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Biological control of damping-off caused by *Pythium ultimum* and *Rhizoctonia solani* using *Trichoderma* spp. applied as industrial film coatings on seeds

Biological control of damping-off

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Abstract

Conidia of seven *Trichoderma* strains were applied on cucumber or radish seeds as a simple methyl cellulose coating or through an industrial film coating process. The seeds were sown in a peat-based soil artificially infested by *R. solani* or *P. ultimum*. Four strains controlled damping-off caused by *R. solani* when applied as a simple coating or as an industrial film-coating. Also, four strains significantly reduced damping-off caused by *P. ultimum* in cucumber. A correlation was found between production of volatile antibiotics in vitro and control of *P. ultimum*. Survival during storage varied according to the strain. Better survival was observed for two strains, with a decrease in conidial viability of one order of magnitude after storage for three and five months at 15 °C and 4 °C, respectively. The results show the feasibility of biocontrol of seedling diseases by some antagonists applied onto seeds through an industrial film-coating process.

Introduction

Trichoderma spp. have been known to control damping-off caused by *Pythium ultimum* [Wolffhechel and Jensen, 1991] or by *Rhizoctonia solani* [Mihuta-Grimm and Rowe, 1986]. Formulations based on actively-growing hyphae on bran [Lewis and Papavizas, 1987], wheat-bran-peat [Sivan and Chet, 1984], or maize perlite media [Wilson *et al.*, 1988] have been successfully applied to the soil. However, such formulations do require large amounts of material which cannot practically be stored and applied because of their bulk. The application of antagonists by seed treatments is thought to be an economical alternative, since only relatively small amounts of inoculum are needed; the effectiveness against *Pythium* and *Rhizoctonia solani* of spores of *Trichoderma* coated on pea or radish seeds using a simple methyl cellulose treatment has been previously claimed [Harman *et al.*, 1981]. The change from treating seeds employing small-scale laboratory methods to large-scale application is complex. To our knowledge, only the successful application of *Trichoderma harzianum* through industrial seed coating against damping-off in sugar beet in the field [Pérez De Algaba *et al.*, 1993] has been reported. At S&G Seeds, industrial film-coating processes are being developed for the application of chemical and biological crop protection agents [Scheffer, 1994.] In this study, effectiveness of *Trichoderma* spp. applied by the industrial film coating process was tested in two model systems: damping-off caused by *Pythium ultimum*, and damping-off caused by *Rhizoctonia solani*. Survival of conidia coated on seeds was also investigated.

Materials and methods

Strains. Strains of *T. harzianum* (ZUM 1302 and 1303), *T. hamatum* (ZUM 1304), and *T. viride* (ZUM 1305, 1306 and T004) were kindly provided by N. Fokkema, IPO-DLO, the Netherlands. A benomyl-resistant mutant of *T. harzianum*, T-95, [Chang *et al.*, 1986] came from the American Type Collection (ATCC#60850). Strains were maintained on potato-dextrose-agar (PDA).

In vitro *antagonism.* The following experiments were performed with two test fungi, *Rhizoctonia solani* and *Pythium ultimum*. *R. solani* strain (ZUM 1611) was isolated in 1985 from a diseased Impatiens plant in the trial field north of S&G Seeds buildings. *P. ultimum* strain (ZUM 1608) was provided by René Gees, Sandoz Agro, and is pathogenic against a range of crops. Production of volatile antibiotics was tested according to the methods described by Dennis and Webster [1971]. Plates containing 15 ml malt-extract agar (MEA) were inoculated centrally with a *Trichoderma* agar plug. After incubation at 22 °C for 7 or 14 days, the lid of each plate was replaced by a bottom containing 15 ml of MEA inoculated with a test fungus. The lids of control plates, not inoculated with *Trichoderma* were replaced in the same way. The colony diameter of the test fungus was measured after a further two or three days incubation. For non-volatile antibiotics experiments, *Trichoderma* was grown seven days in potato-dextrose broth (PDB) at 300 rpm and at room temperature. Cultures were filtered to remove the mycelium and centrifuged to remove the conidia. Filtrates were sterilized by filtration (Acrodisc nitrocellophane membranes, pore size 0.2 μm). Fifty μl of the filtrates was injected into wells (three wells/plate) previously cut into plates containing 15 ml PDA. In control plates, fifty μl water was injected per well. After complete diffusion of the filtrates into the agar, a test fungus was inoculated in the centre of the plates and incubated for two days at 22 °C; the colony diameter was assessed daily. For mycoparasitism experiments, water agar plates were inoculated with *Trichoderma* at one side of the plates. After three days incubation at 22 °C, the test fungus was inoculated on the opposite side of the plate. Hyphal interactions were observed under the light microscope. Initially, experiments were conducted on PDA plates, but abundant mycelium production of the antagonists made the microscopic observation difficult.

Seed treatments. Conidia from 15-day-old plates were washed with sterile distilled water and filtered through 2 layers of cheese-cloth. The final spore concentration applied on seeds was 10^7–10^8/ml, except when the effect of spore density was tested. These suspensions were used for both industrial or simple methyl cellulose (MC) coatings. Only radish seeds were coated through the MC coating, and both cucumber and radish seeds were used for the industrial process. For MC coatings, conidial suspensions were added (1/1) to methylcellulose 2%, and 3 ml of this solution mixed with 15 g radish seeds (*Raphanus sativus* L. 'Saxa Nova'). The seeds were then allowed to dry in the laminar flow cabinet for 12 h. For industrial coatings, 12 ml or 10 ml of the spore suspension with 0.05% of a vinylacetate sticker were respectively used per 50 g radish or cucumber seeds (*Cucumis sativus* L. 'Nevada'). The conidial suspensions were applied with a fluidized bed bottom spray coater. For storage, coated seeds were kept in polythene/aluminium foil pouches at 4 °C or at 15 °C.

Number and viability of conidia on seeds. Ten seeds were suspended in water, shaken for 3 min and dilutions were plated on a *Trichoderma* selective medium [Elad *et al.*, 1981]. The total number of conidia was determined with a haemacytometer.

Preparation of Rhizoctonia solani *inoculum. Rhizoctonia solani* was grown on corn meal agar (CMA) at 22 °C for 2 days. Straw was chopped and sieved to get pieces between 425 and 710 μmm. It was adjusted to 70% relative humidity with water, placed in one-liter flasks (1:1) and three times autoclaved for 20 min. The flasks were inoculated with half a MMA plate of *R. solani* cut into small pieces and incubated for 2 weeks at 24 °C. The hyphal culture was dried in a sterile airstream in a laminar flow cabinet. The dried mixture was kept at 4 °C until use. Fifteen grams of infested straw was mixed with 900 ml water, 120 ml sand and 2.5 kg peat-based potted soil. This mixture was then added to soil at rates from 0.1% to 4%. For an accurate estimation of mycelial biomass added to the soil, a colonization method was used [Papavizas and Lewis, 1985]. Hundred grams of infested soil was adjusted to 50% of its water-holding capacity and mixed with 100 sugar beet seeds (*Beta vulgaris* L. 'Freja'). The samples were incubated one day at 24 °C and the seeds transferred to plates (10 seeds/plate) containing Water Agar Antibiotics medium [AWA, Papavizas and Lewis, 1985]. After 24 h of incubation at 24 °C, the number of colonized beet seeds was counted and infection rate estimated as the percentage infested seeds.

Preparation of Pythium ultimum *inoculum. Pythium ultimum* ZUM 1608 from the S&G Seeds collection was grown on maize meal agar (MMA) at 22 °C for 2 days. MMA-disks (10 mm ϕ) were inoculated into petri-dishes (140 mm ϕ) containing 30 ml vegetable juice medium [Stasz and Harman, 1980]. The plates were incubated at 15 °C in the dark for one week. The medium was removed, the mycelium washed three times with water, and subsequently incubated in distilled water for one week. The culture was fragmented in a blender and the density of non-germinated oospores determined with a haemacytometer. The whole biomass was mixed with talc to a final concentration of 3.10^5oospore/g talc. Survival of oospores after drying was 1%, determined by plating dilutions of talc on RPM medium [Mircetich and Kraft, 1973]. The inoculum talc was kept at 4 °C until use. It was found earlier that 0.75% talc inoculated with *P.ultimum* into the soil induced approximately 50% damping-off 11 days after sowing.

Growth chamber assays. Seeds treated as previously described were planted in peat-based potting soil. For tests with *R. solani*, 25 radish seeds were sown in trays measuring 25×38 cm with a depth of 2.5 cm. For each treatment, six trays were sown, and for statistical analyses of the experiment each tray was considered as a replicate. When the level of disease in the infested control reached about 50%, plants were harvested, and roots cut to allow for precise disease diagnostics. For tests with *P. ultimum*, trays (4 replicates per treatment) containing cells of 31 cm^3 were filled with infested soil (10 g soil/cell), and 50 seeds were planted per tray. After one week, healthy plants were counted. Differences in the treatments compared to the healthy controls refer to non-germinated seeds and diseased plants. For each model, methylcellulose coated seeds or industrial coated seeds with no conidia were used as controls.The temperature was 24 °C, the relative humidity 80%, day/night 12h/12h, and light intensity 30,000 lux (Philips Hpi-T).

Data analysis. Experiments were arranged in completely randomized block designs, and repeated three times, except for the dose-response experiment, which was repeated twice. Data from repeated experiments with similar results were tested by a one-way variance analysis followed by a L.S.D. test.

Table 1. Production of volatile antiboiotics by *Trichoderma* strains

	Growth of *P. ultimum*(a)				Growth of *R. solani*(a)			
	24 h		48 h		24 h		48 h	
Strain	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
1302	98	96	100	93	71	63	68	58
1303	98	96	100	93	97	77	83	66
1304	101	74	100	78	68	30	54	47
1305	55	35	25	28	36	56	33	51
1306	88	50	48	44	92	57	86	45
T95	101	98	100	103	90	81	90	83
T004	100	81	100	76	102	54	96	65

Values are means of triplicates.
(a) Growth of the test figures is expressed as % of the control. *Trichoderma* spp. were grown 7 days (1) or 14 days (2) before inoculation of the pathogens.

Results

Production of volatile antibiotics. Growth reduction of *R. solani* was observed with an inhibition percentage of 40–60% for all strains, except for T95, where only 10 to 20% inhibition was observed (Table 1). No inhibition of *P. ultimum* was observed with strains 1302, 1303 and T95. Inhibition with strains 1305 and 1306 was up to 60–70%. Fourteen-days old plates of strains 1304 and T004 produced volatile compounds which inhibited *P. ultimum*, but seven-days old plates failed to influence growth of the pathogen. These results indicate that the volatile antibiotics production varies with the age of the *Trichoderma* culture. The volatile antibiotics production by young active growing hyphae, which are responsible of the most active antagonistic activity in biological control , might be different from our results based on 7 and 14 days old cultures.

Production of non-volatile antibiotics. The colony diameters of *P. ultimum* and of *R. solani* were determined daily, until entire colonization of the agar which occurred respectively three and four days after the pathogen inoculation. No inhibition of both test fungi was observed on plates with the culture filtrates added. In a dual culture experiment on PDA, macroscopic observations revealed that all *Trichoderma* strains grew over the test fungi. No antibiosis area could be detected (results not shown).

Mycoparasitism. One day and five days after hyphal contact, coiling of *P. ultimum* was observed by one to three strains, although coiling was not observed in

Table 2. In vitro coiling by *Trichoderma* spp.

	P. ultimum			*R. solani*	
	Days after contract			Days after contact	
Strain	1	3	5	1	3
1302	–	±	–	+	+
1303	–	+	±	++	++
1304	±	+	–	+	±
1305	–	+	±	++	+
1306	–	–	–	±	+
T95	–	–	–	–	–
T004	–	+	+	+	±

Plates were observed in triplicates.
(–) no coiling was observed; (±) coiling was observed only on one or two plates; (++) large area of pathogens were coiled.

Table 3. Percentage survival of *Trichoderma* spp. after coating and the number of viable conidia on radish seeds

	Simple MC coating		Industrial coating	
Strain	% Survival	Log cfu/ radish seed	% Survival	Log cfu/ radish seed
1302	5	5.7	1.5	4.2
1303	8	5.8	2.7	4.8
1304	14	5.2	1.6	4.1
1305	7	5	3.5	4.9
1306	13	4.8	0.4	2.8
T95	0.8	4.9	0.2	2.7
T004	20	5.2	2.4	4.4

every triplicate tested (Table 2). Three days after contact, five *Trichoderma* strains out of seven were shown to parasitize *P. ultimum*. Coiling of *R. solani* by all strains was observed at one and three days after hyphal contact, except on plates colonized by T95 where no coiling could be detected.

Effect of Trichoderma *strains on* Rhizoctonia solani. Upon application on seeds as a methyl cellulose treatment, five strains significantly reduced damping-off caused by *R. solani* (T95, ZUM 1306, 1304, 1303 and 1302; Fig. 1). Differences among strains in disease suppressing ability were obvious: T95 protected the seedlings better than ZUM 1306, 1304, 1303 and 1302 did, and strains T004 and 1305 had no effect at all. Strains 1302, 1304, 1306 and T95 were applied on radish seeds through the industrial process. The percentage of conidia able to germinate was lower; the ultimate number of living conidia decreased by one or two orders of magnitude (Table 3). On the other hand, these seed treatments gave significant protection against *R. solani* (Fig. 1) in the same model system. The protection was even higher than with the simple MC coating, particularly with T95, which totally suppressed damping-off, and 80% of healthy plants were recovered with ZUM 1302, 1304, and 1306.

Effect of Trichoderma *strains on* Pythium ultimum. Numbers of viable conidia recovered from the cucumber seeds after the industrial process were similar for each strain. The average was equal to log = 5.35 (standard deviation = 0.56). These seeds were immediately sown for the *P.ultimum* control experiment. A low level of damping-off occurred at seven days, with over 78% of healthy plants in the infested control (Fig. 2). Nevertheless, seedlings were totally protected with T004, and 10% of damping-off occurred with ZUM 1304. At 11 days, these two strains were still effective against *P.ultimum*. On the other hand, strains 1305 and 1306 had a beneficial effect only at 11 days, against post-emergence damping-off. The number of healthy seedlings treated with T95 was higher at 11 days but not significantly different of the infested control.

Effect of different concentrations of conidia on seeds. Concentrated conidial suspensions (10^8-10^9/ml) of strains 1302, 1304, 1306 and T95 were diluted and applied onto seeds through the industrial process. Although over 2.10^6 conidia were applied per seed, the concentrations of viable conidia recovered from seeds were between 10^2 and 10^5 cfu/seed. Seed treatments were tested in the *R. solani* model. Protection was shown for conidial concentrations as low as 10^2 cfu/seed, but increasing numbers of healthy plants were found with increasing numbers of viable conidia on seeds, with correlation coefficients between 0.77 and 0.83 (Fig 3). The number of healthy plants recovered from seeds with autoclaved conidia applied was higher, although not significantly, than the number of plants recovered from the non-treated seeds. Based on the lowest number of conidia on seeds providing acceptable biocontrol against *R. solani*, the strains could be separated into two groups: strains 1302 and 1304 on one hand, and strains T95 and 1306, on the other hand. To achieve 80% of the healthy control, strains 1302 and 1304 had to be applied at a density of 3.10^4–10^5 conidia /seed, while 3.10^3 conidia of strains 1306 and T95 per seed provided the same effectiveness.

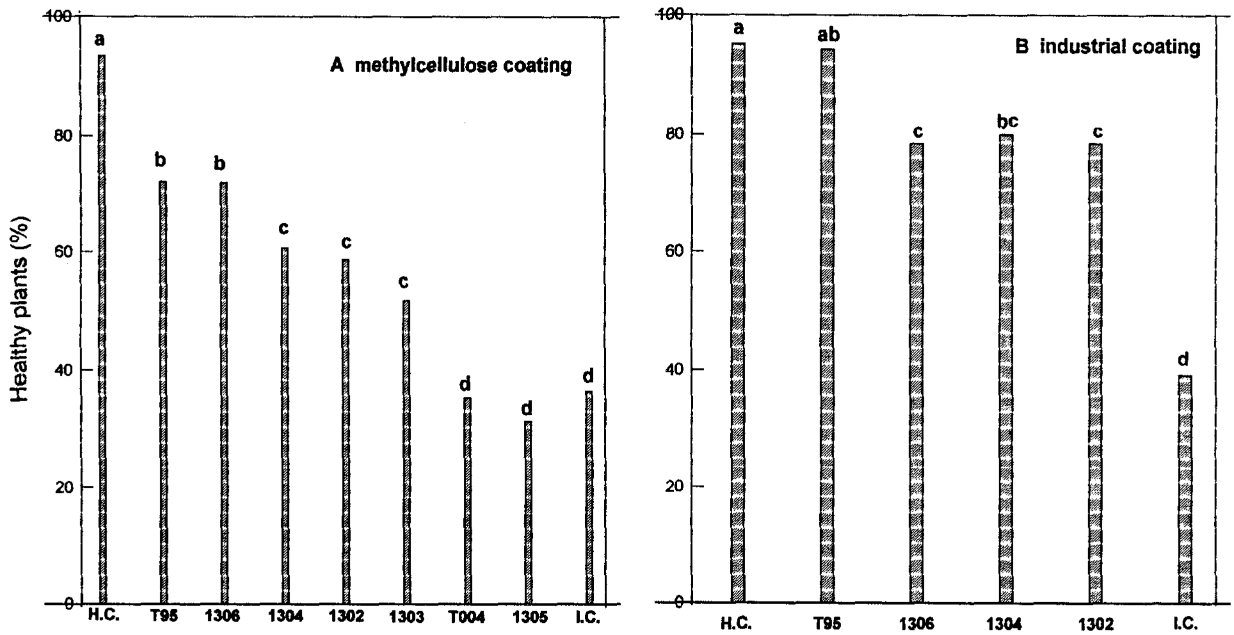

Fig. 1. Effect of radish seed treatments on damping-off caused by *R. solani*. Methylcellulose coated seeds were used as controls and sown in healthy soil (H.C., healthy control) or in soil artificially infested with *R. solani* (I.C., infested control). Bars with dissimilar letters are significantly different (P = 0.05).

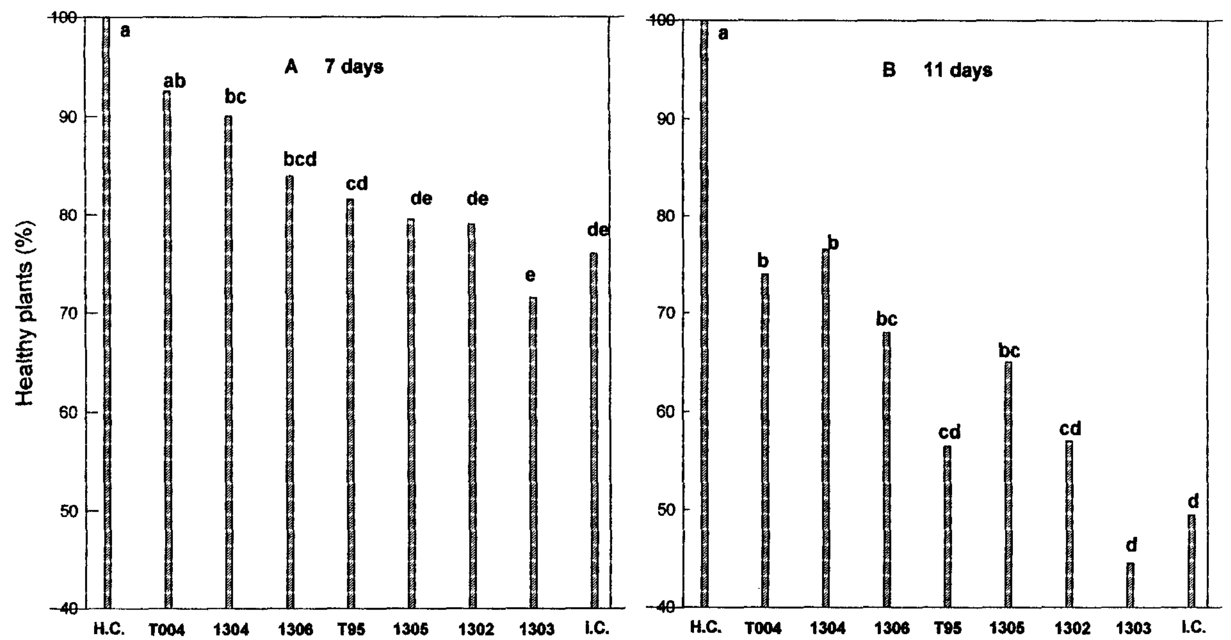

Fig. 2. Effect of cucumber seed treatments on damping-off caused by *P. ultimum*. Conidia were applied through the industrial film-coating process. Seeds without conidia were used as controls and sown in healthy soil (H.C., healthy control) or in soil artificially infested with *P. ultimum* (I.C., infested soil). Bars with dissimilar letters are significantly different (P = 0.05).

Survival of conidia on seeds during storage. At 15 °C, conidial viability declined within 2 months for strain T004 and T95 (Fig. 4), and within 3 months for strain 1306. Conidia of strains 1302 and 1304 survived longer, up to 4–5 months. With regard to the initial number of cfu/seed, strain T004 was the most

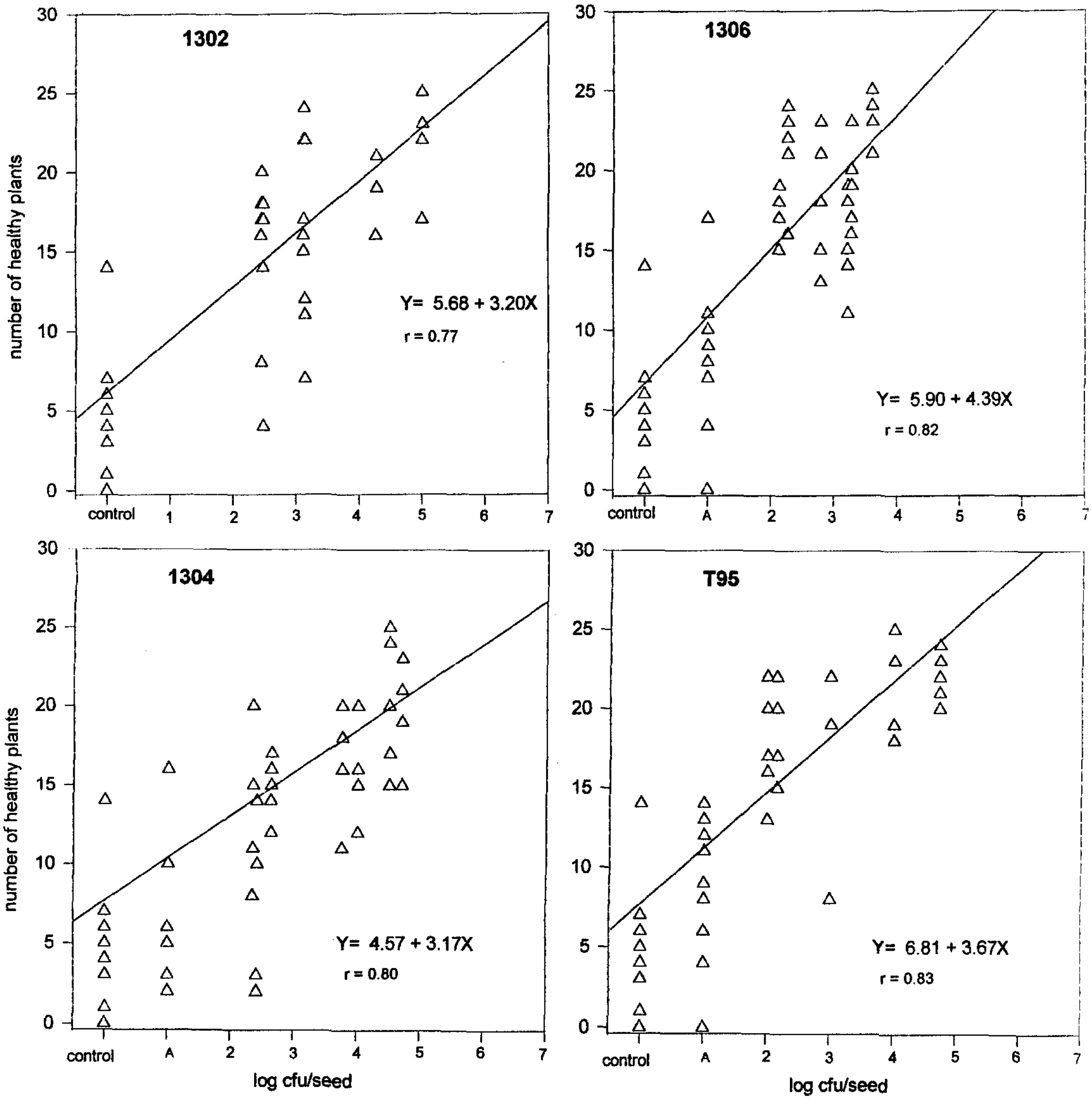

Fig. 3. Effect of the density of cfu/radish seed on damping-off caused by *R. solani* 12 days after sowing. Seeds were coated though the industrial film coating process. **Control**: no conidia on seeds. **A**: autoclaved conidia treatments ($\pm 10^7$/seed). △ Seeds coated with viable conidia. Twenty-five radish seeds were planted by tray, and five trays were sown per treatment.

affected by storage at this temperature. At 4 °C, T004 conidia also lost viability rapidly (Fig. 4). Strains 1302 and 1304 conidial viability of 3.10^4-10^5/seed required to provide 80% of the healthy control was one and two months at 15 °C and 4 °C, respectively. Although the conidial viability required for strain 1306 and T95 was lower (3.10^3/seed) to achieve 80% of healthy control, the conidial viability decreased under this threshold after one month of storage, at 15 and 4 °C.

Discussion

Limited inoculum needed to suppress damping-off is the major advantage of using seed treatments [Harman *et al.*, 1981]. In our experiments, industrial seed film-coatings with some *Trichoderma* spp. isolates were shown to be effective, compared with seed film-coatings with no *Trichoderma*, in controlling damping-off caused by *R. solani* in radish and by *Pythium ultimum* in cucumber. They were even more effective than simple coatings to control *R. solani*, although

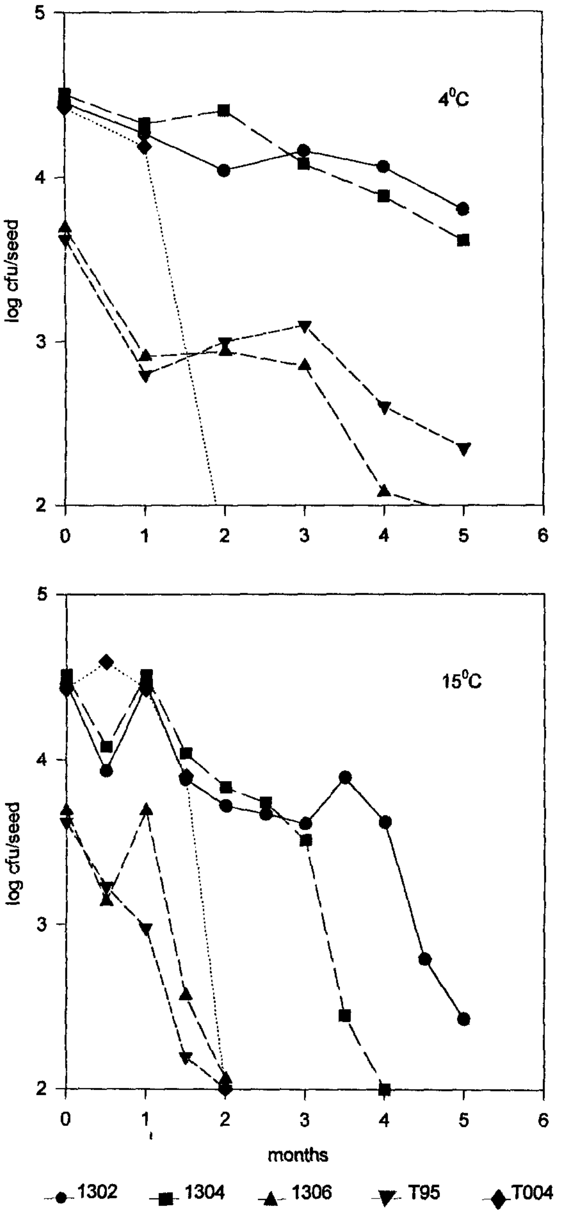

Fig. 4. Survival of *Trichoderma* spp. on radish seeds.

the density of viable spores on the seeds was lower. In the *R. solani* experiments, 80 to 90% of the healthy controls was achieved with industrial seed treatments of strains 1302, 1304, 1306 and T95 at between 10^4 and 10^5 cfu/seed. Based on the regression models, the amount of viable conidia needed to achieve 80% healthy plants varied from 3.10^3 cfu/seed for strains 1306 and T95, to 3.10^4–10^5 cfu/seed for strains 1302 and 1304. When autoclaved and applied on seeds, *Trichoderma* gave also better protection than methylcellulose control coatings, suggesting that the industrial coating may act as a barrier. On one hand, despite the high amount of dead conidia on seeds ($\pm 10^7$/seed), no increase of severity of damping-off was observed, suggesting that the dead conidia were not used by *R. solani*. On the other hand, high densities of dead conidia as reported after the industrial process, may be utilized by *Trichoderma*, as previously claimed for other nutrients, leading to an enhanced control [Harman *et al.*, 1981]. Although survival of conidia is known to be stable, storage of conidia and especially onto seeds, is not well documented. In bran alginate pellets, survival of *Trichoderma* including both chlamydospores and conidia was generally still high at 6 months at 5 °C, but very poor at 25 °C [Lewis and Papavizas, 1985], and two strains out of 12 failed to survive during storage. In our study, numbers of living conidia per radish seed of strains 1302 and 1304 were comparable to these results, and T004 failed to survive both at 4 °C and 15 °C. Studies are being undertaken with osmoticums in the growth medium [cf. Harman *et al.*, 1991] in order to increase survival of *Trichoderma*.

In the growth chamber ZUM 1304 and 1306 were found to be effective against both *P. ultimum* in cucumber and *R. solani* in radish, indicating that these strains have a wide host range. T95 gave the better protection against *R. solani*, but no effect was observed on *P. ultimum*. Strain T004 controlled *P. ultimum*, but failed to protect radish seedlings against *R. solani*. *In vitro* tests were carried out to correlate control data with known mechanisms of biological control, and particularly to understand why the two last *Trichoderma* strains were effective against one pathogen, but not against the other one. For *P. ultimum*, a correlation was found for all strains between the production of volatile antibiotics and the control of this pathogen in the growth chamber. Mycoparasitism was shown to be correlated to disease control only for three strains. In the growth chamber, the production of toxic compounds by *Trichoderma* might be one mechanism of control of *Pythium*, as previously claimed [Lifshitz *et al.*, 1986]. When the test pathogen was *R. solani*, four strains parasitized *R. solani in vitro* and controlled damping-off in the growth chamber; two strains parasitized the pathogen *in vitro*, but no beneficial effect was found in soil. On one hand, *R. solani* control may be due at least in part to mycoparasitism. Since the control depends on the level of hydrolytic enzymes produced, the lack of control observed for some strains in the growth chamber, despite observed hyphal coiling *in vitro*, may be explained by a difference in the ability to produce these enzymes [Elad *et al.*, 1982]. On the other hand, coiling is not a standard criterion of mycoparasitism [Deacon, 1992] and it may even be more indicative of host resistance rather than susceptibility. Volatile compounds secreted by most strains inhibited *R. solani*, as previously described [Dennis and Webster, 1971], and may also take part in the control of *R. solani* in the growth chamber [Claydon *et al.*, 1987; Wilson *et al.*, 1988]. T95 was the only strain which failed to 'parasitize' *R. solani in vitro*, but which was effective in the growth chamber. This phenomenon was previously reported for *Gliocladium virens* mutants [Howell, 1987]. Also, no inhibition by T95 was found against *P. ultimum in vitro* by Wolffhechel and Jensen [1992], who reported a beneficial effect of T95 applied as 1% peat-bran against *Pythium* in soil. However, the variability of effectiveness of T95 cannot be attributed to the formulation used. Applied as a seed treatment, T95 failed to protect cucumber seedlings against *P. ultimum* [Harman *et al.*, 1989] but Ahmad and Baker [1987] found a significant protection in a similar assay. Apparently T95 is not a reliable antagonist of *P. ultimum* in the different systems used, and the mechanism of control, when it occurs, remains unclear. Antibiosis in dual cultures, although the easiest test, does not reflect the antagonism in soil [Papavizas and Lewis, 1983]. Culture filtrates failed to give any information in our tests, probably due to the low volumes pipetted into the plates, compared to those of Sivan *et al.* [1984] and Lifshitz [1986]. Following the hypothesis that the biological control mechanisms of *Trichoderma* spp. against *R. solani* and against *P. ultimum* are dissimilar [Lifshitz, 1986], we suggest to use more than one in vitro test, as mentioned by Whipps [1987]. In combination, in vitro tests should help for the screening of antagonists.

As a conclusion, we demonstrated the effectiveness of some *Trichoderma* applied as an industrial film-coating against damping-off caused by *R. solani* or *P. ultimum*. Furthermore, conidia of some strains applied on seeds were able to survive storage at 4 °C and 15 °C for five and three months, respectively, which seems to make control of damping-off by industrial seed treatment a feasible approach.

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