Biological Control Effects of a New Isolate of *Trichoderma harzianum* on *Pythium aphanidermatum*

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This research was supported by a grant from the United States-Israel Binational Agricultural Research and Development Fund (BARD).
We thank H. Harshemesh, Y. Hadar, R. Govrin, Y. Straschnov, O. Kleifeld, Y. Zvieli, and M. Platt for technical assistance.
Accepted for publication 7 December 1983.

ABSTRACT

Sivan, A., Elad, Y., and Chet, I. 1984. Biological control effects of a new isolate of *Trichoderma harzianum* on *Pythium aphanidermatum*. Phytopathology 74:498-501.

A wheat-bran plus peat mixture (1:1, v/v) was the most efficient of the raw plant material substrates (which included several agricultural plant wastes) found suitable for growing a new isolate of *Trichoderma harzianum* (T-315). The bran/peat preparation of *T. harzianum*, applied to either soil or rooting mixture, efficiently controlled damping-off induced by *Pythium aphanidermatum* in peas, cucumbers, tomatoes, peppers, and gypsophila. Disease reduction of up to 85% was obtained in tomatoes. *T. harzianum* applied in a seed coating mixture containing 5×10^9 conidia per milliliter was as effective in sandy soil as the broadcast application of wheat bran/peat preparation. However, the broadcast application was superior to seed coating for protecting tomato seedlings in an infested peat/vermiculite rooting mixture. When germinated at low temperature (22 C) pea seeds coated with conidia of *T. hamatum* were better protected from *P. aphanidermatum* than seeds coated with *T. harzianum*, but this was not the case at 30 C. Extracellular filtrate from cultures of *T. harzianum*, added to a synthetic medium, inhibited linear growth of *P. aphanidermatum* by 83% compared with 8% inhibition by a culture filtrate of *T. hamatum*. Substances excreted by *P. aphanidermatum* into the growth medium enhanced the linear growth of *T. harzianum* by 34%, but not that of *T. hamatum*.

Additional key words: biocontrol, damping-off, fungal antagonism.

The major problem of applying antagonists to soil is their inability to become established in the ecosystem, and to overcome the resistance of soil microflora to the introduction of new microorganisms (1,3). In recent years, several attempts have been made to overcome this obstacle by applying fungal biocontrol agents, grown on suitable food bases, to the soil (2,11,18). The application of wheat bran colonized by *T. harzianum* to soils infested with *Rhizoctonia solani* and *Sclerotium rolfsii* reduced the incidence of disease caused by these pathogens in beans (8,11). Survival and activity of *T. harzianum* was improved if it was applied in a wheat bran preparation than in a fungal conidial suspension (8). *T. harzianum* in a wheat bran preparation persisted for >3 mo in field soil infested with *S. rolfsii* (9).

Biological control of soilborne plant pathogens can also be achieved by seed treatment with antagonists. Liu and Vaughn (14) succeeded in controlling *P. ultimum* in table beet seedlings by coating the seeds in a suspension of conidia. Similarly, Harman et al (12) recently reported the biocontrol of *R. solani* and *Pythium* spp. by coating radish and pea seeds with *T. hamatum* (Bon.) Bain.

In the present study we tested different substrates as food bases for *Trichoderma* spp. Broadcast and seed treatment applications were tested for a new isolate of *T. harzianum* capable of controlling *P. aphanidermatum*.

MATERIALS AND METHODS

Soilborne fungi. *Pythium aphanidermatum* (Edson) Fitz. was isolated from diseased cucumber seedlings by using a medium selective for *Pythium* species (17). *Rhizoctonia solani* Kühn and *Sclerotium rolfsii* Sacc. were isolated from diseased bean plants on a solidified synthetic medium (SM) (15). *Trichoderma* spp. were isolated on a medium selective for *Trichoderma* (TSM) according to Elad et al (6). *T. harzianum* (T-315), isolated from soil naturally infested with *P. aphanidermatum*, was compared with an isolate of *T. hamatum* (Bon.) Bain (12) as a biocontrol agent. Test plants were: cucumber (*Cucumis sativus* L. 'Alma'), tomato (*Lycopersicum esculentum* Mill 'Rehovot 13'), pepper (*Capsicum annuum* L. 'Maor'), pea (*Pisum sativum* L. 'Perfection'), bean (*Phaseolus vulgaris* L. 'Brittle-Wax'), and gypsophila (*Gypsophila paniculata* 'Bristol-Fairy').

Growth of *T. harzianum* on agricultural plant waste substrates. The growth potential of *T. harzianum* on organic food bases, including several agricultural wastes, was studied, using: wheat bran, wheat straw compost, ground wheat straw, ground cotton straw, peat and a wheat bran/peat mixture (1:1, v/v). Test tubes, each containing 5 g of substrate, were autoclaved for 1 hr. Substrate moisture was adjusted to 50% (w/w) with sterile distilled tap water. Each medium was inoculated with 0.1 ml of a conidial suspension containing 2×10^4 conidia of *T. harzianum* per milliliter and incubated for 7 days at 30 C. The number of germinating propagules was assessed by the dilution method on TSM.

Production of inhibitory substances by *Trichoderma* species. *T. harzianum* and *P. aphanidermatum* were grown separately on cellophane membranes placed on SM in petri dishes. The membranes were removed after 48 hr and a fungal disk of *P. aphanidermatum* was placed in the center of each plate. Linear growth was determined 24 hr later.

Excretion of inhibitory substances in a liquid culture was tested by using 250-ml Erlenmeyer flasks each containing 50 ml of SM, inoculated with either one of the *Trichoderma* spp. and shaken on a rotary shaker at 150 rpm, for 48 hr at 30 C. The mycelium was then washed with sterile tap water and separated by centrifugation at 9,150 *g* for 20 min at 4 C. The supernatant was filtered through Whatman No. 1 filter paper, passed through a 0.45-μm Millipore filter, mixed with SM containing 3% agar (1:1, v/v) and then poured into petri dishes. SM supplemented with sterile tap water served as control. A mycelial disk of *P. aphanidermatum*, *T. harzianum*, or *T. hamatum* served as inoculum. Linear growth rate of the tested fungi was determined 24 hr after inoculation.

The same technique was used to determine the effect of culture filtrate of *P. aphanidermatum* on the linear growth of *Trichoderma* spp.

Greenhouse and nursery experiments. Biological control studies of *P. aphanidermatum* were carried out in sandy loam soil (82.3% sand, 2.3% silt, 15% clay, and 0.4% organic matter, pH 7.4;

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moisture-holding capacity, 12.2%) or in a spent rooting mixture (from a commercial nursery and containing peat and vermiculite [1:1, v/v]) infested with *P. aphanidermatum*. Tests with *R. solani* and *S. rolfsii* were performed in an alluvial-vertisol field soil (27% sand, 17.0% silt, 55.5% clay, and 0.5% organic matter, pH 7.95) that was already infested with both pathogens. Experiments in soil were set up in plastic boxes (9 × 9 × 10 cm) containing 0.5 kg of soil sown with 10 seeds of the test plant. Nursery experiments were carried out in rooting mixture in Speedling-type, preformed trays consisting of 84 conical compartments (each 3.5 × 3.5 × 7 cm). Each compartment was sown with one seed of the test plant. The biological control experiment of *P. aphanidermatum* and *R. solani*, in gypsophila cuttings during their rooting period, was carried out under misting conditions. In the first 2 wk of rooting the cuttings were maintained in the greenhouse under natural light conditions and were misted for 9 sec every 6 min and later for 10 sec every 30 min.

Soil inoculation with *P. aphanidermatum*. Roux bottles (1,000-ml), each containing 100 ml of carrot medium (16), were seeded with mycelial disks from a 48-hr-old culture of *P. aphanidermatum* and incubated for 14 days at 30 C. The mycelium was washed, suspended in sterile tap water, and homogenized in a Waring blender for 5 min. Ten milliliters of the homogenized culture, containing 5×10^6 oospores per milliliter, were mixed with 1 kg of sandy-loam soil in an electrically powered soil mixer.

Broadcast application of *Trichoderma* species. Wheat bran/peat mixture (1:1, v/v) adjusted to 40% moisture (w/w) was autoclaved in autoclavable polyethylene bags (50 × 50 cm) for 1 hr at 121 C on three successive days. The substrate mixture was inoculated with a conidial suspension of each of the two isolates of *Trichoderma* and incubated in an illuminated chamber for 14 days at 30 C. This preparation of *Trichoderma* was mixed with soil (5 g/kg soil) or rooting mixture (20% preparation by volume) before sowing seeds of the test plants.

Seed treatment with *Trichoderma* species. *T. harzianum* was grown in Erlenmeyer flasks (500 ml) each containing 200 ml of PDA (Oxoid, London, England). Conidia were separated by adding 10 ml of 0.001% Tween-20 (J. T. Baker, Phillipsburg, NJ 08865) solution in sterile tap water and 10 glass 3-mm-diameter beads to each Erlenmeyer flask and then shaking the flasks on a rotary shaker at 400 rpm for 10 min. The conidial suspension, adjusted to 5×10^9 conidia per milliliter, was supplemented with 0.2 g of the polysaccharide Pelgel (Nitragin, Milwaukee, WI 53209) as an adhesive. Three milliliters of this suspension were used to coat 150 g of pea, 50 g of cucumber, or 10 g of tomato seeds and were immediately dried by warm ventilation.

All experiments were carried out with nontreated seeds. The fungicide prothiocarb (Previcur, *S*-ethyl *N*-(3-dimethyl-aminopropyl) thiocarbamate, 70% W.P. Schering, A.G., Berlin, Germany), was used alone or combined with the inoculum preparation of *T. harzianum* for the control of *P. aphanidermatum* in gypsophila.

The incidence of disease caused by *P. aphanidermatum, R. solani,* and *S. rolfsii* were expressed as the percentage of diseased plants in a population.

Greenhouse experiments were carried out at 27–33 C. Treatments in all experiments were replicated six times and were set in randomized blocks. All tests were repeated at least twice. Statistical significance of the data was determined by analysis of variance, $P = 0.05$.

RESULTS

Wheat bran and a wheat bran/peat mixture were found to be the most suitable media for growth and sporulation of *T. harzianum* (Table 1). Seven days growth on wheat bran, wheat straw compost, cotton straw, and wheat bran/peat mixture resulted in an increase in population density level ranging from 5×10^5 to 1.2×10^7. The best survival was achieved on wheat-bran/peat mixture tested after 1 yr of incubation at 25 C.

The pH of wheat bran was 6.5 initially, but after 10 days of growth that of *T. harzianum* increased to 8.0 (Table 1). By applying 250 mM citrate-phosphate buffer a low pH level of 5.5 was maintained even after 10 days, but this resulted in a population density of *T. harzianum* decreasing from 3.8×10^9 to 8.1×10^6 colony-forming units (cfu) per gram of substrate. Adding peat to wheat bran effectively controlled the pH level throughout the growth period, without decreasing the population of *Trichoderma* (Table 1). This medium was therefore chosen to be the food base for the broadcast application of *Trichoderma* spp.

Production of inhibitory substances by *Trichoderma* species. Assays using solid SM plates on which *T. harzianum* was previously grown demonstrated, 48 hr after incubation, that linear growth rate of *P. aphanidermatum* decreased to 3 mm/day compared with 26 mm/day in the control.

Culture filtrates of both isolates of *Trichoderma* (*T. hamatum* and *T. harzianum*) were separately supplemented to SM. Results (Fig. 1) show that the linear growth of *P. aphanidermatum* was inhibited by the culture filtrate of *T. harzianum* more than by that of *T. hamatum* (83 and 8% inhibition, respectively). Both *T. harzianum* and *T. hamatum* were inhibited by extracellular culture

TABLE 1. Growth and survival of *Trichoderma harzianum* on natural substrates[u]

Substrate	CFU[v]/g substrate (×10⁶) after incubation time of:		Substrate pH	
	7 days[w]	1 year[x]	Before inoculation	After 10 days growth
Wheat bran	4,000 b[y]	12.00 b	6.5	8.0
Peat	450 c	7.30 b	4.5	5.0
Wheat bran/peat[z]	4,900 a	450.00 a	5.0	5.5
Compost	590 c	0.04 d	7.8	8.0
Wheat straw	490 c	0.22 c	7.0	7.5
Cotton straw	210 d	0.21 c	7.5	7.5

[u] Initial population of *Trichoderma* was 400 conidia per gram of substrate.
[v] Colony-forming units on a medium selective for *Trichoderma* (TSM).
[w] Incubation temperature = 30 C.
[x] Incubation temperature = 25 C.
[y] Numbers of each column followed by the same letter are not significantly different ($P = 0.05$).
[z] Mixture (1:1, v/v).

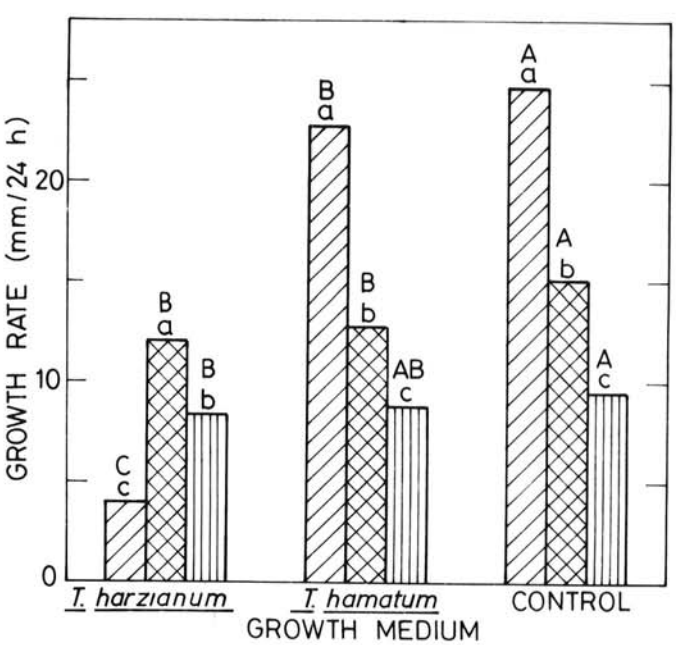

Fig. 1. Effect of culture filtrates of *Trichoderma* spp. supplemented to SM (1:1, v/v) on the linear growth of *P. aphanidermatum* (▨), *T. harzianum* (T-315) (▩), and *T. hamatum* (▥) 24 hr after inoculation. Columns of each fungus followed by a common letter are not significantly different ($P = 0.05$).

filtrate of *T. harzianum* (20 and 14% inhibition, respectively). However, while *T. hamatum* was not affected by its culture filtrate, *T. harzianum* was, by 15.2%.

The same technique indicated that the filtrates of the pathogen enhanced the growth rate of *T. harzianum* by 34%, but did not affect the linear growth of *T. hamatum*.

Application of *T. harzianum* to soil. Wheat bran/peat preparation of *T. harzianum* mixed with loamy sand (5 g/kg soil) artificially infested with *P. aphanidermatum*, significantly reduced disease incidence caused by this pathogen in cucumbers, peas and tomatoes (69, 81, and 85%), respectively. The conidial suspension

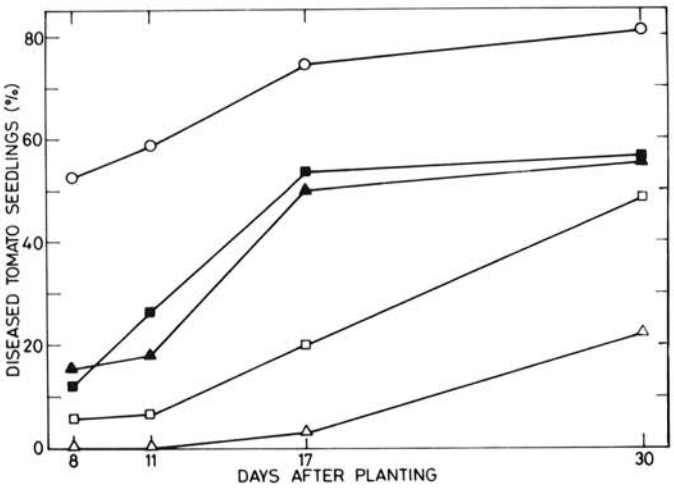

Fig. 2. Biological control of *Pythium aphanidermatum* in tomato plants in a nursery by application of *T. harzianum* (△, ▲) or *T. hamatum* (□, ■) either by mixing wheat bran/peat preparations with rooting mixture (20% by volume) (△, □), or as seed coating (▲, ■). Infested, untreated rooting mixture served as control (O).

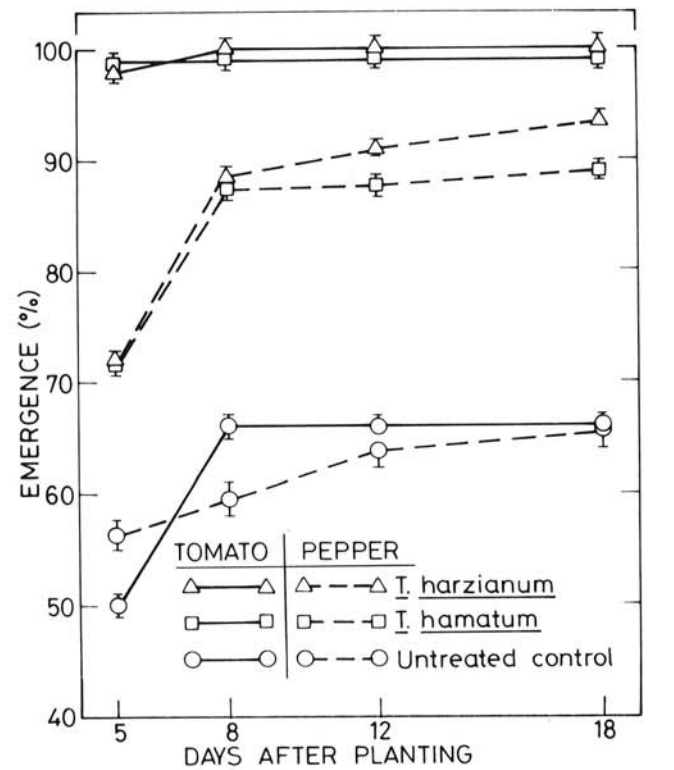

Fig. 3. Effect of *Trichoderma harzianum* and *T. hamatum* on emergence percentage of tomato and pepper seedlings planted in a rooting mixture naturally infested with *Pythium aphanidermatum*. Isolates of *Trichoderma* were applied as wheat bran/peat preparations mixed with rooting mixture (20% by volume). Infested and untreated rooting mixture served as control.

of *T. harzianum* applied to seeds was as effective as the broadcast application of wheat-bran/peat preparation, reducing diseased cucumber, pea, and tomato seedlings by 76, 88, and 87%, respectively. Single and double coating of pea seeds with the adhesive polysaccharide alone resulted in an increase in damping-off percentage to 56 and 66%, respectively, compared with 37% in the control (sown with untreated seeds). A double coating of the conidial suspension of *Trichoderma* containing the same concentration of the adhesive did not improve the results.

Studying the effect of temperatures on antagonism showed that at low temperature conditions (22 C), *T. hamatum* reduced disease incidence by 79% compared with a 44% reduction by *T. harzianum*. However, at 30 C *T. harzianum* reduced disease incidence by 91% (from 37% in the untreated control to 3%). This compared with a reduction of only 73% by *T. hamatum*.

Application of *Trichoderma* species in a plant nursery rooting mixture. Planting tomatoes in a rooting mixture, naturally infested with *P. aphanidermatum*, resulted in a high damping-off percentage (81%, 30 days after planting) (Fig. 2). *T. hamatum* and *T. harzianum* applied either as wheat bran/peat preparation to the rooting mixture (20% by volume) or as a seed coating, increased the number of healthy seedlings by 63–76% and 57–58%, respectively (Fig. 2). Eleven days after planting, no damping-off symptoms were observed in the *T. harzianum* mixture, whereas ~60% of the seedlings in the untreated control were already killed. Preemergence disease incidence was reduced by the seed coating treatment (74% reduction), whereas the broadcast application resulted in 100% reduction. Similarly, broadcast application of *T. harzianum* reduced damping-off incidence by 90.3% in pepper seedlings. This isolate was significantly ($P = 0.05$) greater than that with *T. hamatum* (77%) 30 days after planting.

Wheat-bran/peat preparation of *T. harzianum* mixed with a naturally infested rooting mixture resulted in an increase of 96 and 27% in emergence of tomato and pepper seedlings, respectively, 5 days later (Fig. 3). No preemergence damping-off could be detected in the *Trichoderma* treatments 18 days after planting, compared with 31 and 22% preemergence damping-off in the control for tomato and pepper seedlings, respectively. At the end of rooting period (35 days after planting) total loss of seedlings in the control due to pre- and postemergence damping-off was 45% in tomatoes and 43% in peppers. The *T. harzianum* preparation, however, reduced the disease incidence by 87 and 90%, respectively.

Gypsophila cuttings were planted in a commercial nursery rooting mixture infested with *P. aphanidermatum* and *R. solani*. Two weeks after planting, the percentage of completely rotted cuttings reached 54 and 44%, respectively. Application of the fungicide prothiocarb reduced the incidence of disease caused by *Pythium* by 67%; however, this treatment resulted in a 42% increase in the number of plants infected with *R. solani*, compared with the untreated control. Incorporating *T. harzianum* preparation into the rooting mixture (20% by volume) delayed the appearance of the disease by 3 days and reduced both *P. aphanidermatum* and the incidence of disease caused by *R. solani* by 54 and 16%, respectively. Integrated application of prothiocarb and the wheat bran preparation of *T. harzianum* reduced the incidence of disease caused by *Pythium* by 88%. Thirty days after planting, most of the gypsophila cuttings, except for those treated with both chemical and biological agents, were infected by either *P. aphanidermatum* or *R. solani*.

Our *T. harzianum* isolate, T-315, successfully controlled not only *P. aphanidermatum*, but also *R. solani* and *S. rolfsii* in beans. Twenty days after planting, percentages of plants infected by *R. solani* and *S. rolfsii* were 34 and 42%, respectively, in the untreated control, and 12% in the presence of the wheat-bran/peat preparation of *T. harzianum*.

DISCUSSION

This study demonstrates the usefulness of a new carrier for growth and delivery of *Trichoderma* spp. into pathogen infested soils.

A wheat bran preparation was used as a food base for the growth

and application of *T. harzianum* to soil as a biocontrol agent by Chet et al (5). During the fungal growth period its pH level increases from 6.5 to 8.0. Geypens and Feys (10) reported that pH modification affected the antagonistic activity of *Trichoderma* spp. Chet and Baker (4) have found that pH values higher than 6.5, 6.0, and 5.2 are needed for maximal linear growth, conidiophore formation, and conidium germination, respectively, of *T. harzianum*.

Our study reveals that acidification of wheat bran by chemical means was unsuccessful, whereas the addition of peat maintained the growth mixture at an optimal pH value of 5.0. The acidified medium was also beneficial in preventing bacterial contaminations. Hence, the wheat bran/peat mixture was chosen as the food base and carrier of *Trichoderma* spp. for broadcast application. Moreover, grown on this mixture, *Trichoderma* viable population persisted more than on any other tested substrate.

Harman et al (12,13) reported the biological control of *Pythium* spp. and *R. solani* by *T. hamatum* as seed coating. Biocontrol of *Pythium* spp. was achieved only in peas, whereas our isolate was capable of controlling this pathogen in cucumbers, tomatoes, peas, peppers, and gypsophila. They reported that 25 C was the optimal temperature for biological control of *Pythium* spp. in peas. We have found that at relatively higher temperatures *T. harzianum* was superior to *T. hamatum* and may serve as a better biocontrol agent in warm regions.

Elad et al (7) reported the lytic activity of extracellular enzymes of *T. harzianum*, when grown on fungal cell wall components of pathogenic soilborne fungi, as a sole carbon source. However, this may not be the only biological control mechanism.

Our study shows that culture filtrates of our selected isolate of *T. harzianum* (T-315) have an inhibitory effect on the linear growth of *P. aphanidermatum*. This inhibition was observed when using either a solidified or a liquid SM. Comparison between *T. hamatum* and *T. harzianum* (T-315) reveals that the filtrate from the latter is more inhibitory. Moreover, culture filtrate of *P. aphanidermatum* enhanced the linear growth of *T. harzianum* but not that of *T. hamatum*. This perhaps explains the superiority of *T. harzianum* (T-315) in controlling *P. aphanidermatum*.

The same new isolate of *T. harzianum* was also capable of controlling *S. rolfsii* and *R. solani* in beans.

Applying this isolate to the rooting mixture of gypsophila cuttings protected the plants from both *P. aphanidermatum* and *R. solani*. *T. harzianum* (T-315) was found to be as effective as the commercial fungicide prothiocarb in controlling *Pythium*. However, the use of this fungicide significantly increased *R. solani* disease incidence. Integrated control, by combining *T. harzianum* and prothiocarb, resulted in the best control of both pathogens. Apparently the use of the fungicide combined with *Trichoderma*, increased the efficiency of the latter against *R. solani*.

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