for maximum biocontrol activity of *T. hamatum* when it was used as a seed treatment. Thus, it was established that effective control of damping-off may not be achieved in cold (<15 C) soils (Fig. 2). While the biocontrol agent also was ineffective above 37 C, soils in temperate regions during seeding are not often at this temperature. In the absence of amendments, conidial concentrations of less than $10^6$ per milliliter in the seed coating suspensions were ineffective. Soil pH influenced the efficiency of biocontrol; acid conditions favored the activity of *Trichoderma* spp. in protection of seeds against damping-off and soils became suppressive to *R. solani* in a shorter period of monoculture (4,5). Thus, the acid pH of Pelgel in seed coating mixtures is propitious.

Manipulation of edaphic factors may increase the efficiency of biocontrol. Furthermore, an additional increment of control was achieved by creating a nutritional environment conducive to *T. hamatum*. This microorganism is a parasite of both *Pythium* spp. and *R. solani*. It produces β-1,3 glucanase, chitinase, and cellulase (5,6)—enzymes capable of degrading cell walls of these pathogens. Therefore, the addition of preparations of fungal cell walls and the polymers found associated in their structure seemed appropriate for favoring the activity of *T. hamatum*. Indeed, increased efficiency in control of damping-off was realized when chitin or the cell walls of *R. solani* were added to seed coatings containing conidia of *T. hamatum* (Table 1). In addition, population density of the antagonist in soils containing seeds coated with a mixture of chitin and/or cell walls of *R. solani* and *T. hamatum* was significantly higher than in soils not containing seeds with coatings containing these amendments. We interpret this phenomenon as an enhancement of biocontrol activity of *T. hamatum* through the establishment of a food base compatible to the mycoparasite.

Chitin was an effective amendment for control of soilborne pathogens (12,13) but, particularly with pythiaceous fungi, addition of unbleached chitin (as used in this study) may increase disease (8). In this study, we applied chitin to seeds (rather than soil) with a biocontrol agent capable of using this material to provide a food base primarily for the antagonist. Thus, there was less dependency on the chitin-degrading microflora indigenous to the soil to provide biocontrol.

Cellulose had no significant influence on disease incidence relative to treatment only with *T. hamatum* (Table 1). Similar results were obtained in studies in which *Chaetomium globosum* was used as a seed protectant (J. Hubbard and G. E. Harman, *unpublished*). The reason for this is not clear; however, cellulose, unlike chitin, can be degraded by both *R. solani* and *Pythium* spp. (1,2).

The effect of peat differed from that of other amendments placed on seed coats; it had no effect on the efficacy of *T. hamatum* in controlling the seedling diseases, but it did stimulate the production of propagules of the antagonist in soil (Table 1). *Trichoderma* spp. may not have enzyme systems capable of degrading and utilizing the complex organic compounds in peat. Thus, this substance would not be an appropriate food base. Subsequently, however, peat should provide an environment of lower pH (than that of the original soil [9]) and higher organic matter similar to the soil from which *T. hamatum* was originally isolated (6).

Establishment of *T. hamatum* in soil in sufficiently large numbers to induce suppressiveness (at least $10^4$ propagules per gram of soil [4,6]) is important if this agent is to be used to control pathogens attacking plants in later stages of growth or in subsequent crops. Increase of the biocontrol agent over one order

TABLE 1. Influence of seed treatments on numbers of *Trichoderma hamatum* propagules in soil and on disease induced by *Rhizoctonia solani* or *Pythium* spp. seedings of radish or pea, respectively

| Treatment[a] | Diseased seedlings[b] (%) | | Population density[b,c] (propagules per gram of soil of *Trichoderma* (×$10^3$) in soil planted with: | |
|---|---|---|---|---|
| | Pea | Radish | Pea | Radish |
| None | 65 A | 52 AB | 0.5 D | 0.01 D |
| *T. hamatum* | 55 A | 44 B | 17.0 C | 9.8 C |
| *T. hamatum* + cellulose | 60 A | 75 A | 7.3 C | 1.8 D |
| *T. hamatum* + chitin | 14 B | 20 C | 59.0 A | 31.0 A |
| *T. hamatum* + *R. solani* cell walls | 28 AB | 12 C | 28.0 B | 16.0 B |
| *T. hamatum* + nonpathogenic *R. solani* | 47 AB | 20 C | 7.7 CD | 7.0 CD |
| *T. hamatum* + peat | 63 A | 37 B | 55.0 A | 38.0 A |

[a] Seeds were treated with suspensions containing $10^5$ *T. hamatum* conidia per ml in 10% (w/v) Pelgel®. Amendments were added at the rate of 3% (w/v) except peat, which was added in sufficient quantity to give a 25% (w/v) suspension.

[b] Numbers in each column followed by different letters are significantly different, $P = 0.05$.

[c] Numbers of *Trichoderma* spp. were determined by dilution plate techniques utilizing a *Trichoderma*-selective medium (11) 9 days after seeding. The soil that was used contained $10^2$ propagules per gram before planting.

of magnitude in soil by use of appropriate amendments suggests that techniques can be devised whereby proliferation of the antagonist, after its introduction on seeds, can be achieved leading to long-term control of soilborne pathogens.

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