Evaluation of *Trichoderma koningii* and *T. harzianum* from New York Soils for Biological Control of Seed Rot Caused by *Pythium* spp.

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ABSTRACT

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Some soils with low iron levels are suppressive to *Trichoderma* spp. because of the activity of pseudomonads and their siderophores. Isolates of *Trichoderma* were obtained from a soil suppressive to these fungi. The abilities of these isolates to protect pea seeds against *Pythium* spp. were determined. The two most effective isolates were identified as *T. koningii* (T8) and *T. harzianum* (T12). Both isolates grew well on seeds, and neither was affected by seed-colonizing pseudomonads. T8 grew better than T12 at 10–20 C. The optimal pH for both isolates was 4.5, and both grew slowly at pH 2 and 8. Both isolates were inhibited by the partially-purified fluorescent pigment from a *Pseudomonas* sp., but T12 was less inhibited than T8. T12 grown on low-iron (King's B) medium produced a fluorescent pigment that was precipitated by the addition of $FeCl_3$, but T8 did not. T8 produced 28% as much biomass on a medium with very low (17 ng/ml) iron as on a less deficient medium, but T12 produced only 7.5% as much. Both isolates protected seeds against seed rots in soils naturally infested with *Pythium* spp. when applied either as a seed coating in various adhesives or when applied in gels used for fluid seed drilling. Polyvinyl alcohol tended to give less disease than other adhesives tested. Among gels, Poly Surf C (a hydroxyethyl cellulose) and Polytran A (a glucan) gave better results than the silica-based Laponite 508. In field trials, T8 consistently protected peas and beans against seed rots. Materials tested as spreader-stickers for seed treatments or as gel carriers used in fluid seed drilling differ in suitability as delivery systems for biocontrol agents; polyvinyl alcohol works well as a spreader sticker, and Poly Surf C is useful in fluid seed drilling systems.

Additional key words: antagonism, *Cucumis sativus*, *Phaseolus vulgaris*, *Pisum sativum*.

There have been many reports of successful use of antagonistic fungi to control soilborne pathogenic fungi (2,4,5,7,8,12,15,20), but no agent has been used extensively, partly because of the large amount of material required (4,8,16,20) and also because of the variability in performance between locations and seasons (9,12,20).

The application of antagonists by seed treatments or during fluid seed drilling procedures (6) are attractive methods for the introduction and establishment of a biocontrol agent in the infection courts of the host. Both methods require smaller amounts of inoculum than either broadcast or in-furrow treatments (5,7,10).

Seed treatment with the biological control agent *Trichoderma hamatum* (Bon) Bain protects seeds and seedlings of radish and peas from attack by *Pythium* spp. or *Rhizoctonia solani* in Colorado (7). The same antagonist protects cotton seeds from *R. solani* under field conditions in Israel (5). However, it is ineffective when applied on seeds planted in New York soils that have low levels of available iron (9); these soils contain *Pseudomonas* spp. that compete with the biocontrol agent for iron (9).

The objectives of this work were to isolate, characterize, and test biocontrol agents that would control Pythium seed rot in these soils. Additionally, we tested the suitability of various materials as spreader-stickers in seed treatments, and as gel carriers for agents in fluid seed drilling operations.

MATERIALS AND METHODS

Microorganisms. *Trichoderma* spp. were isolated from soil on a Trichoderma-selective medium (TSM) (3) and transferred to malt extract agar (Difco Laboratories, Detroit, MI 48232) for identification. The soil was an Arkport fine sandy loam (9) which has little available iron (approximately 1 μg of extractable iron per gram of soil) (9).

Other organisms used were *T. harzianum* (T-Co) (originally described as *T. hamatum*) isolated from a soil from Columbia (South America) suppressive to *Rhizoctonia* (2). It was effective as a seed treatment against *Pythium* spp. and *R. solani* in Colorado (7) and Israel (5), but not in New York (9). An ultraviolet-induced mutant of this isolate (T95) and an isolate of *T. hamatum* (382) isolated from a hardwood bark compost were also tested. Isolates T95 and 382 were used because their introduction into various planting media induces suppressiveness to *R. solani* (R. Baker, *unpublished*, and E. B. Nelson and H. A. J. Hoitink, *unpublished*).

Interaction of fluorescent pigments from *Pseudomonas* spp. with isolates of *Trichoderma*. Fluorescent pigments were obtained from a *Pseudomonas* (isolate 10) described earlier (9). This isolate is classified in the group characterized by *P. putida* and *P. fluorescens*. This bacterium was grown under continuous agitation for 30 hr at 25 C on a salts medium containing succinic acid as a carbon source (13). Bacterial cells were removed by centrifugation at 5,900 *g* for 20 min, and the pigment was partially purified by the acetone precipitation procedure described by Misaghi et al (13). To test effects of the pigment on growth of *Trichoderma* spp., in a medium with low iron levels, 4 ml of King's B medium (11) and aliquots of purified pigments were added to 25-ml flasks and volumes were brought to 5 ml. The flasks were then inoculated with 100 μl of a suspension containing 10^5 spores of *Trichoderma* per milliliter of water, incubated at 25 C with shaking on a reciprocating shaker at 60 strokes per minute for 48 hr, and the dry weight of the mycelium was determined after drying at 70 C. A medium containing sucrose and asparagine (18) was used for the production of pigments from *Trichoderma* spp. The purification method was the same as for the bacterial pigment (13). Tests were conducted to determine whether these pigments were produced in the presence of $FeCl_3$ and whether these pigments gave typical

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reactions for siderophore-like compounds (eg, precipitation, quenching of fluorescence, and color changes caused by the addition of $FeCl_3$) (13). All experiments contained three replicates of each treatment, and each experiment was performed at least twice.

Seed treatments. Seeds were coated with a conidial suspension of *Trichoderma* spp. containing 10^8 conidia per milliliter. Conidia were scraped from 9- to 14-day-old colonies grown on petri dishes on Bacto potato-dextrose agar (PDA) (Difco, Inc., Detroit, MI 48233). Antagonists were applied to seeds of pea (*Pisum sativum* L. 'Venus'), snap beans (*Phaseolus vulgaris* L. 'Thor'), and cucumber (*Cucumis sativus* L. 'Slice Master'). Aqueous spore suspensions were applied in 2% (w/v) Methocel A4C Premium (Dow Chemical Co., Midland, MI 48640), 20% (w/v) polyvinyl alcohol type II (PVA) (Sigma Chemicals Co., St. Louis, MO 63178), or 2% (w/v) Polytran N (Jetco Chemicals, Inc., Corsican, TX 75110). Aliquots (0.4 ml) of spore suspensions were added to 100 bean or pea seeds while 0.3 ml were applied to 100 cucumber seeds. These mixtures were shaken in Erlenmeyer flasks until spores were distributed over the seed surfaces. After shaking seeds in water and plating them on PDA, 10^4–10^5 propagules of *Trichoderma* per seed were recovered. Other seeds were treated with captan at the rate of 1.6 mg a.i. per gram of seed. Antagonists also were applied to seeds in aqueous gels containing either 0.8% (w/v) Poly Surf C (Hercules, Inc., Wilmington, DE 19899), 1.5% (w/v) Laponite 508 (Laporte Inc., Hackensack, NJ 07601), or 0.6% (w/v) Polytran N. Gels were applied at rates of 50 ml per 100 seeds for both pea and cucumber seeds.

Microscopy. Small sections (1- to 2-mm square) were cut from seed coats of peas germinating in naturally-infested Arkport soil. Examinations were made 24 and 48 hr after the planting of nontreated seeds, seeds treated with the agents in Methocel, or seeds treated with Methocel alone. They were stained and examined by using our modification (9) of Anderson and Slinger's (1) procedure.

Iron removal and determination. Iron was removed from media by complexing with 8-hydroxyquinoline according to the method described by Waring and Werkman (19). Iron concentrations were determined with a Plasma 100 spectrometer (Instruments Laboratory Inc., Wilmington, MA 01887).

Temperature and pH experiments. Growth of *Trichoderma* spp. at various temperature and pH levels was determined in vitro. Growth at various temperatures in incubators was determined by the increase in radial growth on PDA in petri dishes. Determination of the growth of isolates at various pH levels was determined in King's B broth adjusted to various levels between pH 2 and 8. Inoculation and incubation conditions were as described for studies of the interactions between *Trichoderma* spp. and fluorescent pigments produced by *Pseudomonas* sp. Growth was determined 48 hr after inoculation by removing hyphae by filtration and determining dry weight after drying overnight at 70 C.

Growth chamber experiments. An Arkport fine sandy loam naturally infested with *Pythium* spp. was used for all experiments (9). Soil was stored in covered containers prior to use. High levels of pathogen propagules were obtained in these soils by growing a mixture of wheat, barley, and oats for 3 wk in the greenhouse, incorporating the plants into the soil, and incubating it for an additional 2 wk (9). Numbers of propagules of *Pythium* were determined on Mircetich's medium (14). For growth chamber experiments, planted soils were screened and mixed with unamended soil to give levels of *Pythium* capable of rotting about 80% of nontreated seeds. This usually required 50–100 propagules of *Pythium* per gram of soil.

Five pea seeds or 10 cucumber seeds were planted in 10 × 10 × 6-cm boxes, at a depth of approximately 1 cm. All incubations were at 25 C. Each box was considered a replication and there were five boxes per treatment. Stand counts were taken 5 and 8–9 days after planting; values reported are those obtained at the latter time. All experiments were conducted at least twice.

Field experiments. Two field experiments were conducted at different locations in the spring of 1982. Environmental and soil conditions during the course of the experiment are given in Table 1. Randomized complete block designs with five replications were used for both tests. Each row was 4 m long and received 100 seeds. Soil samples were taken from the root system of plants 4–6 wk after emergence. These soil samples were taken from the root zone of plants in each replication and pooled. Density of propagules of *Trichoderma* in these samples was determined by using the soil dilution method on TSM (3).

RESULTS

Isolation and identification. *Trichoderma* spp. were isolated from the Arkport sandy loam. Forty different cultures were tested for ability to control pea seed rot caused by *Pythium* in Arkport soil. The two antagonists most effective in controlling the disease were identified as *Trichoderma koningii* Oud and *T. harzianum* Rifai, according to the criteria of Rifai (17). The *T. koningii* (isolate T8) had phialides that measured 7.5–10 × 2.5–3.5 μm. The phialospores were mostly elliptic and measured 3.0–4.8 × 1.9–2.8 μm. *T. harzianum* (isolate T12) phialides measured 5–7 × 3.0–3.5 μm and the phialospores measured 3.0–3.6 × 2.5–3 μm. Both isolates have green smooth spores, and grow rapidly on malt extract agar.

Factors affecting the biocontrol agents in culture. The optimal growth temperature for both isolates was 25–30 C; however, T8 grew faster than T12 between 10–20 C (Fig. 1). Optimal growth occurred at pH 4.5 for both isolates while both grew slowly at pH 2 and 8 (*unpublished*). The isolates differed in ability to grow in the presence of partially purified fluorescent pigment from a *Pseudomonas* sp.; T8 was more sensitive to the bacterial pigment (Fig. 2). Growth inhibition, as measured by comparing the dry weights of hyphal mats, could be reversed by adding 100 μg/ml $FeCl_3$ to the culture medium. The inhibitory activity of the pigment was affected by pH. At pH 3, the growth of both isolates was unaffected by the presence of the bacterial pigment (*unpublished*).

T12 grown in either the King's B or succinate-salts culture medium produced a brown fluorescent pigment. This pigment, when partially purified, was precipitated with $FeCl_3$ and was not produced if $FeCl_3$ was added to either the King's B or succinate-salts medium. Production of pigments by T8 was not observed. When King's B medium was extracted with 8-hydroxyquinoline, iron levels were reduced from 0.38 to 0.017 μg/ml. At the low iron level, T8 produced 28% as much biomass as at the higher level,

TABLE 1. Environmental conditions and soil characteristics in field experiments

Location[x]	Soil temperature 4 cm deep (C)[y] Minimum Range	Minimum Avg.	Maximum Range	Maximum Avg.	Rainfall during experiment[x] (mm)	Soil characteristics pH	P (μg/g)	K (μg/g)	Ca (μg/g)	Mg (μg/g)	Mn (μg/g)	Fe (μg/g)	Zn (μg/g)	Organic matter (%)
Phelps	3–15	9	13–31	24	28	5.9	64	380	1,820	460	26	17.4	4.6	2.2
Geneva	12–20	15	17–34	26	108	5.8	26	280	4,200	480	70	26	3.6	1.5

[x] Field planting done in a Palmyra gravelly loam near Phelps, NY, and in a Honeoye fine sandy loam at the Vegetable Research Farm near Geneva, NY. Near Phelps, the planting date was 23 April 1982 and near Geneva 21 May 1982. Final stand counts were taken 25 May and 20 June near Phelps and Geneva, respectively. Rainfall data is the cumulative total between the planting data and the data when final stand counts were determined.

[y] Data given in the range column are ranges of daily minimum or maximum temperatures, while that in the average columns are mean high and low temperatures for the period of the experiment.

while T12 produced only 7.5% as much. When 10 μg of $FeCl_3$ per milliliter was added to the low-iron medium, the growth of both fungi was similar to that in nontreated media.

Microscopic observations. When seeds were treated with T8 or T12 in Methocel, conidia of both T8 and T12 germinated on treated pea seed coats 18–22 hr after planting in Arkport soil. Twenty-four hours later, dense hyphal mats were observed on seed coats together with bacterial colonies. Hyphae of T8 and T12 growing through and near bacterial colonies were not damaged. Four days after planting, sporulation of *Trichoderma* was observed on the seed coats. Conversely, hyphae of T-Co were severely damaged under these conditions (cf, 9). Nontreated seeds were covered mainly with bacterial colonies and only a few hyphae were observed.

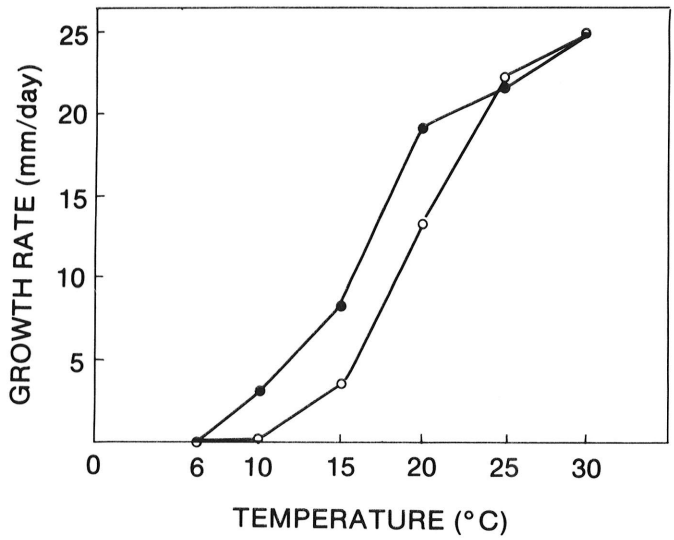

Fig. 1. Growth of *Trichoderma koningii* (T8) (•) and *T. harzianum* (T12) (o) at different temperatures.

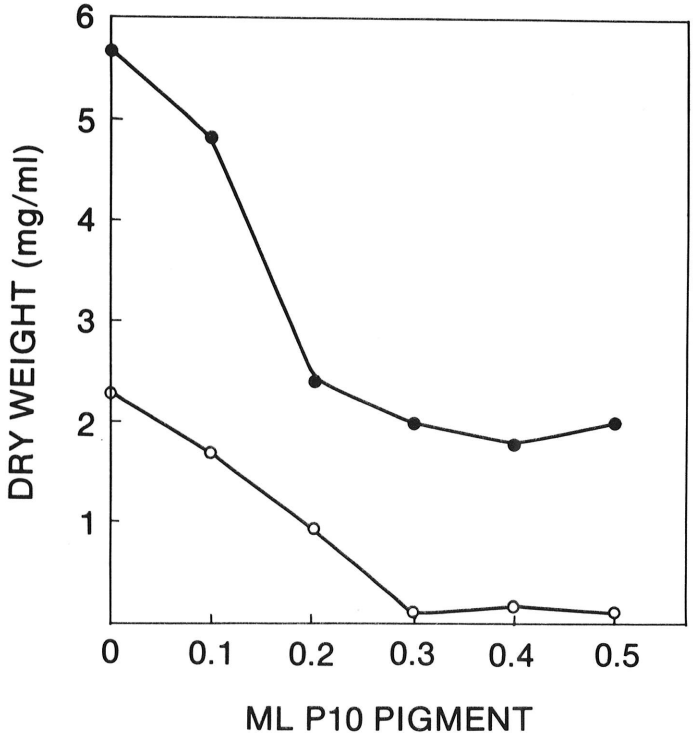

Fig. 2. Dry weight of *Trichoderma koningii* (T8) (o) and *T. harzianum* (T12) (•) after two days growth in King's medium in the presence of various amounts of a partially purified fluorescent pigment from a *Pseudomonas* sp.

Growth chamber experiments. Seed rot and damping-off caused by *Pythium* in peas and cucumbers was reduced by applying conidia of T8 or T12 to seeds. Application of T-Co in Methocel did not reduce seed rot relative to the Methocel check (*unpublished*; cf, 9). While significant disease control was obtained with both isolates (Table 2), the greatest stands were obtained with captan-treated seeds. Stands from seeds treated with different adhesives did not differ significantly, although seeds treated with PVA tended to have less disease (Table 2). Cucumber seeds treated with T8 or T12 were protected from both preemergence and postemergence damping-off (Fig. 3).

Pea and cucumber seeds were mixed with a gel (Poly Surf C) containing different concentrations of T8 spores and planted in soil infested with *Pythium*. In both crops, high levels of *Trichoderma* (10^8–10^9 spores per milliliter of gel) were necessary to obtain

TABLE 2. Reduction of *Pythium* seed rot by *Trichoderma koningii* (T8) and *Trichoderma harzianum* (T12) applied to pea seeds in growth chamber experiments[x]

Adhesive	Protectant	Healthy seedlings[z] (%)
None	None	24 d
None	captan	92 a
Polyvinyl alcohol	None	40 d
Polyvinyl alcohol	*T. koningii*	88 ab
Polyvinyl alcohol	*T. harzianum*	72 abc
Polytran N	None	24 d
Polytran N	*T. koningii*	68 bc
Polytran N	*T. harzianum*	64 c
Methocel A4C	None	24 d
Methocel A4C	*T. koningii*	84 abc
Methocel A4C	*T. harzianum*	68 bc

[x]Seeds were treated with aqueous suspensions containing 10^8 spores per milliliter, and 0.4 ml of suspension was used to treat 100 seeds. The concentration of polyvinyl alcohol was 20% w/v, while that of Polytran N and Methocel was 2% w/v. Seeds were planted in Arkport fine sandy loam infested with *Pythium* spp.

[y]Numbers followed by a common letter are not statistically different according to Duncan's multiple range test ($P = 0.05$).

[z]Each value is an average of five replicates with five seeds planted per replicate.

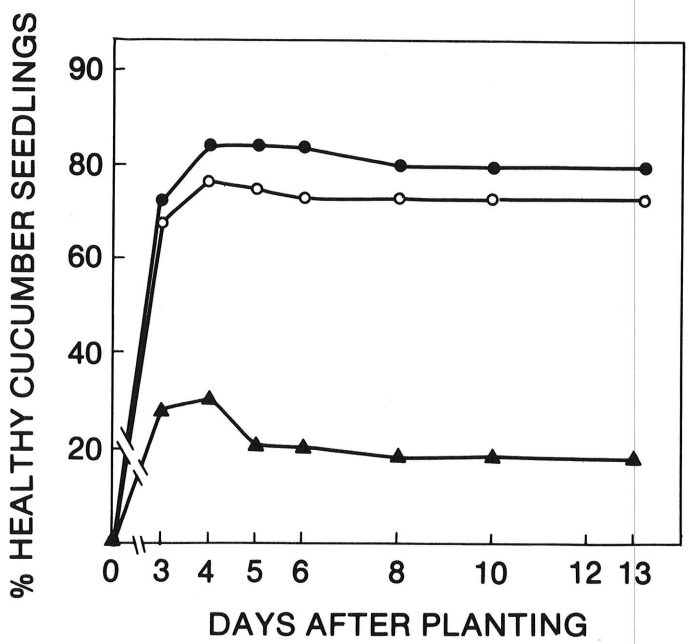

Fig. 3. Percentage of healthy cucumber seedlings produced from nontreated seeds (▲), seeds treated with *Trichoderma koningii* (T8) (•), or seeds treated with *T. harzianum* (T12) (o) at various lengths of time after planting in soil infested with *Pythium*.

efficient control (Fig. 4). Three different gels were compared as carriers for T8 or T12 in pea plantings. The *Trichoderma* spp. were effective when applied in Poly Surf C, or Polytran N, but these fungi were less effective when applied in Laponite 508 (Table 3).

When the experiments shown in Table 2 and Fig. 3 were completed, soils from replicates of each treatment were mixed. These soils were replanted with nontreated seeds, stand counts were taken 5–7 days later, and the populations of *Trichoderma* then determined by dilution plating on TSM. The soil did not become suppressive to *Pythium*; and disease incidence did not differ between the control plants and those treated with *Trichoderma*. Population levels of *Trichoderma* in these soils were $2–5 \times 10^2$ CFU per gram of soil in the control and $5 \times 10^4–2 \times 10^5$ CFU per gram in the soil treated with *Trichoderma*. When the soils were treated with a spore suspension of T8 or T12, 10^8 conidia per gram of soil were necessary to achieve significant control of *Pythium* with both isolates.

TABLE 3. Reduction of *Pythium* seed rot of pea seeds with *Trichoderma* applied in gels in laboratory experiments[x]

Gel	*Trichoderma*	Healthy seedlings[y] (%)
None	None	15 d[z]
Poly Surf C	None	48 b
	T. koningii	72 a
	T. harzianum	76 a
Polytran N	None	44 b
	T. koningii	76 a
	T. harzianum	64 ab
Laponite 508	None	24 cd
	T. koningii	56 b
	T. harzianum	44 bc

[x]Spore concentration was 3.5×10^6 per milliliter of gel, and 0.5 ml of gel was used per seed. Seeds were planted in Arkport fine sandy loam infested with *Pythium* spp.
[y]Each value is an average of five replicates with five seeds planted per replicate.
[z]Numbers followed by a common letter are not statistically different according to Duncan's multiple range test ($P = 0.05$).

TABLE 4. Reduction of seed rot of peas under field conditions (Phelps) by application of spores of various strains of *Trichoderma* spp. in 2% Methocel

Seed treatment	Healthy plants[y] (%)	Healthy cotyledons (%)	CFU/g soil *Trichoderma*
Nontreated	53 d[z]	17 c	6×10^3
Captan	80 a	72 a	2×10^4
T. koningii (T8)	68 bc	82 a	3×10^4
T. harzianum (T12)	54 d	22 c	6×10^3
T. harzianum (T95)	63 cd	50 b	4×10^4
T. harzianum (T-Co)	57 cd	···	···

[y]Each value is an average of five replicates; 100 seeds were planted per replicate.
[z]Numbers followed by a common letter within a column are not different according to Duncan's multiple range test ($P = 0.05$).

Field experiments. Peas and snap beans coated with several isolates of *Trichoderma* or with captan were sown at two locations in soil naturally infested with *Pythium* spp. In all experiments, the best control was observed with captan-treated seeds (Tables 4 and 5). T8 was the most efficient biocontrol agent and significant disease reduction was observed in both locations and with both crops. The results with the other isolates were variable between locations and crops tested. In the experiment at the Phelps site, the percent of pea seedlings with nonrotted cotyledons was recorded 3 wk after planting. A correlation between the number of healthy seedlings and plants with cotyledons was observed. Populations of *Trichoderma* were higher in soil near plants that grew from seeds treated with captan or *Trichoderma* than in soil near plants growing from nontreated seeds (Tables 4 and 5).

In both field locations, soils were dry (Table 1). Plants emerged from soil over a period of several weeks. Soil pH was 5.8–5.9 (Table 1).

DISCUSSION

Isolates of *Trichoderma* native to a soil may be better adapted to it than introduced isolates, and may be more able to coexist with native soil microflora. *T. koningii* (T8) and *T. harzianum* (T12) provided significant biological control against seed rot induced by *Pythium* under growth chamber conditions in Arkport fine sandy loam. These isolates were obtained from the Arkport soil which contains low levels of available iron. Other isolates, including T-Co, T95, and 382 which rendered other soils suppressive to

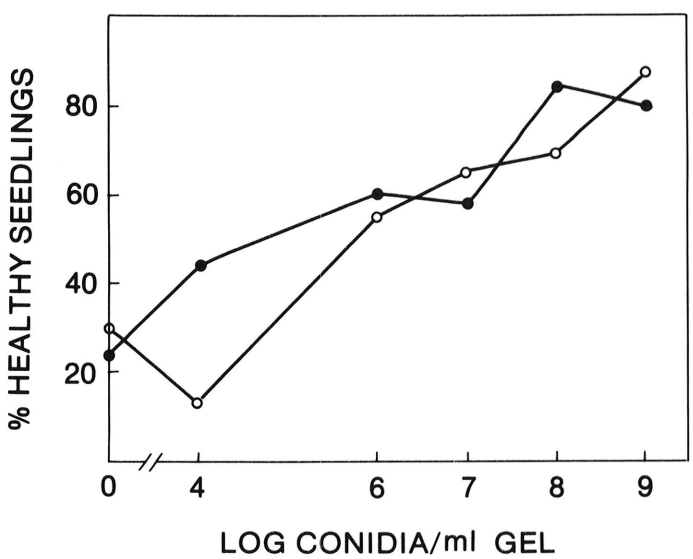

Fig. 4. The relationship between numbers of conidia of *Trichoderma koningii* (T8) applied as seed treatments in gels containing 0.8% (w/v). Poly Surf C and percentage of healthy pea (•) or cucumber (o) seeds.

TABLE 5. Reduction of seed rot of beans and peas under field conditions (Geneva) by application of spores of strains of *Trichoderma* spp. in 2% Methocel

Seed treatment	Beans		Peas	
	Healthy seedlings[y] (%)	*Trichoderma* (CFU/gm soil)	Healthy seedlings[y] (%)	*Trichoderma* (CFU/gm soil)
Nontreated	74 d[z]	6×10^2	65 de	$<10^2$
Captan	96 a	2×10^3	87 a	6×10^4
T. koningii (T8)	95 a	2×10^3	75 bc	4×10^3
T. harzianum (T12)	77 cd	5×10^3	75 bc	4×10^4
T. harzianum (T95)	83 bc	6×10^4	63 e	7×10^4
T. harzianum (T-Co)	85 b	5×10^4	69 cde	···
T. hamatum (382)	86 b	4×10^4	62 e	···

[y]Each value is an average of five replicates with 100 seeds planted per replicate.
[z]Numbers followed by a common letter within a crop are not statistically different according to Duncan's multiple range test ($P = 0.05$).

Pythium or *R. solani* (2,5,7; R. Baker, E. B. Nelson, and H. A. J. Hoitink, *unpublished*), were ineffective in this soil. Isolate T-Co was unable to compete for iron or to grow on seed coats in the presence of *Pseudomonas* spp. (9); however, T8 and T12 grew and sporulated well in their presence. T12 was less sensitive than was T8 to the partially purified pigment of *Pseudomonas*. T12 produced a fluorescent pigment that was precipitated with iron, but which was not produced when iron was present. Conversely, T8 did not produce any pigments, but was less severely inhibited than T12 at very low iron levels (17 ng/ml).

Factors other than iron and pseudomonads probably also affect efficacy of *Trichoderma* spp. In field experiments, T8 consistently gave significant stand improvements relative to nontreated seeds. Other isolates gave improvements in some experiments, but not in others, even though all isolates parasitize *Pythium* spp. and create suppressiveness in some soils or planting media (2,5,7; R. Baker, E. B. Nelson, and H. A. J. Hoitink, *unpublished*). Soils used in these trials contained enough iron (>10 μg/g) so pseudomonad siderophores should not have inhibited growth (9) or effectiveness of any isolate; other factors apparently prevented their activity. Field plantings were in cool soils, and T8 grows better than T12 between 10 and 20 C. Temperature may have been a factor in its superior performance relative to other agents.

Ability of an agent to suppress disease depends on factors other than the presence of large numbers of propagules of effective *Trichoderma* spp. In the Arkport fine sandy loam, suppressiveness required 10^8 conidia of *Trichoderma* per gram of soil, while only 10^4–10^5 conidia are required for the same effect in Fort Collins clay loam (2,7). Nelson et al (15) have made analogous observations; some planting media containing peat or fresh hardwood bark seldom become suppressive, even though *Trichoderma* spp. grow well or large numbers of effective isolates are added. Apparently, interactions of *Trichoderma* spp. with the environment strongly affect their performance.

Seed coating required fewer spores and lower volumes of material for similar levels of disease reduction than did application in gels. Of the seed-coating materials, PVA seemed superior, since less disease occurred when seeds were treated with PVA than when seeds were treated with Methocel or Polytran N. Among the various gels, more disease occurred with Laponite 508 than with the other gels. Laponite 508 has a pH of 9, which is less favorable to *Trichoderma* spp. than the pH 6–7 of Polytran N or Poly Surf C. Additionally, Laponite 508 is an inert, silica-based compound that is not a food source, while Polytran N is a mixture containing a glucan gum produced by *Sclerotium glucanicum* and Poly Surf C is a hydroxyethyl cellulose. Both compounds can serve as carbon sources for *Trichoderma* spp.

In all field experiments, T8-treated seeds produced significantly more healthy seedlings than nontreated seeds, but the best protection from seed rot usually was obtained by treating seeds with captan. Higher populations of *Trichoderma* spp. were observed near plants arising from captan-treated seeds than from plants derived from nontreated seeds. Many isolates of *Trichoderma* are resistant to this fungicide (*unpublished*) and this treatment may limit growth of fungi competitive to *Trichoderma*. Integration of biological seed protectants with fungicidal treatments that eliminate competitors may enhance the establishment of desired biocontrol agents and provide better control of seed and seedling disease than either used separately (10,16).

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