European Journal of Plant Pathology **106:** 215–225, 2000.

Substrate colonization, strain competition, enzyme production *in vitro*, and biocontrol of *Pythium ultimum* by *Trichoderma* spp. isolates P1 and T3

C. Thrane[1,*], D. Funck Jensen[1] and A. Tronsmo[2]
[1]*Department of Plant Biology, Plant Pathology Section, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark;* [2]*Department of Chemistry and Biotechnology, Agricultural University of Norway, N-1432 Aas, Norway;* [*]*Present address: Department of Ecology, Section of Genetics and Microbiology, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark (Fax: +35 282606)*

Accepted 27 November 1999

Key words: antagonism, biological control, cell wall degrading enzymes, GUS-transformant, monitoring

Abstract

The antagonistic *Trichoderma* spp. isolates P1 and T3 differed in their ability to colonize and to compete in sphagnum peat moss and on wood chips. In peat supplemented with straw, isolate T3 produced twice as many colony forming units (cfu) as isolate P1. On wood chips, the two isolates formed a similar number of cfu. When the two *Trichoderma* isolates were cultivated together approximately 85–90% of the cfu were from T3 on both substrates. The presence of *Pythium ultimum* in peat amended with straw did not influence the number of *Trichoderma* cfu formed. The two *Trichoderma* isolates produced different amounts of hydrolytic enzymes both in liquid cultures and in peat. Seven different enzyme activities were tested. Enzyme production by *T. harzianum* isolate T3 was less influenced by the type of carbon source amendment than that of isolate *T. atroviride* P1. Culture filtrates of isolate P1 grown on complex carbon sources were high in endochitinase activity, whereas cellulase and endo-1,3-β-glucanase activities were more pronounced in filtrates of isolate T3. There was no significant difference between the two isolates in their ability to protect cucumber seedlings against *P. ultimum* while the combination of the two fungi resulted in significantly less biocontrol than each isolate alone.

Abbreviations: Cellobiohydrolase – 1,4-β-D-glucan cellobiohydrolase (EC 3.2.1.91); Cellulase – 1,4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase (EC 3.2.1.4); Chitobiosidase – enzyme activity resulting in hydrolysis of 1,4-β-glucosidic linkage in chitin releasing chitobiose from the non-reducing end of the chitin chain (Tronsmo and Harman, 1993); Endo-1,3-β-glucanase – 1,3-(1,3;1,4)-β-D-glucan 3(4)-glucanohydrolase (EC 3.2.1.6); Endochitinase – poly (1,4)-(N-acetyl-β-D-glucosaminide) glycanohydrolase (EC 3.2.1.14); β-glucosidase – β-D-glucoside glucohydrolase (EC 3.2.1.21); NAG′ase – β-N-acetyl-D-hexosaminide N-acetylhexosaminohydrolase (EC 3.2.1.52); Peat – sphagnum peat moss.

Introduction

Many reports published on the biocontrol agent *Trichoderma* spp. indicate that there is a large variation in physiological properties and biocontrol activity among different isolates. The antagonistic mechanisms and the level of biocontrol achieved depend on the species and isolate of the plant pathogen, the crop, and whether the disease occurs in the field, in the greenhouse, or in storage (post harvest).

Extracellular hydrolytic enzymes produced by *Trichoderma* spp. are considered important determinants of the antagonistic ability of these fungi. The role of these enzymes in biocontrol is supposed to be connected either to their saprophytic lifestyle (Lynch, 1989; Sivan and Chet, 1989b; Sivan and Harman,

1991) or to their direct action against plant pathogens (Elad et al., 1981; Sivan and Chet, 1989a; Chérif and Benhamou, 1990; Lorito et al., 1993; Migheli et al., 1998; Woo et al., 1999). Endo-1,3-β-glucanase and cellulase appear to be involved in the antagonism of *Pythium* by *Trichoderma* (Thrane et al., 1997).

Regardless of the mode of action, ecological fitness of the antagonist may be the most critical attribute for reliable disease control (Deacon, 1991; Hayes, 1992). Competition for nutrients is probably the most general mechanism in biocontrol and other mechanisms only serve as facilitating mechanisms (Paulitz, 1990; Deacon and Berry, 1992). A GUS-transformed isolate of *T. harzianum* has been used to study root colonization and competition (Thrane et al., 1995) in order to distingush this specific isolate from other *Trichoderma* isolates and to visualize actively growing mycelia in soil and on plant roots (Green and Jensen, 1995; Thrane et al., 1997).

The main objective of the present study was to test whether the *in vitro* production of seven different hydrolytic enzymes produced by two isolates of *Trichoderma* spp. isolate P1 and T3 were related to the natural habitat of the strains and to the pathogens they control. *Trichoderma* P1 was isolated from wood chips (Tronsmo, 1989) and has been used, for example, in control of *Botrytis cinerea* in storage of fruits (Tronsmo, 1991), whereas *Trichoderma* T3 was isolated from *Pythium* suppressive sphagnum peat moss (Wolffhechel, 1989) and controls *Pythium* spp. damping-off (Wolffhechel and Jensen, 1992). The ability of the two isolates to establish and compete in peat and on wood chips was thus studied by the use of the GUS-transformant of *T. harzianum* isolate T3 (Thrane et al., 1995) and by isozyme activity gels (Thrane et al., 1996). Finally, the efficiency of the isolates to control *Pythium* damping-off was compared. Recently and after the end of this study isolate P1 which was formerly identified as a *T. harzianum* (Tronsmo, 1989) was redesignated as *T. atroviride* (Lübeck et al., 1997).

Materials and methods

Fungal strains, plant cultivar, and media

T. atroviridae isolate P1 (Lübeck et al., 1997) (*T. harzianum* Rifai P1 (ATCC 74058)) was originally isolated from wood chips and selected for iprodione resistance (Tronsmo, 1989). *T. harzianum* Rifai isolate T3, *T. virens* (*Gliocladium virens*) isolate G2 and 62-6 were isolated from a *Pythium ultimum* suppressive sphagnum peat moss from Sweden (Wolffhechel, 1989). *Clonostachys rosea* Shroers, Samuels, Seifert and Gams (previously *Gliocladium roseum*) IK726 was isolated from a barley seedling (Knudsen et al., 1995). *T. harzianum* T3a is a GUS-transformed isolate T3 (Thrane et al., 1995). This GUS-transformant was used in the colonization studies to distinguish it from isolate P1. *P. ultimum* Trow isolate HB2 was isolated from Højbakkegård field station, Denmark. Cucumber, *Cucumis sativus* L. cv. 'Langelands Kæmpe-Gigant' (Dæhnfeldt, Denmark) was used as the test plant. Cultures were maintained on potato dextrose agar (PDA, Difco laboratories, Detroit). The media used for determination of *Trichoderma* cfu were either PDA or *Trichoderma* selective medium (TSM) (Elad et al., 1981) modified according to Green and Jensen (1995).

Protein determination

Total protein was determined by using the Biorad Protein Assay Kit II (Biorad) with BSA as a standard.

Nomenclature and determination of chitinase, chitobiohydrolase, and NAG'ase activity

Enzyme nomenclature and activity assays were carried out according to Tronsmo and Harman (1993).

Nomenclature and determination of cellulase and endo-1,3-β-glucanase activity

Enzyme nomenclature is according to Webb (1992). The activity determinations were carried out according to Thrane et al. (1997).

Nomenclature and determination of β-glucosidase and cellobiohydrolase activity

Enzyme nomenclature is according to Webb (1992). The synthetic substrates p-nitrophenyl β-D-glucopyranoside (Sigma N-7006) and p-nitrophenyl cellobioside (Sigma N-5759) were used to assay β-glucosidase and cellobiohydrolase activity according to Claeyssens and Aerts (1992). Test samples (30 µl) were added to each well of a 96-well microtiter test plate. Fifty µl substrate solution (300 µg/ml) dissolved in 50 mM KPO_4 buffer (pH 6.7) containing 0.02% NaN_3 was added to each well, and plates were incubated at 37 °C for 15 (β-glucosidase) or 30 min

(cellobiohydrolase), respectively. Reactions were terminated by addition of 50 µl 0.4 M Na_2CO_3 to each well. Absorbance at 410 nm was measured using a microtiter plate reader (Ceres UV 900 HDI, Bio-Tek Instruments, Inc., Winooski, VT, USA).

Time course study of enzyme activities from liquid cultures

Erlenmeyer flasks (300 ml) containing 80 ml medium were inoculated with 5×10^6 spores/ml. The following medium was used: 100 ml vegetable juice (Campbell's V8), 0.68 g KH_2PO_4, 0.1 g KCl, 2 g $NaNO_3$, 0.5 g sucrose, and 900 ml of water, pH 6.0. C-source amendments used were 2% glucose, 1% cellulose (SIGMA C-6663) or 1% chitin (SIGMA C-3387). The fungi were cultured at 25 °C in darkness with shaking at 200 rpm. In both experiments, 1.5 ml samples were taken daily for up to 10 days from each flask (3 flasks/treatment) and stored with 0.05% NaN_3 (final concentration) at 4 °C until analyzed. The mycelium was pelleted by centrifugation ($8.000 \times g$ (Sigma-3MK, Laborzentrifugen GmbH, Osterode, West Germany) and the supernatant was used as the crude enzyme extract. Three flasks were used for each treatment representing three replicates. The experiment was repeated. The data shown are from one trial. In the repeated experiment the levels of enzyme activity were different, but the pattern between the production of enzymes by isolate P1 and T3 was similar.

Enzymes produced in peat cultures

The still cultivation was carried out in 250 ml Erlenmeyer flasks with 39 g sphagnum peat (final dry weight 40%) supplemented with 1 g straw. The medium was autoclaved twice on two successive days (120 °C for one hour). The flasks were finally inoculated with three agar plugs from non-sporulating cultures grown on PDA. There were three flasks per treatment. Samples from each flask were analyzed individually representing one replicate. Thus there were three replicates (flasks of each treatment). The flasks were incubated at room temperature (21–23 °C) in the dark for 14 days. The contents were mixed daily. Extracts were made from the cultivations by addition of 50 mM Na-phosphate buffer (pH 6.8) containing 0.04% NaN_3. The liquids were separated from the solids by centrifugation ($8.000 \times g$) followed by sterile filtration as above. This was used as the crude enzyme extract for the enzyme activity assays. For analysis on activity gels the extracts were concentrated by Centricon 10 ultrafiltration (Amicon Inc., Beverly, MA, USA) and subjected to isoelectric focusing electrophoresis (IEF) using the Pharmacia Phastsystem (Pharmacia LKB Biotechnology, Uppsala, Sweden). Visualization of enzyme activity on IEF gels was detected as described by Thrane et al. (1997). The experiment was repeated. The data shown are from one trial. In the repeated experiment the levels of enzyme activity were different, but the pattern was similar with respect to the time point of the maximum activity, and differences between treatments and fungal isolates.

Competitive ability and cellulase activity of the Trichoderma *isolates in peat amended with straw with or without the presence of the plant pathogen* P. ultimum

Fungal culture conditions were as described above for the still culture experiment. There were seven different treatments with different combinations of fungal inocula: 1: *P. ultimum*, 2: Isolate P1, 3: Isolate T3, 4: Isolate P1 and *P. ultimum*, 5: Isolate T3 and *P. ultimum*, 6: Isolate P1 and isolate T3, and 7: Isolate P1, isolate T3, and *P. ultimum*. For each treatment three flasks were inoculated. Flasks were inoculated with six agar plugs of the *Trichoderma* isolates (in the mixed cultivations, three plugs of each isolate was used) and three agar plugs of *P. ultimum* HB2. In this experiment the GUS-transformed isolate of T3 was used (T3a). After 14 days of incubation, colonization of the substrate by the fungi and the competitive ability of the two *Trichoderma* isolates under these conditions were evaluated by measuring the colony forming units (cfu) on modified TSM. The use of the GUS-transformed T3a isolate made it possible to distinguish between P1 and T3a by GUS-testing of the colonies according to Green and Jensen (1995). Sterile water (150 ml) was added to each flask for extraction of spores by vortexing and shaking by hand. A one ml sample of this suspension was used for dilution plating. Inoculum from each dilution was spread on three plates and an average of the colonies formed on these plates was calculated representing one replicate. Three such replicates were made from each treatment by extracting from three individual flasks. After three days colonies were counted and tested for GUS-activity. After the sample for dilution plating was removed, 16.5 ml 500 mM Na-phosphate buffer (pH 6.8) and 0.6 ml 10% NaN_3 was added to

the flasks to give final concentrations of 50 mM Na-phosphate buffer and 0.04% NaN_3. The flasks were vortexed and shaken with this solution. The liquid was separated from the solids as described above. Enzyme assays were made and IEF was carried out on concentrated extracts followed by detection of enzyme activities on activity gels according to Thrane et al. (1996a). The experiments were carried out twice with three replicates (one analysis of each flask) represented by three flasks of each treatment.

Competitive ability of the Trichoderma *isolates on sterile wood chips*

Portions (39 g) of the following mixture were placed in Erlenmeyer flasks (250 ml) and autoclaved twice: 200 g conifer wood chips (Jiffy a/s, Ryomgaard, Denmark), 200 ml basic solution (Thrane et al., 1996b), and 300 ml water. Each flask was inoculated with six agar plugs of non-sporulating cultures of isolates P1 and T3 from PDA (the mixed cultivations were inoculated with three agar plugs of each isolate). Incubation, plate dilution, cfu determination, and GUS-assay were measured as described above, except PDA was used for the plate dilution assay. The experiment was carried out twice at room temperature (21–23 °C).

Growth chamber assay for biocontrol of cucumber damping-off caused by P. ultimum

The purpose of the bioassay was to test biocontrol efficiency of the different treatments when the healthy plants were exposed to diseased plants. The experiment was carried out essentially according to Green (1996). Cucumber seeds were surface sterilized in 70% ethanol and subsequently in 2.5% sodium hypochlorite and pregerminated for two days at 25 °C on PDA (1/2 concentration of PDA, 2% agar). The growth medium contained 40% (dry weight) sphagnum peat moss and 60% (dry weight) vermiculite. This was mixed with water to a final dry weight of 40% and was steamed three times for 67 min. The pregerminated cucumber seeds were placed in rhizoboxes (Green, 1996) containing the growth medium. The inoculated rhizoboxes were placed in pots containing growth medium. Two different sets of pots were set up: The first set of pots (1) were used to obtain *Pythium* infested plants for use in the second set up (2), which was the actual biocontrol experiment. In both experiments, the pregerminated cucumber seeds were placed in the pots (5 seedlings in each pot). They were watered with 0.1% Hornum nutrient solution (P. Brøste Industri a/s, Lyngby, Denmark) to a final dry weight of 20%. The pots were all incubated at 18 °C (16 h light/8 h dark). (1) *Pythium* inoculum was added to the rhizoboxes as agar plugs from a three-day-old culture from a PDA plate. These pots were kept in plastic bags to increase disease pressure. (2) In other pots peat-bran inocula of the *Trichoderma* isolates, prepared according to Sivan et al. (1984) were mixed with the sphagnum/vermiculite to a final concentration of 10^8 cfu/g dry weight. After three days the plants in the rhizoboxes inoculated with *Pythium* showed disease symptoms (1). The diseased plants were placed within the growth medium 4 cm from the healthy seedlings in the other pots (2). After 12 days the number of diseased and healthy plants were counted. The experiment was carried out twice. In one experiment there were three replicates of each treatment and in the other there were six replicates. The results from the two experiments were combined for the statistical analysis. SAS PROC GLM (SAS Institute, Cary, NC) was used for the statistical analysis. The Duncan test was used to distinguish between statistically significant results.

Results

Time course study of enzyme activities from shake flask cultures

The production of endo-1,3-β-glucanase by *T. harzianum* isolate T3 was generally higher than that of *T. atroviride* isolate P1. Isolate T3 produced significant amounts of this enzyme even on glucose (Figure 1A). Isolate T3 produced similar amounts of endo-1,3-β-glucanase on cellulose and chitin whereas isolate P1 produced more endo-1,3-β-glucanase on chitin.

In media amended with 2% glucose as C-source, little or no activity of β-glucosidase, cellobiohydrolase, and cellulase were produced by either isolate (Figure 1B–D). Isolate T3 produced equal amounts of β-glucosidase and cellobiohydrolase but higher amounts of cellulase on media amended with 1% cellulose compared to cultivation on 1% chitin. Isolate P1 produced high amounts of β-glucosidase and cellobiohydrolase on cellulose (Figure 1B and C) but only low and barely quantitatively detectable cellulase activity on any of the substrates used (Figure 1D).

Generally production of chitinolytic enzymes by isolates P1 and T3 was stimulated by amendments of 1%

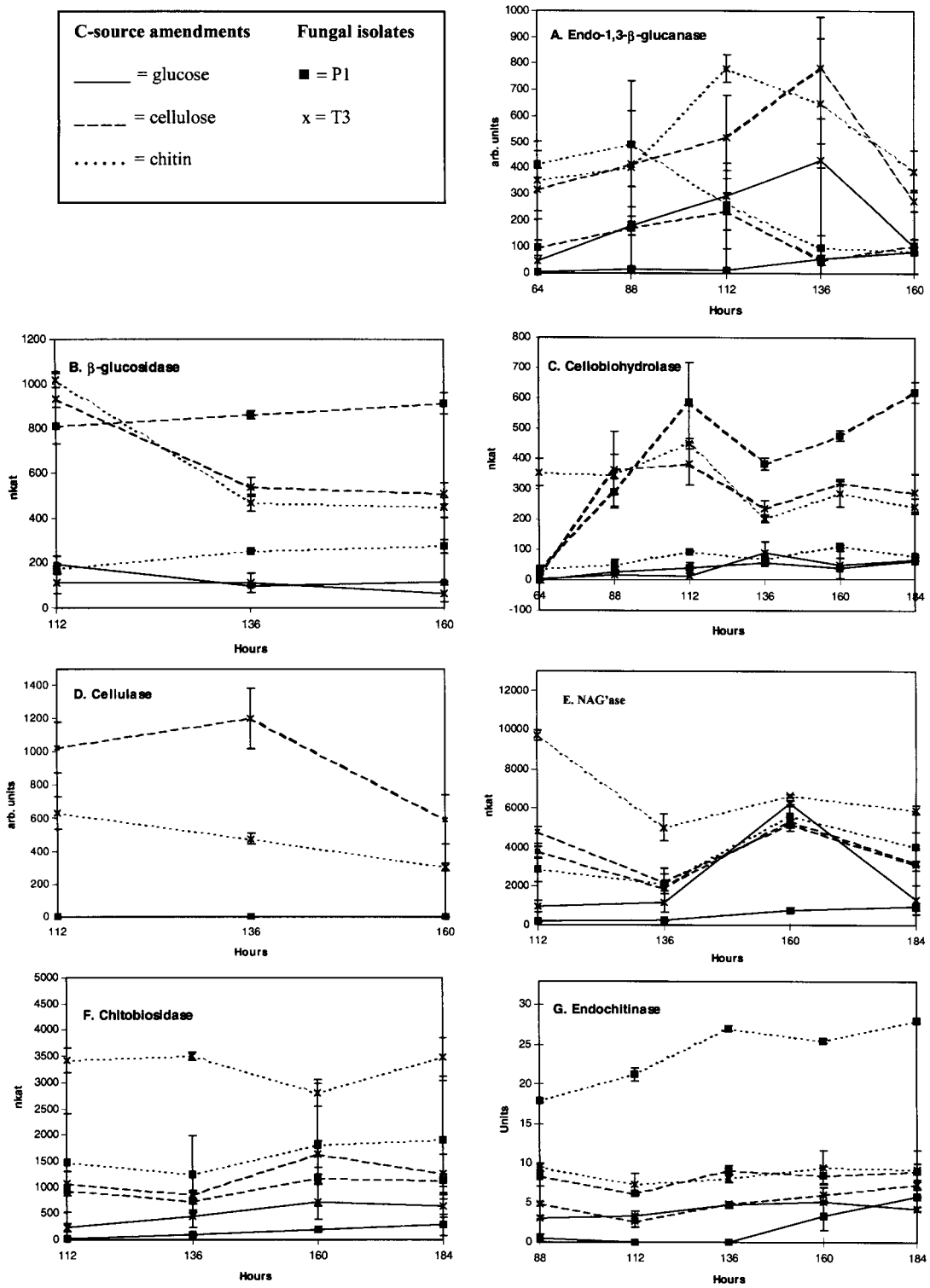

Figure 1. Enzyme activities in culture filtrates of *Trichoderma* isolates P1 and T3 grown in media amended with glucose, cellulose or chitin. Enzyme activities were measured daily over an incubation period of 184 h. (A) Endo-1,3-β-glucanase. (B) β-glucosidase. (C) Cellobiohydrolase. (D) Cellulase. (E) NAG′ase. (F) Chitobiosidase. (G) Endochitinase. The data shown are the average of three replicates per treatment (± standard error).

cellulose and 1% chitin but not by glucose (Figure 1E–G). There were equal amounts of NAG′ase production by the two isolates (Figure 1E) whereas isolate T3 produced more chitobiosidase in media amended with 1% chitin than did isolate P1 (Figure 1F). However, on the same substrate isolate P1 produced more endochitinase than did T3 (Figure 1G).

Table 1. Enzyme activities of *Trichoderma* spp. isolates P1 and T3 in filtrates of liquid cultures with 1% cellulose as C-source amendment (112 h) and extracts from peat still cultures[x]

Medium	Fungal isolate	Endo-β-1,3-glucanase	Cellulase	Endochitinase
Liquid	P1	233 B[y]	0 B	6.2 A
	T3a	516 A	1027 A	2.7 B
Peat	P1	72 A	207 A	2.9 A
	T3a	103 A	860 B	2.8 A

[x]The data shown are from one trial with three replicates.
[y]Values followed by different letters are statistically different ($P < 0.05$) according to Duncan's MRT. Statistical comparison of enzyme activities was carried out for each culture medium.

Comparison of enzymes produced in vitro *in liquid culture and in peat cultivations*

In the culture filtrate from the liquid cultures amended with 1% cellulose as carbon source endo-1,3-β-glucanase and especially cellulase activities of T3 were significantly higher than of P1 (Table 1). However, isolate P1 produced much more endochitinase. In peat supplemented with straw, cellulase activity was still higher for isolate T3. Chitinase and endo-1,3-β-glucanase activities measured in peat extracts were not statistically different between the two isolates.

Extracts from the peat cultivations and culture filtrates were subjected to isoelectric focusing and subsequent staining for cellulase activity (Figure 2). A preliminary study compared cellulase activity banding patterns after isolelectric focusing of five different antagonistic isolates (Figure 2A). Although the banding patterns of isolates P1 and T3 isolates were similar, they were sufficiently different to be used as markers for the presence of each isolate. The *T. viride* isolates were apparently identical. *G. roseum* cellulase banding pattern differed significantly from *T. atroviride, T. harzianum* and *T. viride*. Staining for cellulase activity showed that in liquid cultures of isolates P1 and T3 grown with cellulose or in peat supplemented with straw, many different cellulases were produced by T3. However, there seemed to be some differences among the isomers produced on the two substrates (Figure 2B). For P1, several bands were seen in the extracts of the peat cultivation, whereas fewer and weaker bands of cellulase activity were detected in the culture filtrate. This showed that this IEF-analysis was more sensitive than quantitative measurements (Figure 1D).

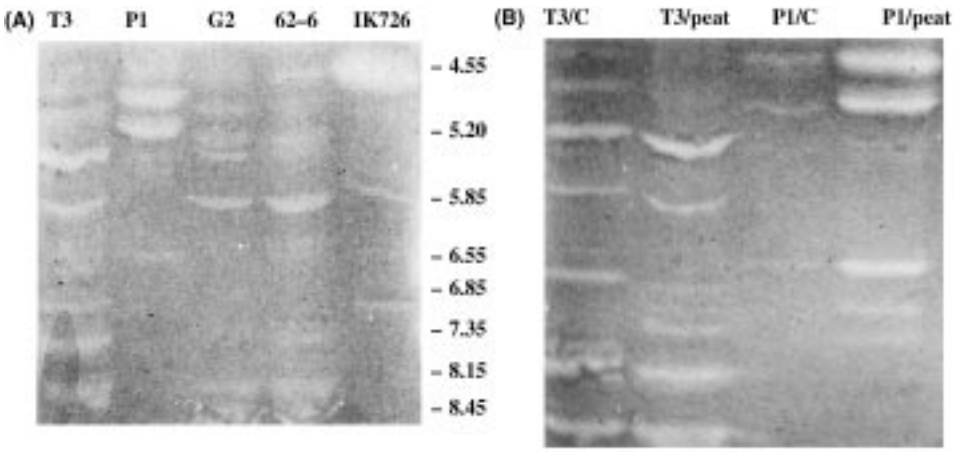

Figure 2. Cellulase activity gels. Cellulase activities are seen on an IEF gel (pI 3.5–9). pI-values are shown next to the gels. (A) Enzyme sources were extracts from peat cultures of *T. harzianum* isolate T3 (lane 1), and *T. atroviride* P1 (lane 2), *T. virens* isolates G6 (lane 3) and 62-6 (lane 4), and *Gliocladium roseum* IK726 (lane 5). (B) Enzyme sources were extracts from peat cultures (peat) and filtrates from liquid cultures amended with cellulose (C) of *Trichoderma* isolates P1 and T3. T3/liquid culture (lane 1). T3/peat (lane 2). P1/liquid culture (lane 3). P1/peat (lane 4).

Competitive ability and enzyme activities of the Trichoderma *isolates in peat amended with straw with or without the presence of the plant pathogen* P. ultimum

Determination of number of cfu of T3a (GUS-active colonies) and P1 cultivated in peat supplemented with straw showed that isolate T3a formed twice as many cfu as did isolate P1, when cultivated individually with or without the presence of actively growing *P. ultimum* (Table 2, treatments 2–5). In treatments where P1 and T3a were combined (treatments 6 and 7), T3a constituted 85–90% of the cfu. In these treatments, the total number of cfu was intermediate between the numbers of cfu produced when the isolates were cultivated separately. The presence of *P. ultimum* did not significantly influence the number of *Trichoderma* cfu in any of the repeats (Table 2).

The amount of extractable proteins from the different cultivations generally followed the same pattern as the cfu (data not shown). Cellulase activity measurements are shown in Table 2. Apparently there was no specific increase of cellulase production by the presence of *Pythium*. The level of cellulase activity was higher whenever isolate T3 was present. The extracts from the cultivations were subjected to isoelectric focusing and subsequent staining for cellulase activity (Figure 3). The signals were stronger in the extracts of the cultivations where T3 was present. There were differences in the pI values of the cellulase isomers of the two isolates, which were sufficient to distinguish the two isolates. In the combined cultivations of the *Trichoderma* isolates (treatments 6 and 7) the majority of the activity came from isolate T3a. Staining for cellulase activity of P1 filtrates indicated that the presence of *Pythium* did not change the banding pattern on the gel.

Table 2. Colony forming units (cfu) on TSM of the *Trichoderma* isolates P1 and T3a (GUS-transformant) and cellulase activity from cultivation on peat supplemented with straw for 14 days[x]

Treatment/ fungi	cfu (10^8)	% GUS-active cfu (= T3a)	Cellulase activity (arb.units)
1. *P. ultimum*	0.0 D[y]	0 C	0.6 D[z]
2. P1	4.9 C	0 C	8.3 C
3. T3a	10.1 A	100 A	27.8 A
4. P1/*P. ultimum*	4.6 C	0 C	9.8 C
5. T3a/*P.ultimum*	10.8 A	100 A	25.9 A
6. P1/T3a	8.1 B	88 B	20.0 AB
7. P1/T3a/*P.ultimum*	7.5 B	90 B	20.1 AB

[x]The percentage of cfu isolate T3a (total cfu) is shown as GUS-active cfu. Results from first and second repeat trials are combined.
[y]Values followed by different letters are statistically different ($P < 0.0001$) according to Duncan's MRT.
[z]Values followed by different letters are statistically different ($P < 0.05$) according to Duncan's MRT.

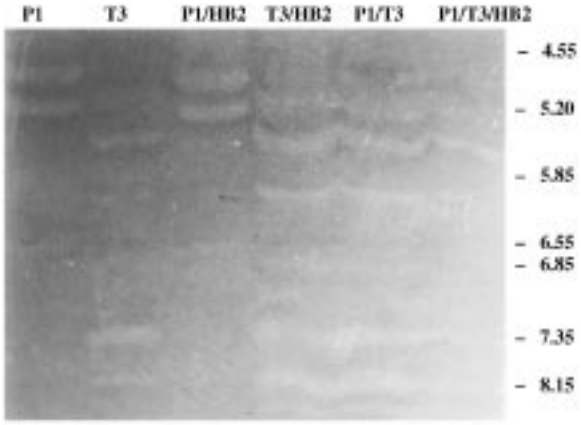

Figure 3. Cellulase activities after isoelectric focusing (pI 3.5–9) of extracts from the peat cultivations with *Trichoderma* isolates P1 and T3 with or without the presence of *P. ultimum*. pI-values are shown next to the gel. Isolate P1 (lane 1). Isolate T3 (lane 2). Isolate P1 and *P. ultimum* (lane 3). Isolate T3 and *P. ultimum* (lane 4). Isolate P1 and T3 (lane 5). Isolate P1 and T3, and *P. ultimum* (lane 6).

Table 3. Colony forming units (cfu) on PDA of the *Trichoderma* isolates P1 and T3a (GUS-transformant) from the cultivation experiment in wood chips[x]

Fungal treatment	cfu ($\times 10^6$)	% GUS active (=T3a)
P1	11.2[y]	0
T3a	14.1	97
P1/T3a	13.5	85

[x]The percentage of cfu isolate T3a (total cfu) are shown as GUS-active cfu. Results are combined from the first and second repeat trials.
[y]There was no statistical difference between the treatments ($P > 0.05$).

Competitive ability on wood chips

On wood chips, the two *Trichoderma* isolates formed equal numbers of cfu (Table 3). In the mixed cultivation, averages of 85% of the colonies in the two trials were GUS-active and originating from isolate T3a.

Table 4. The biocontrol efficacy of the *Trichoderma* spp. isolates P1 and T3 against *P. ultimum* on cucumber seedlings in the growth chamber

Fungal treatment	Diseased plants per pot
Control	4.3 A
P1a	1.3 BC
T3	1.0 C
P1a/T3	2.9 AB

Values followed by different letters are statistically different ($P < 0.001$) according to Duncan's MRT. There were five seedlings in each pot.

Biocontrol of cucumber damping-off caused by P. ultimum

In the control pots, 86% of the plants died. Both *Trichoderma* isolate P1 and T3 significantly improved plant survival (Table 4). There was no statistical difference in the biocontrol of *Pythium* damping-off between isolate P1 and isolate T3, but the combination of the two *Trichoderma* isolates resulted in significantly less biocontrol than the isolates used individually.

Discussion

This study confirmed that *in vitro Trichoderma* isolate P1 (recently redesignated as *Trichoderma atroviride* (from *T. harzianum*) based on universal primer PCR (UP-PCR) analysis (Lübeck et al., 1997)) is primarily a chitinolytic isolate (Harman et al., 1993; Lorito et al., 1994), at least in comparison to isolate T3. Results also showed that *T. harzianum* isolate T3 was more cellulolytic and 1,3-β-glucanolytic than *T. atroviride* isolate P1. These findings are in agreement with the cell wall composition of the pathogens controlled by the two antagonists. Thus, isolate P1 is used to control *Mycocentrospora acerina* and *Rhizoctonia carotae* on carrots in storage (Tronsmo, 1989) and *Botrytis* on carrots, apples and grapes (Tronsmo, 1989; Tronsmo, 1991). Isolate T3, originally isolated from *Pythium* suppressive peat (Wolffhechel, 1989), controls *Pythium* damping-off of cucumber in the growth chamber (Wolffhechel and Jensen, 1992). *In vitro* assays on purified enzymes from these two isolates have indicated a role in antagonism (Lorito et al., 1993; Tronsmo and Harman, 1993; Thrane et al., 1997). Further, Woo et al. (1999) have shown that an endochitinase null-mutant of isolate P1 was significantly reduced in biocontrol activity.

Endo-1,3-β-glucanase production by isolate T3 seemed to be less affected by high glucose levels than that of isolate P1. This suggests that isolate T3, which has been shown to have activity on roots of cucumber (Thrane et al., 1997), may retain the ability to produce this cell wall degrading enzyme in the presence of easily fermented carbon components of plant exudates. The high amounts of endo-1,3-β-glucanase produced on glucose could also be due to a morphogenetic function of this enzyme (Peberdy, 1990) because biomass production was greater on glucose. However, it is more likely that enzymes involved in morphogenesis work intracellulary and therefore would not be excreted and thus not measured by the methods used in this study. In the case of cellulase, enzyme activity was not increased when the glucose was consumed in liquid cultures (Ilmén et al., 1996; Thrane et al., 1997).

The presence of the pathogen did not promote a quantitative induction of cellulase activity. However, in another peat cultivation without added straw, there was an increase in cellulase activity by T3 when *P. ultimum* was present (Thrane et al., 1997). In this study, it is probably not possible to separate the effect of the cellulolytic activity induced by *Pythium*, because another cellulose source, straw, was present in much higher amounts. For *T. longibrachiatum*, a strong indication for the role of cellulase in the control of *Pythium* spp. has been shown by Migheli et al. (1998).

A GUS-transformant of T3 was used to facilitate distinction between isolates P1 and T3 on plates when monitoring relative competitiveness (Green and Jensen, 1995). The results indicate that isolate T3 was more competitive in peat amended with straw (Table 1). This may have something to do with the fact that isolate T3 was originally isolated from peat (Wolffhechel, 1989) and the pronounced cellulolytic activities of this isolate observed in this study probably facilitated its colonization of straw. The GUS-mutant of isolate T3 also substantially colonized the dead parts of cucumber roots (Thrane et al., 1997), indicating high cellulase activity. On wood chips, from which isolate P1 was originally isolated (Tronsmo and Dennis, 1978), the two isolates were equally competitive.

The second method used for monitoring the isolates was based on indigenous markers, the pI values of extracellular isozymes. The methods used in these experiments could prove to be valuable for monitoring a certain isolate or species in a mixed population, on the basis of its activity and not simply by the presence of fungal biomass (e.g. by dilution plating). In biocontrol, this tool would be especially valuable for

addressing the question whether a specific enzyme is involved in an antagonistic interaction. In this study pI-banding patterns of the cellulases of four of the isolates (the four different species) differed sufficiently to be useful in monitoring these isolates in different combinations. The same method has been applied to endo-1-3-β-glucanases from the same fungal isolates (Thrane et al., 1996). Similarily, Schickler et al. (1998) used 2-D electrophoresis and chitinase activity gels to identify different *Trichoderma* isolates. Further, the pI-methods were used in a preliminary study testing a field soil with and without enrichment of a naturally occurring *Penicillium* isolate. In extracts of field soils enriched with this fungus a β-glucosidase activity banding pattern (max. activity band at approx. pI 4.5) similar to that of a pure culture was seen on isozyme gels for detection of that specific enzyme activity (C. Thrane and S. Elmholt, unpublished).

Combination of antagonistic *T. koningii* and fluorescent pseudomonads has been reported to result in enhanced suppression of *Gaumannomyces graminis* var. *tritici* causing take-all disease on wheat (Duffy et al., 1995). However, Harman et al. (1989) showed that there was no enhanced effect in combining effective strains of *T. harzianum*. In our experiments, the combination of the two *Trichoderma* spp. isolates resulted in less biocontrol of *Pythium* than with the isolates used alone (Table 4). This is probaly due to competition and incompatibility between the two closely related isolates, whereas Duffy et al. (1995) showed compatibility between *T. koningii* and different pseudomonads strains. Gomez-Caballero et al. (1995) have characterized karyotypes of several *T. harzianum* isolates. Isolates belonging to the same karyotype were resistant to each other's metabolites and isolates with different karyotypes were sensitive to each other's metabolites. Competition or even inhibition between isolates P1 and T3a is indicated by the results obtained in the peat and wood-chips cultivation experiments, since the total amount of *Trichoderma* cfu were lower in the mixed cultivation than when isolate T3a was cultivated by itself. In both experiments T3a was always dominant. Jeffries and Young (1994) mention resistance to competition from other antagonists as one criterion of a good biocontrol agent. Because of the specific but less complex environment under storage conditions, resistance to other antagonists may be a less important character. Isolate P1, which has a growth rate approximately twice that of isolate T3 under storage temperature (4 °C) (C. Thrane, unpublished) has been used for biocontrol of storage rot (Tronsmo, 1989).

Unexpectedly, a high level of biocontrol of *P. ultimum* was obtained in the pot experiment with isolate P1. Isolate P1 has been isolated from a very different ecological niche and was selected as a biocontrol agent against pathogens with chitin-containing cell walls. There might be two reasons for this. First, it is possible that the treatments would be more comparable in the biocontrol assay if equal amounts on peat-bran weight basis of the two *Trichoderma* inocula had been used. In this experiment the amount of inoculum was based on equal numbers of cfu's in the peat bran and therefore less peat bran by weight was added for isolate T3. Secondly, the inoculation method (broadcast application) likely resulted in pre-emptive exclusion of the pathogen by nutrient depletion before it was able to attack the plant (Paulitz, 1990). This is in line with the findings of Harman et al. (1989), who stated that the quantity and the quality as well as the location of the bioprotectant was more important for the achieved biocontrol than its genetic ability to antagonize pathogens *per se*. However, Deacon (1991) has pointed out that the long prehistory of unsuccessful employment of fungal antagonists is due to a lack of focus on the ecological attributes of the isolates. Ecological characters are particularly important under physiologically extreme conditions such as storage of vegetables and fruits at low temperature (Tronsmo, 1989; Tronsmo, 1991), or above the soil level on fruits or leaves where the antagonist is exposed to the weather, UV-light and so forth (Tronsmo, 1991). Indeed our results show significant differences between the two *Trichoderma* isolates studied with respect to several ecologically relevant characteristics.

Acknowledgements

This work was supported by the Danish Agricultural and Veterinary Research Council (SJVF), grant no 13-4591. We are very grateful to Dr. L. Hjeljord for commenting on the manuscript. The technical assistance by K. Olesen is highly appreciated. Nordic Academy for Advanced Study (NorFa) supported C. Thrane's scientific visits to A. Tronsmo's laboratory.

References

Chérif M and Benhamou N (1990) Cytochemical aspects of chitin break down during parasitic interaction of a *Trichoderma* spp. on *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Phytopathology 80: 1406–1414

Deacon JW (1991) Significance of ecology in the development of biocontrol agents against soil-borne plant pathogens. Biocontr Sci Technol 1: 5–20

Deacon JW and Berry LA (1992) Modes of action of mycoparasites in relation to biocontrol of soilborne plant pathogens. In: Tjamos ES, Papavizas GC and Cook RJ (eds) Biological Control of Plant Diseases (pp 157–165) Plenum Press, New York

Duffy BK, Simon A and Weller DM (1996) Combination of *Trichoderma koningii* and fluorescent pseudomonads for control of take-all on wheat. Phytopathology 86: 188–194

Elad Y, Chet I and Henis Y (1981) A selective medium for improving quantitative isolation of *Trichoderma* spp. from soil. Phytoparasitica 9: 59–67

Gomez-Caballero I, Chet I and Herrera-Estrella A (1995) Genome organization and strain compatibility in *Trichoderma harzianum*. Abstract from the 18th Fungal Genetics Conference, March, 1995. Fung. Genet. Newsl. 42A–1995 supplement

Green H (1996) Ecology of *Trichoderma* spp. in relation to biocontrol of plant diseases caused by *Pythium ultimum*. New technologies for autoecological investigations. Ph.D. thesis. Plant Pathology Section, Department of Plant Biology, The Royal Veterinary and Agricultural University, Denmark

Green H and Jensen DF (1995) A tool for monitoring *Trichoderma harzianum*: II. GUS transformants used for ecological studies in the rhizosphere. Phytopathology 85: 1436–1440

Harman GE, Taylor AG and Stasz TE (1989) Combining effective strains of *Trichoderma harzianum* and solid matrix priming to improve biological seed treatments. Plant Disease 73: 631–637

Harman GE, Hayes CK, Lorito M, Broadway RM, Di Pietro A and Tronsmo A (1993) Chitinolytic enzymes of *Trichoderma harzianum*: Purification of chitobiosidase and endochitinase. Phytopathology 83: 313–318

Hayes CK (1992) Improvement of *Trichoderma* and *Gliocladium* by genetic manipulation. In: Tjamos EC, Papavizas GC and Cook RJ (eds) Biological Control of Plant Diseases. Progress and Challenges for the Future (pp 277–286) Plenum Press, New York.

Ilmén M, Thrane C and Penttilä M (1996) The glucose repressor gene *cre1* of *Trichoderma*: Isolation and expression of a full length and a truncated mutant form. Mol Gen Genet 251: 451–460

Jeffries P and Young TWK (1994) Interfungal Parasitic Relationships. CAB International, Wallingford, UK

Knudsen I, Hockenhull J and Jensen DF (1995) Biocontrol of seedling diseases of barley and wheat caused by *Fusarium culmorum* and *Bipolaris sorokiniana*: Effects of selected fungal antagonists on growth and yield components. Plant Pathology 44: 467–477

Lorito M, Harman GE, Hayes CK, Broadway RM, Tronsmo A, Woo SL and Di Pietro A (1993) Chitinolytic enzymes produced by *Trichoderma harzianum*: Antifungal activity of purified endochitinase and chitobiosidase. Phytopathology 83: 302–307

Lorito M, Hayes CK, Di Pietro A, Woo SL and Harman GE (1994) Purification, characterization, and synergistic activity of a glucan 1,3-β-glucosidase and an N-acetyl-β-glucosaminidase from *Trichoderma harzianum*. Phytopathology 84: 398–405

Lübeck M (1997) Marker genes and PCR based approaches as tools for monitoring fungi in soil: With emphasis on UP-PCR and fungi from the genera *Trichoderma* and *Gliocladium*. Ph.D. thesis. Plant Pathology Section, Department of Plant Biology, The Royal Veterinary and Agricultural University, Denmark

Lynch JM (1989) Environmental potential of the *Trichoderma* exocellular enzyme system. In: Lewis NG and Paice MG (eds) Plant cell wall polymers. Biogenesis and biodegradation. (pp 608–618) American Chemical Society. Washington DC

Migheli Q, Gonzáles-Candelas L, Dealessi L, Camponogara A and Ramón-Vidal D (1998) Transformants of *Trichoderma longibrachiatum* overexpressing the β-1,4-endoglucanase gene egl1 show enhanced biocontrol effect on cucumber. Phytopathology 88: 673–677.

Paulitz T (1990) Biochemical and ecological aspects of competition in biological control. In: Baker RR and Dunn PE (eds) New Directions in Biological Control. Alternatives for Suppressing Agricultural Pests and Diseases (pp 713–724) Alan R. Liss, Inc., New York

Peberdy JF (1990) Fungal cell walls – A review. In: Kuhn PJ, Trinci APJ, Jung MJ, Goosey MW and Copping LG (eds) Biochemistry of Cell Walls and Membranes in Fungi (Chapter 2) Springer-Verlag, Brelin

Schickler H, Danin-Gelali BC, Haran S and Chet I (1998) Electrophoretic characterization of chitinases as a tool for the identification of *Trichoderma harzianum* strains. Mycol Res 102: 373–377

Sivan A and Chet I (1989a) Degradation of fungal cell walls by lytic enzymes of *Trichoderma harzianum*. J Gen Microbiol 135: 675–682

Sivan A and Chet I (1989b) The possible role of competition between *Trichoderma harzianum* and *Fusarium oxysporum* on rhizosphere colonization. Phytopathology 79: 198–203

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

Sivan A and Harman GE (1991) Improved rhizosphere competence in a protoplast fusion progeny of *Trichoderma harzianum*. J Gen Microbiol 137: 23–29

Thrane C, Lübeck M, Green H, Degefu Y, Thrane U, Allerup S and Jensen DF (1995) A tool for monitoring *Trichoderma harzianum*: I. Transformation with the GUS gene by protoplast technology. Phytopathology 85: 1428–1435

Thrane C, Tronsmo A and Jensen DF (1996) Monitoring the activity of different *Trichoderma* isolates by the isoelectric points (pI) of their extracellular enzymes. In: Jensen DF, Jansson H-B and Tronsmo A (eds) Monitoring Antagonistic Fungi Deliberately Released into the Environment (pp 105–111) Kluwer Academic Publishers, Netherlands

Thrane C, Tronsmo A and Jensen DF (1997) Endo-1,3-β-glucanase and cellulase from *Trichoderma harzianum*: Purification and partial characterization, induction by and biological activity against plant pathogenic *Pythium* spp. Eur J Plant Pathology 103: 331–344.

Tronsmo A (1989) *Trichoderma harzianum* used for biological control of storage rot on carrots. Nor J Agric Sci 3:157–161

Tronsmo A (1991) Biological and integrated controls of *Botrytis cinerea* on apple with *Trichoderma harzianum*. Biol Cont 1: 59–62

Tronsmo A and Dennis C (1978) Effect of temperature on antagonistic properties of *Trichoderma* species. Trans Br Myc Soc 71: 469–474

Tronsmo A and Harman GE (1993) Detection and quantification of N-acetyl-β-D-glucosaminidase, chitobiosidase, and endochitinase in solutions and on gels. Anal Biochem 208: 74–79

Webb EC (1992) Enzyme Nomenclature. International Union of Biochemistry and Molecular Biology. Academic Press, Inc., New York

Wolffhechel H (1989) Fungal antagonists of *Pythium ultimum* isolated from a disease suppressive sphagnum peat. Växtskyddsnotiser 53: 7–11

Wolffhechel H and Jensen DF (1992) Use of *Trichoderma harzianum* and *Gliocladium virens* for the biological control of post-emergence damping-off and the root rot of cucumbers caused by *Pythium ultimum*. J Phytopathol 136: 221–230

Woo SL, Donzelli B, Scala F, Mach R, Harman GE, Kubicek CP, Del Sorbo G and Lorito M. (1999) Disruption of the *ech42* (endochitinase-encoding) gene affects biocontrol activity in *Trichoderma harzianum* P1. Mol Plant–Microbe Int 12: 419–429