European Journal of Plant Pathology **103:** 331–344, 1997.

Endo-1,3-β-glucanase and cellulase from *Trichoderma harzianum*: purification and partial characterization, induction of and biological activity against plant pathogenic *Pythium* spp.

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Accepted 9 December 1996

Key words: antagonism, biological control, fungicide, GUS-transformant, root colonization, synergism

Abstract

There were indications that endo-1,3-β-glucanase (1,3-(1,3;1,4)-β-D-Glucan 3(4)-glucanohydrolase (EC 3.2.1.6)) and cellulase (1,4-(1,3;1,4)-β-D-Glucan 4-glucanohydrolase (EC 3.2.1.4)) activity of *Trichoderma harzianum* Rifai isolate T3 were induced in sphagnum peat moss cultivations and dual culture experiments by the presence of *Pythium ultimum*. Further, *P. ultimum* stimulated the germination of *Trichoderma* conidia. Endo-1,3-β-glucanase and cellulase were purified from *T. harzianum* isolate T3, known to control *Pythium* damping-off of cucumber seedlings. The enzymes were purified from the culture filtrate of the fungus by gel filtration and isoelectric focusing. The purified endo-1,3-β-glucanase was a small protein with a molecular mass of 17 kilodaltons and a pI of 5.0. Two cellulases were purified to homogeneity and had molecular masses of 40 and 45 kilodaltons respectively, and pI's of 6.4 and 7.6 respectively. Germination of encysted zoospores and elongation of germ tubes of a plant pathogenic *Pythium* isolate were inhibited by low concentrations of the purified enzymes. A strong synergistic effect was observed on the inhibition of cyst germination by a combination of the endo-1,3-β-glucanase and the fungicide Fongarid. Finally, a time-course study of colonization of the rhizosphere of cucumber seedlings showed that the active fungal mycelial biomass of a GUS-transformant of *T. harzianum* isolate T3 increased over four weeks. *Trichoderma* appeared to colonize healthy roots only superficially, whereas the mucilage of the root hairs and of distal parts of wounded areas or broken parts of the roots, were extensively colonized.

Abbreviation: Peat – sphagnum peat moss.

Introduction

Long-standing evidence indicates that mycoparasitism associated with the action of chitinases enable *Trichoderma harzianum* to control many plant pathogens that have cell walls containing a chitinous microfibrillar skeleton (Chet et al., 1981; Chérif and Benhamou, 1990; Lorito et al., 1994). In the case of oomycetes such as *Pythium* spp. that only possess small quantities of wall bound chitin (Dietrich, 1973), biological control by *T. virens* (*Gliocladium virens*) is believed to be dependent on antibiosis (Howell, 1982; Jones and Hancock, 1988). However, *T. harzianum* also produces various secondary metabolites (Claydon et al., 1987) that are likely to be involved in antagonism (Schirmböck et al., 1994), but competition (Lifschitz et al., 1986) and hydrolytic enzymes (Elad et al., 1982) have also been suggested as possible mechanisms of controlling *Pythium* spp.

The ability of *Trichoderma* spp. to utilize organic substrates such as plant debris and to colonize the roots and rhizosphere of plants (Ahmad and Baker, 1987; Sivan and Chet, 1989; Sivan and Harman, 1991) has been related to biocontrol efficiency and the potential production of cellulase and hemicellulase. In this way, cellulases likely serve several functions in *Tricho-*

derma isolates used in the biocontrol of *Pythium*. The lytic properties of cellulase may be connected to direct inhibition of the pathogen, or to colonization of the plant roots, and establishment in the soil on plant debris.

The *T. harzianum* isolate T3, was originally isolated from *Pythium*-suppressive peat (Wolffhechel, 1989) and it showed a consistently good biocontrol effect in the growth chamber of damping-off disease caused by *Pythium* spp. on cucumber seedlings (Wolffhechel and Jensen, 1992). The aim of this study was to identify extracellular enzymes produced by *T. harzianum* isolate T3 that could be related to the antagonistic properties of the fungus. As the major cell wall components of *Pythium* are 1,3-β-glucans (and 1,6-β-glucan) covering fibrillar cellulose (Sietsma et al., 1969), cultivation experiments in peat and on enzyme indicative agar were set up to determine whether *Trichoderma* endo-1,3-β-glucanase and cellulase activities increased in the presence of the pathogen. Endo-1,3-β-glucanase and cellulase were further purified and tested alone and in combination with the greenhouse fungicide Fongarid for their effect on the germination rate of encysted *Pythium* zoospores and elongation of germ tubes. Finally, activity of *T. harzianum* isolate T3 on roots of cucumber seedlings over a five-week period was carried out by using our GUS-transformant of this isolate (Thrane et al., 1995).

Materials and methods

Fungi and plant. Trichoderma harzianum Rifai isolate T3 was isolated from a *Pythium* suppressive sphagnum peat moss from Sweden (Wolffhechel, 1989). *T. harzianum* T3a is a GUS-transformed isolate of T3 (Thrane et al., 1995). *Pythium ultimum* Trow isolate HB2 (var. *ultimum*) was isolated from the Hoejbakkegaard field station in Denmark. *Pythium* isolate P11 (*Pythium* 'Group F' according to van der Plaats-Niterink (1981)) was isolated in Denmark from diseased pepper plants grown in a hydroponic system by C. Rosendahl. Cucumber, *Cucumis sativus* L. cv. 'Langelands Kaempe-Gigant' (Daehnfeldt, Denmark), was used as the test plant.

Media and growth conditions in liquid culture. For enzyme production, *T. harzianum* T3 was grown on a synthetic basal medium (SM) (Nelson et al., 1988) that contained 0.68 g KH_2PO_4, 0.87 g K_2HPO_4, 0.2 g KCl, 1.0 g NH_4NO_3, 0.2 g $CaCl_2$, 0.2 g $MgSO_4 \times 7$ H_2O, 2 mg $FeSO_4$, 2 mg $MnSO_4$, 2 mg $ZnSO_4$, 1 g polyvinylpyrrolidone (PVP) (Polyclar AT, GAF Corp., Germany), 100 ml V8 juice (Campbell Soup. Co., Cambden, NJ, USA), and 900 ml H_2O. The pH was adjusted to 6.7. Carbon sources tested were sucrose, chitin (Sigma C-3387, Sigma Chemical Co., St. Louis, MO, USA), cellulose (Sigma C-6663), or cell walls of *P. ultimum* HB2. *T. harzianum* isolate T3 was grown in 100 ml SM in 300 ml Erlenmeyer flasks. The flasks were inoculated with conidia from PDA plates (final concentration $5*10^6$ conidia ml^{-1}) and placed on a reciprocal shaker at 150 rpm at 21 °C. After 6 days, the biomass was separated from the extracellular liquid by filtration through a GF/B glass microfiber filter (Whatman Int. Ltd, Maidstone, England), and this liquid is hereafter designated culture filtrate. Before storage at 4 °C, 0.02% NaN_3 was added to the culture filtrates.

P. ultimum HB2 cell walls were prepared by still cultivation of agar plugs of fungal mycelia in petri dishes containing 10 ml potato carrot broth for 3 days in the dark at room temperature. The mycelia were harvested by filtration through 4 layers of cheese cloth, washed in sterile water, and subsequently freeze-dried. The dry mycelia were finally ground in liquid nitrogen and autoclaved.

For the studies of possible glucose repression of enzyme production, the following growth medium was used: 100 ml V8 juice, 6.8 g KH_2PO_4, 0.1 g KCl, 2 g $NaNO_3$, 0.5 g sucrose, 900 ml H_2O. The pH was adjusted to pH 6.0. Conidia ($5*10^6$/ml) were added to 300 ml erlenmeyer flasks with 80 ml substrate. Four different carbon source amendments were used. a: 2% glucose (with addition of another 2% glucose after 40 h of incubation); b: 1% cellulose (with addition of 2% glucose after 40 h of incubation); c: 1% cellulose; d: 1% cellulose and 2% glucose. The fungus was grown at 25 °C in the dark, with shaking at 200 rpm. Samples (1–2 ml) were taken daily for protein and enzyme activity measurements. There were three replicates of each treatment. The experiment was repeated. In the repeated experiment, the level of enzyme activities was different, but the pattern was similar. Data from the first repeat trial are shown.

Enzyme indicative agar. In dual culture experiments, agar plates were prepared by mixing equal amounts of dialyzed Blue substrates (see below) and 2% bacto-agar. The *T. harzianum* isolate T3 and the *P. ultimum* isolate HB2 were inoculated on the agar and incubated at room temperature for 1–3 days. Indications of enzyme activity (clearing zones) were scored on

the plates. Detection of enzyme activities on activity gels after electrophoresis were carried out according to Thrane et al. (1996).

Extraction and determination of enzyme activities from peat cultivation experiments with T. harzianum *and* P. ultimum. Erlenmeyer flasks (250 ml) were inoculated with three agar plugs of young cultures of the fungi in 39 g sterile peat (35% d.w.). Extracellular proteins were extracted after 8 days of cultivation at 25 °C, using 80 ml 50 mM Na-phosphate buffer (pH 6.7) containing 0.04% NaN3, and vortexing and shaking by hand. The liquid phase was centrifuged ($8.000 * g$) (Sigma-3MK, Laborzentrifugen, GmbH, Osterode, West-Germany) to pellet the peat debris. The supernatant was sterile filtered and used as crude enzyme extract. There were three replicates in each experiment which was repeated twice.

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

Determination of cellulase and endo-1,3-β-glucanase activity. Blue substrates (Loewe Biochemica GmbH no. 04100 and no. 04101) were used for determination of cellulase (E.C. 3.2.1.4) and endo-1,3-β-glucanase (E.C. 3.2.1.6) (Webb, 1992). Enzyme units were defined on the basis of a standard curve obtained by measuring enzyme activity in dilutions of the purified enzymes. According to Wirth and Wolf (1992), the substrates exclusively detect and quantify endo-acting enzymes. Relative activities in percentage of maximal activity were used to determine pH and temperature profiles as well as the effect of different chemicals on enzyme activities. For cellulases a total of 100 μl undiluted or diluted enzyme solution was mixed with 300 μl 50 mM phosphate buffer (pH 6.7) and 100 μl blue substrate in eppendorf tubes. The tubes were incubated at 37 °C for 60 min. The reaction was stopped by addition of 300 μl 1 M HCl followed by incubation at 4 °C for 20 min. Excess substrate was pelleted by centrifugation for 10 min ($10.000 * g$). The absorbance at 600 nm was measured either in 1 ml cuvettes in a spectrophotometer ((Ultrospec III, Pharmacia) or in a microtiter plate reader (Ceres UV 900 HDI, Bio-Tek Instruments, Inc., Winooski, VT, USA). For endo-1,3-β-glucanase the assay was the same except that a total of 200 μl enzyme solution was used.

Germination of Trichoderma *conidia on water agar in response to* Pythium ultimum. Water agar (1.8% agar) was mixed at 50 °C with *Trichoderma* conidia from a 5-day-old PDA culture to a final conidia concentration of $2 * 10^5$. Agar plugs of *P. ultimum* isolate HB2 were placed on the water agar in one treatment and not in the control treatment. The plates were incubated for 24 h at 20 °C in the dark. The numbers of conidia that had germinated in the presence or absence of *P. ultimum* were counted under a microscope. The experiment was carried out on two individual days with three or six replicates of each treatment. 100–200 conidia were counted in each replicate. The data are represented as the mean of the two experiments. A minimum of 100 conidia were counted in each replicate.

Enzyme purification. Culture filtrates were concentrated in dialysis tubes placed in polyethylene glycol (Sigma P-2139, 8000 Mol. Wt.) at 4 °C until the volume was reduced 5-10 fold. The dialysis tubes were changed and the samples were dialysed overnight against distilled water at 4 °C. If the resulting dialysed, concentrated culture filtrate was to be stored, 0.02% NaN_3 was added as a preservative and the material stored at 4 °C until use. Subsequently it was injected into a chromatography column (5×60 cm) packed with Sephacryl S-300 HR (Pharmacia LKB Biotechnology, Uppsala, Sweden). The column was equilibrated and eluted with 50 mM potassium phosphate buffer, pH 6.7, containing 200 mM NaCl and 0.04% NaN_3. Samples and elution buffer were pumped from the bottom of the column at a rate of 6 ml/min, and fractions were collected every 2 min.

The fractions were analysed for enzymatic activity. Fractions exhibiting the same activity were pooled, concentrated in dialysis tubes against polyethylene glycol, and dialysed overnight against distilled water. These pooled fractions were further purified by electrofocusing using 2% Bio-lyte 3/10 or 3/5 on a Rotofor apparatus (Bio-Rad Laboratories, Richmond, CA, USA) according to the directions of the manufacturer.

Several similar purifications were conducted for the enzymes, and the elution profiles and activities in the figures and tables are typical examples of the results obtained.

Characterization of molecular weight and isoelectric point of the enzymes. Polyacrylamide gel electrophoresis (PAGE) was used to assess purity of enzyme preparations and to partially characterize purified proteins. All electrophoresis was done on a Phast-

System (Pharmacia LKB Biotechnology, Uppsala, Sweden). Native and SDS electrophoresis were conducted using 12.5% homogenous gels, while isoelectric focusing (IEF) was done on gels with a range of pH 4–6.5, pH 5–8, and pH 3–9. Electrophoresis and silver staining of gels were carried out according to the protocols supplied with the Phast System. For SDS and IEF electrophoresis the protein standards provided by Pharmacia were used for determination of molecular weights and isolelectric points; for SDS the molecular weights of the six standard proteins ranged from 14.4 to 94 kD, and for IEF the isoelectric points of the 12 standard proteins ranged from pH 3.5 to 9.3. Isoelectric points were also determined in the Rotofor system.

Temperature characteristics of the enzymes. To determine the optimum temperature for the enzymatic reactions of the purified endo-1,3-β-glucanase and cellulase, the reactions were performed at temperatures ranging from 20 °C to 65 °C at 5 °C intervals. The stabilities of the purified enzymes were tested by incubating the enzymes at temperatures ranging from 20 °C to 65 °C at 5 °C intervals for 60 min, and then testing the activity at the usual test temperature (37 °C). There were two replicates in each experiment, which was carried out twice.

pH characteristics of the enzymes. Enzyme activities of the purified endo-1,3-β-glucanase and cellulase at various pH levels were determined in a 50 mM citric acid potassium phosphate buffer mixture at pH 3 to 7 using the enzyme assays described above.

Effect of different chemicals on enzyme activity. The following chemicals were tested in the noted final assay concentration for their effect on the enzyme activity of the purified endo-1,3-β-glucanase and cellulase: 10 mM NaCl, 10 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM $MnSO_4$, 2 mM $FeSO_4$, 2 mM $ZnSO_4$, 2 mM $Hg(II)SO_4$, 1% SDS, and 1% β-mercaptoethanol. The enzyme assays were as above except water was used instead of Na-phosphate buffer. The experiment was repeated three times with two replicates in each experiment.

Preparation of zoospores from P. ultimum *isolate P11*. For the production of zoospores from the *P. ultimum* isolate P11, the fungus was grown for 3–4 days (20 °C) on plates containing the following medium: 200 ml vegetable juice (V8), 3 g $CaCO_3$, 20 g agar, and 800 ml H_2O. Zoospore production was induced by adding 40 ml of basal solution to each plate, which was then incubated for 5–20 h at 20 °C. The basal solution was made up as following: Stock solution A (1000 ml): 5.605 g NH_4NO_3, 51.365 g $Ca(NO_3)_2$ $4H_2O$, 1.820 g KNO_3, 13.720 g $Mg(NO_3)_2$ $6H_2O$, 0.125 g H_3BO_3, 0.0185 g Na_2MoO_4 $2H_2O$. Stock solution B (750 ml): 6.67 g KH_2PO_4, 7.395 g $MgSO_4$ $7H_2O$, 0.118 g $MnSO_4$ $1H_2O$, 0.0215 g $ZnSO_4$ $7H_2O$, 0.0185 g $CuSO_4$ $5H_2O$. Stock solution C (500 ml): 3.725 g Na_2-EDTA (Titriplex III) and 2.785 g $FeSO_4$ $7H_2O$. To the final medium 50 ml solution A, 37.5 ml solution B, and 12.5 ml solution C were added to 10 l H_2O. To encyst the zoospores, the solutions of the zoospores were vortexed briefly 5–10 times. Immediately after encystment, the cysts were used for the bioassays. The concentration of encysted zoospores used in the bioassays was $5*10^4$–$5*10^5$ ml^{-1}.

Biological activity of purified enzymes on cyst germination and germ tube growth. The biological activity of the purified enzymes was tested on germination of encysted zoospores and growth of germ tubes from the cysts of *Pythium* isolate P11. In a total volume of 15 ml per replicate the following was mixed: zoospores (1.5–$3.0*10^4$ spores ml^{-1}), basal solution, 25 mM MES-buffer (pH 5.5) and enzymes to give the following final concentrations: 180 μg ml-1 endo-1,3-β-glucanase: or 33 μg ml^{-1} cellulase. In experiments where both enzymes were used: 180 μg ml^{-1} endo-1,3-β-glucanase: and 33 μg ml^{-1} cellulase:, or 90 μg ml^{-1} endo-1,3-β-glucanase: and 16.5 μg ml^{-1} cellulase. Water was used in the control treatments. The concentrations of enzymes used were chosen as they gave a 50 to 100% reduction in numbers of germ tubes that were longer than 100 mm compared to the control treatment. The 15 μl aliquots were incubated in eppendorf tubes, gently shaking in the dark at room temperature. After 1.5–4 h of incubation the zoospores were examined under a microscope. The germination rate of the first 50–100 zoospores seen on a microslide, was observed and the lengths of the observed germ tubes were categorized. Each experiment was carried out two to four times. There were three replicates in each experiment. When the experiment was repeated, the level of inhibition by the enzymes was different, but the pattern was similar. Data from one repeat trial is shown. The Sigma cellulase (C-8546) was also used in a bioassay carried out under the same conditions as described above of a final concentration of 400 μg ml^{-1} (d.w.).

Effect of purified enzymes in combination with the fungicide Fongarid on cyst germination and germ tube growth. The experiments were essentially as above. The greenhouse fungicide Fongarid (furulaxyl) (Fongarid 25wp, Ciba-Geigy a/s, Copenhagen, Denmark) was tested for its effect on the encysted zoospores alone and in combination with the enzymes. In this case the following enzyme concentrations were used: 60 μg ml^{-1} endo-1,3-β-glucanase and 14 μg ml^{-1} cellulase. In each experiment there were three replicates. The experiment was repeated five times. When the experiment was repeated, the level of inhibition by the compounds was different, but the pattern was similar. Data from one repeat trial is shown. We used the following (Limpel's) formula according to Lorito et al. (1994) to determine synergistic interactions between the enzymes and Fongarid: E_e = (X+Y–XY/100). E_e is the expected effect from additive responses of two inhibitory compounds. X and Y are the percentages of inhibition obtained with each of the two compounds.

Colonization of Trichoderma *on cucumber roots*. Cucumber seeds were surface sterilized in 70% ethanol (20 sec) and 2.5% sodium hypochlorite (10 min), and washed three times in distilled water. The seeds were pregerminated on 50% PDA (final conc. 2% agar) for 3 days at 25 °C. The cucumber seedlings and agar plugs of the T3a GUS-transformant were inoculated in the pots containing a sterile (autoclaved twice) potting mixture of peat/vermiculite (60%/40%) which is a common growth medium for vegetables in the greenhouse. The pots were then watered to a final d.w. of 30%. The pots were watered every week with Hornum nutrient solution (P. Brøste Industri a/s, Lyngby, Denmark). Cultivation was carried out in the growth chamber (16 h light and 8 h dark) at 18 °C. Samples were taken after 7, 21, 28, and 35 days. The roots were incubated in Na-phosphate buffer (pH 6.7), containing 0.04% NaN_3 and 4 mg ml^{-1} 5-bromo-4-chloro-3-chloro β-D-glucuronide (Sigma B-0522) for 16 h at 37 °C to stain the hyphae of the GUS-transformed *T. harzianum* on the roots of cucumber. After staining, the fungal mycelia could then be distinguished from the plant tissue by its greenish-blue colour. The roots were viewed in the light microscope and the stereo microscope.

Table 1. Comparison of endo-1,3-β-glucanase and cellulase activities in extracts of cultivations of *Trichoderma harzianum* isolate T3 with or without the presence of simultaneously inoculated growing mycelium of *Pythium ultimum* in sterile sphagnum for 8 days

Treatment	endo-1,3-β-glucanase (arb. units)	Cellulase (arb. units)
(A) First repeat trial		
T3	132 +/– 47	105 +/– 23
Pythium + T3	185 +/– 94	147 +/– 74
(B) Second repeat trial		
T3	50 +/– 10	9 +/– 2
Pythium + T3	61 +/– 15	19 +/– 10

Results

Induction of endo-1,3-β-glucanase and cellulase activity in the interaction between T. harzianum *isolate T3 and* Pythium. On agar indicative of endo-1,3-β-glucanase activity a clearing zone was visible in the zone of interaction between *T. harzianum* isolate T3 and *Pythium* (result not shown). In the zone of interaction, the *Pythium* hyphae appeared lysed. A similar test for cellulase activity did not show a clearing exactly in the interaction zone, but rather, as the cultures were overgrowing each other, scattered clearings appeared in the mixed mycelia of the two fungi. In the experiment where *Pythium* and *Trichoderma* were grown in sterile peat, and inocula of the fungi were added simultaneously as actively growing mycelium (Table 1), there were increases in both endo-1,3-β-glucanase and cellulase activity when *Pythium* was present.

Germination of Trichoderma conidia *on water agar in response to* P. ultimum. On water agar 94% of the *T. harzianum* isolate T3 conidia germinated when actively growing *P. ultimum* mycelium was present. Without the presence of *Pythium*, there was a significant reduction in the germination rate, as only 75.7% germinated ($P < 0.0001$).

Identification of hydrolytic enzymes produced by T. harzianum *isolate T3 in liquid culture*. The fungus was grown in shake flasks in liquid medium supplemented with different carbon sources. After six days, the culture filtrates from these liquid cultivations were assayed for the amount of extracellular proteins and for endo-1,3-β-glucanase and cellulase activities. Extra-

cellular protein was highest in cultivations without addition of easy fermentable sucrose. Endo-1,3-β-glucanase activity was highest in culture filtrates of *T. harzianum* isolate T3 grown in cellulose ammended with sucrose and the cellulase activity was highest in culture filtrates containing either cellulose or purified *Pythium* cell walls as carbon sources. On the IEF activity gels of culture filtrates from the various carbon sources one band (pI 5.0) of endo-1,3-β-glucanase activity was detected on all media. There was much more variation in the pI-values and the intensity of the bands of the cellulase activity detected in the different culture filtrates. On the IEF/activity gel there were six major activities (pI 4.8, 5.2, 5.7, 6.4, 7.6, and 8.3) and several minor activities of cellulases detected in the culture filtrate from the liquid culture with *Pythium* cell walls as carbon source.

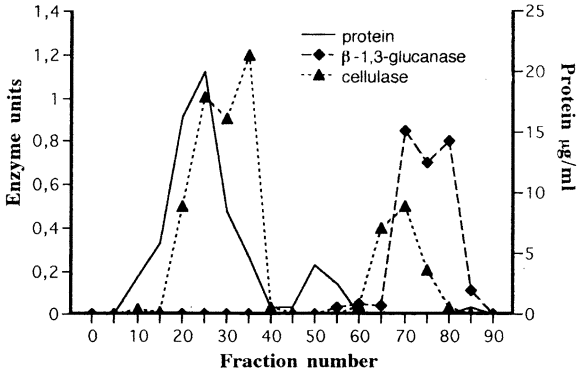

Figure 1. Enzyme activity and protein determination in fractions from the gel-filtration chromatography on the Sephacryl column. A mixture of concentrated culture filtrates with sucrose and cellulose and cellulose as carbon sourses was used. Total protein, endo-1,3-β-glucanase actvity, and cellulase activity are shown in the figure.

Purification of endo-1,3-β-glucanase and cellulase from T. harzianum. In Table 2, data from the purification of endo-1,3-β-glucanase and cellulase on the Sephacryl fractionation column and the isoelectric focusing separation (Rotofor) are shown. Ninetyseven percent of the protein was recovered after the gel filtration. Apparently endo-1,3-β-glucanase and some cellulases bind to the Sephacryl column, as these enzymes were only present in the very last fractions where relatively little total protein was present (Figure 1). After the Sephacryl fractionation, the total recovery of β-1,3-glucanase activity was high (88%) and somewhat lower for cellulase (69%). The Sephacryl fractions high in specific activity for the endo-1,3-β-glucanase and endocellulases were pooled and further purified by isoelectric focusing (Rotofor). The endo-1,3-β-glucanase IEF-elution curve shown in Figure 2A consisted of the fractions 60–78 from the gel-filtration shown in Figure 1. There was a sharp major peak of endo-1,3-β-glucanase activity eluting at approximately pH 5.0 (Figure 2A). Fractions 12–15 from this IEF-separation were pooled and analyzed by IEF-electrophoresis. Silver staining of the IEF-gel (Figure 3A) showed that the endo-1,3-β-glucanase was purified to homogeneity migrating to a pI of 5.0. For the purification of cellulase, the culture filtrate of *T. harzianum* T3 grown on *Pythium ultimum* cell walls was used. The fractions 60-80 from this sephacryl fractionation (see Table 2) were pooled and subjected to IEF separation on the Rotofor. The major peak of cellulase activity eluted at pH 6.4–8.9 (Figure 2B). These fractions (12–16) were pooled and analyzed on an IEF-gel. The silver stained gel showed two bands (Figure 3B) with pI values of 6.4 and 7.6. Subsequent staining for cellulase activity of a similar IEF-gel showed that both bands had cellulase activity.

Characterization of the purified enzymes. The molecular weight mass of the endo-1,3-β-glucanase determined by silver staining on a SDS-gel was 17 kDA. The two cellulases with the pI's of 6.4 and 7.6 had molecular weight masses of 40 and 45 kDA respectively. The temperature optima and stabilities were similar for the β-1,3-glucanase and the cellulases. The temperature optimum was 40 °C for endo-1,3-β-glucanase and 37 °C–40 °C for cellulase. The stability of the enzymes dramatically decreased above 35 °C for the endo-1,3-β-glucanase and above 40 °C for cellulases. The pH optima for the endo-1,3-β-glucanase was 5.0–5.5 and 4.0–5.0 for the cellulases. The effect of different ions on the enzyme activity was assayed. The only significant effects were caused by Hg(II), SDS, and β-mercaptoethanol. Mercury ions totally inhibited the enzyme activities. SDS nullified enzyme activity of the endo-1,3-β-glucanase and reduced the activity of the cellulases to 13%, whereas β-mercaptoethanol had no effect on cellulase activity, but reduced endo-1,3-β-glucanase activity to 25%. In Figure 4, data are shown from a timecourse study to test whether glucose repression was operating on endo-1,3-β-glucanase and cellulase production. A dramatic reduction in cellulase activity was seen after the 40 h glucose supplement. For endo-1,3-β-glucanase the glucose repression was less pronounced.

Table 2. Purification of endo-1,3-β-glucanase and cellulases from *Trichoderma harzianum* isolate T3. The fungus was grown in shake flasks with *Pythium ultimum* cell walls as the carbon source for 6 days. The culture filtrate (300 ml) was separated from the mycelium by filtration and used as the enzyme source

Enzyme step	Total protein (μg)	Enzyme activity (arb. units)	Specific activity (arb. units/mg)	Purification (fold)	Yield (%)
endo-1,3-β-glucanase					
Culture filtrate[1]	7.4	186.0	25.1	1	100
Gel filtration	0.16	149.0	931.3	37.0	80
Isoelectric focusing	0.08	25.0	312.5	12.5	13
Cellulase					
Culture filtrate[1]	6.1	158.0	26.0	1	100
Gel filtration	0.16	46.7	291.9	11.3	30
Isoelectric focusing	0.05	6.5	130.0	5.0	4

[1] Concentrated and dialyzed.

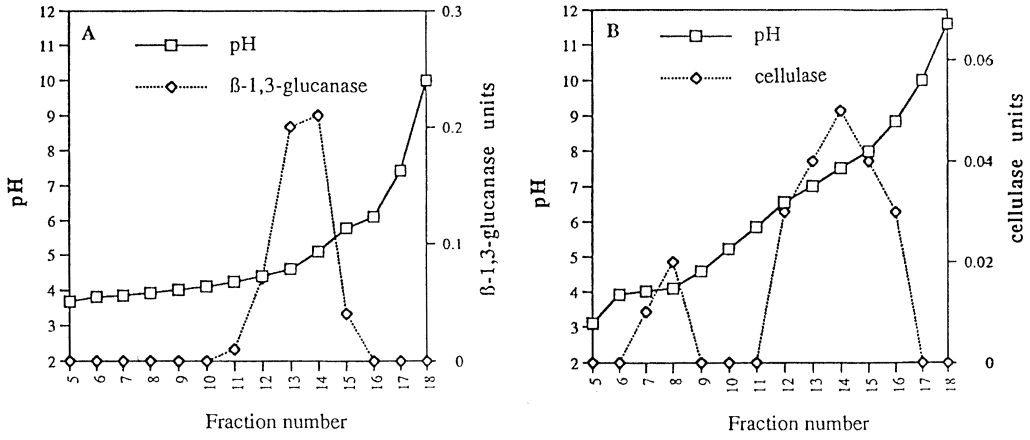

Figure 2. pH gradient and enzyme activity in pooled fractions from the Sephacryl column separated by isoelectric focusing on the Rotofor apparatus. (A) Endo-1,3-β-glucanase (separation range pI 3-5) purified from the sephacryl fractions 60–78 described in Figure 1 and in Table 2. The Rotofor fractions 12–15 were pooled and analyzed on an IEF gel (see Figure 3). (B) Cellulase (separation range pI 3-9) purified from the sephacryl fractions 60–80 of concentrated culture filtrate from a cultivation with *Pythium ultimum* cell walls as carbon source (see Table 2). Rotofor fractions 12–16 were pooled and analyzed on an IEF gel (see Figure 3).

Biological activity of purified enzymes on cyst germination rate and germ tube growth. There were significant effects of the purified endo-1,3-β-glucanase and cellulase on cyst germination rate and on the growth of germ tubes (Figure 5). This effect was enhanced when both enzymes were added, compared to the effect of the individual enzymes separately (result not shown). In general, long germ tubes only developed in the control treatments (H_20) whereas when treated with enzymes, the cysts either developed short germ tubes or did not germinate. Cotton blue, which stains cell wall polymers, hardly stained enzyme treated cell walls of *Pythium*, but controls were stained dark blue.

Antifungal activity of the purified enzymes in combinations with a fungicide. There were significant effects of the purified β-1,3-glucanase and cellulase in combination with the fungicide Fongarid on cyst germination rate and the growth of germ tubes compared to treatments with the fungicide or the enzymes by themselves (Figure 6). According to Limpel's formula there was a strong synergistic effect of the combination of β-1,3-glucanase and Fongarid.

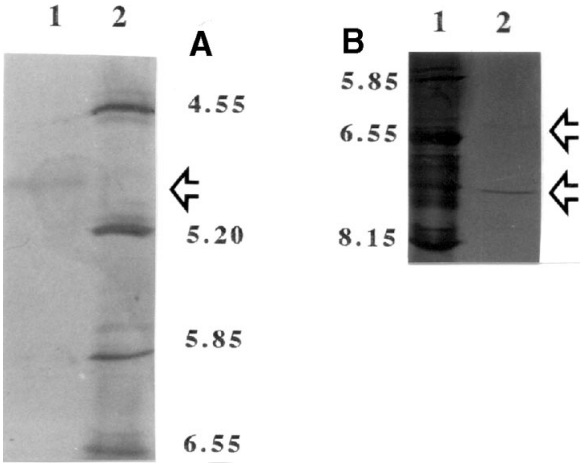

Figure 3. IEF gel electrophoresis and silver staining of the purified enzymes after isoelectric focusing separation (Rotofor) (A) Lane 1: endo-1,3-β-glucanase (pI 5.0). Lane 2: pI markers 4.55, 5.20, 5.85, and 6.55. (B) Lane 1: pI markers 5.85, 6.55, and 8.15. Lane 2: Cellulase (pI 6.4 and 7.6).

Colonization of Trichoderma *on cucumber roots*. The colonization of cucumber roots by *T. harzianum* isolate T3a (GUS-transformant) was studied over a five-week period. There was an increase during the first four weeks of the amount of mycelium on the roots stainable with 5-bromo-4-chloro-3-chloro-β-D-glucuronide, a synthetic substrate for the GUS-enzyme, which stains the fungal mycelium greenish-blue. The staining of the fungal mycelium has previously been shown to be correlated with actively growing mycelium (Green and Jensen, 1995). On the tap root, germinated conidia were detected after 7 days (Figure 7A), and after four weeks the tap root (Figure 7B) and the whole root system was densely colonized by *T. harzianum* isolate T3 (Figure 7C). The seed coat and root hairs were colonized early and throughout the whole time-course experiment by actively growing T3a (not shown). Heavy colonization of the roots by T3 after four weeks was primarily restricted to wounds (Figures 7C–D) and the distal parts of broken roots (Figure 7E). On apparently healthy looking roots the colonization appeared to be superficial (outer epidermis layers) (Figures 7B–D). The cell layers that detach in the zone where lateral roots emerge from the parent root, were also superficially colonized (result not shown). After five weeks there was less fungal mycelia stained on the roots, but on the lower part of the stalk mycelium of *T. harzianum* isolate T3 was detected by blue-staining (result not shown).

Discussion

The peat cultivations and dual cultures showed that endo-1,3-β-glucanase and cellulase activities were induced in *T. harzianum* isolate T3 by the presence of *Pythium*. Further, purified endo-1,3-β-glucanase and cellulase from *Trichoderma* were inhibitory to the germination and growth of encysted *Pythium* zoospores. The combination of the endo-1,3-β-glucanase and cellulase, or the combination of the purified enzymes individually with a fungicide, enhanced this effect.

We did not observe penetration of *Pythium* hyphae by *T. harzianum* isolate T3 on PDA. No obvious indications of antibiotic production in the interaction between the two organisms have been seen in other culture experiments on agar (Wolffhechel and Jensen, 1992). Although not conclusive, our data indicate that endo-1,3-β-glucanase and cellulase play an important role in the antagonism of *Pythium* by *T. harzianum* isolate T3. The results suggest that endo-1,3-β-glucanase acts on *Pythium* by contact or close proximity between the two interacting organisms. The mechanism is likely hyphal interference as that has also been suggested by Jeffries and Young (1994). This type of antagonism occurs, despite a physical distance between the interacting organisms, through the action of diffusible metabolites. On the other hand, cellulases might not work on the living *Pythium*, but rather, this enzyme helps *Trichoderma* to establish in the same ecological niche as the pathogen by degradation of moribound *Pythium* mycelia.

Endo-1,3-β-glucanase was purified to homogeneity and two cellulases were co-purified to homogeneity by gel filtration and subsequent isoelectric focusing. This is the first report on the purification of an acidic endo-1,3-β-glucanase and of cellulases from *T. harzianum*. The size of the *T. harzianum* isolate T3 endo-1,3-β-glucanase was smaller (17 kDA) and the pI much lower (5.0) than a endo-1,3-β-glucanase from another *T. harzianum* isolate (Cruz et al., 1995). The pH optimum for the enzyme activity (pH 5.0–5.5) was in the same range as a purified exo-acting 1,3-β-glucanase from *T. longibrachiatum* (Tangarone et al., 1989). The temperature optimum and stability were lower than those of the exo-acting 1,3-β-glucanase from *T. longibrachiatum* (Tangarone et al., 1989) and the endo-1,3-β-glucanase from a *T. harzianum* isolate (Cruz et al., 1995), but in the range of exo-acting 1,3-β-glucanases from a *T. harzianum* isolate (Kitamato et al., 1987; Lorito et al., 1994).

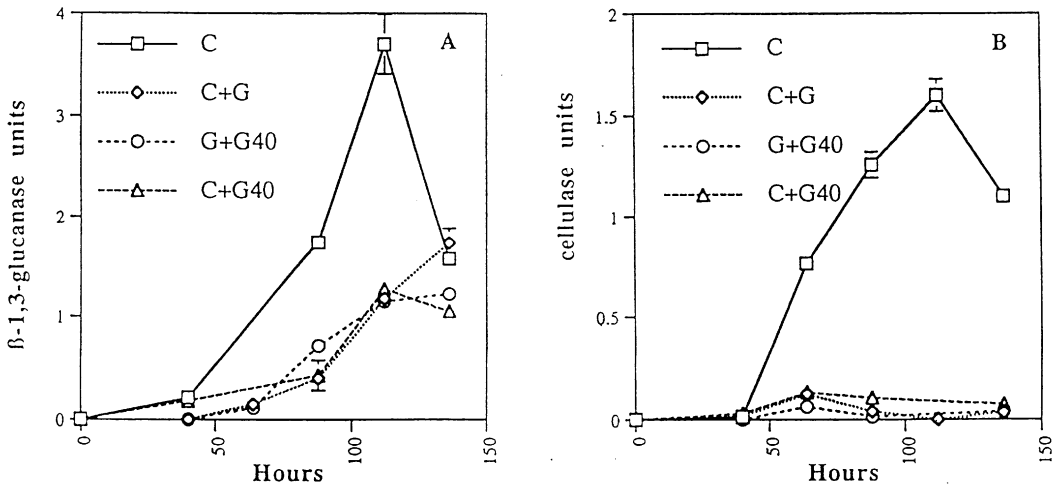

Figure 4. Time course study of glucose repression of enzyme production of *T. harzianum* isolate T3. The following carbon sources were used: a: 1% cellulose (C). b: 1% cellulose and 2% glucose (C+G). c: 2% glucose (amendment of another 2% glucose after 40 h of incubation) (G+G40) d: 1% cellulose (amendment of another 2% glucose after 40 h of incubation) (C+G40). (A) endo-1,3-β-glucanase activity, (B) cellulase activity. Error bars indicate standard deviations.

The purified cellulases were of higher molecular weights (as determined by electrophoresis) than expected from the gel filtration elution profile. The molecular masses of the cellulase (40 and 46 kDA) are comparable in size to *T. reesei* EGI cellulase (van Arsdell et al., 1987). Further, Western blotting of proteins from a culture filtrate from isolate T3 gave a signal at approximately 60 kDA with antibodies raised against *T. reesei* QM9680 cellulase EGI (C. Thrane and M. Ilmén, unpubl.), indicating some interspecies homology between cellulases. The pI of our cellulases are close to neutral and have the same range of optimal pH for enzyme activity (pH 4.5–5.0) as *T. reesei* cellulase (Hayn et al., 1993). The optimal temperature and temperature stability however, are considerably lower than that of *T. reesei* (Hayn et al., 1993) and of bacterial cellulases (Aa et al., 1994). The organisms from which these cellulases were purified were all isolated from habitats where intensive microbial cellulose degradation takes place. The difference in temperature optima between our cellulases and the others might reflect that the temperatures in the soil where biocontrol takes place are lower than in compost (Aa et al., 1994).

There are a few examples of the direct use of antifungal enzymes to control plant pathogens, as adding chitinase to the circulating water in the greenhouse (Shapira et al., 1989) or spraying a rice field with endo-1,3-β-glucanase and chitinase (Tanaka et al., 1970; Poulose 1992). However, there seem to be differences in the biological activity against plant pathogens of enzymes from different sources. This is reported by Di Pietro et al. (1993), who found that the activities of chitinases purified from *T. virens* and *T. harzianum* were very different, despite similarities in their sizes and in the nucleotide sequence of their genes (G.E. Harman, pers. comm.). We found that a commercial *T. reesei* cellulase preparation from Sigma had no effect on the growth of *Pythium*. This is in agreement with Meyer et al. (1976), who found no degradative effect of a Sigma cellulase on *Phytophthora* mycelium.

An approach to reduce input of pesticides in agricultural production is to combine low doses of fungicides with biocontrol agents, as shown by Howell (1991) and Tronsmo (1991). They demonstrated that it was possible to obtain a synergistic effect of *T. virens* or *T. harzianum* respectively in combination with low doses of fungicides in biocontrol of *Pythium* in cotton or *Botrytis cinerea* on apple fruits respectively. We also found an increased effect of the purified enzymes together with a fungicide used in the greenhouse for the control of *Phytophthora* and *Pythium*. The active component of the fungicide is furulaxyl, which is believed to interfere with the RNA polymerase I-template complex, inhibiting ribosomal RNA synthesis (Griffith et al., 1992). In our studies, *Pythium* was at least 10 times more sensitive to the fungicide when it was used together with the purified endo-1,3-β-glucanase or cellulase. Fongarid showed a high degree of synergism especially in combination with endo-1,3-

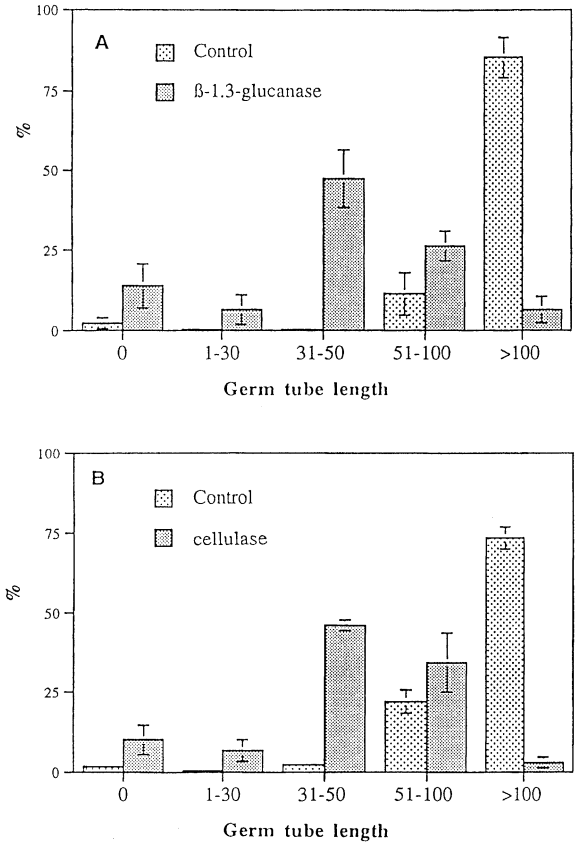

Figure 5. Bioassay of the effect of purified enzymes from *Trichoderma harzianum* isolate T3 and H_2O (control) on the germination rate of encysted zoospores and growth of germ tubes of *Pythium* P11. Values on the Y-axis are the percentage of spores that are ungerminated (shown as 0 on the X-axis), or germinated with germ tube lengths of 1–30 μm, 31–50 μm, 51–100 μm, and respectively more than 100 μm. (A) endo-1,3-β-glucanase: 180 μg ml^{-1}. (B) cellulase: 33 μg ml^{-1}. Error bars indicate standard deviations.

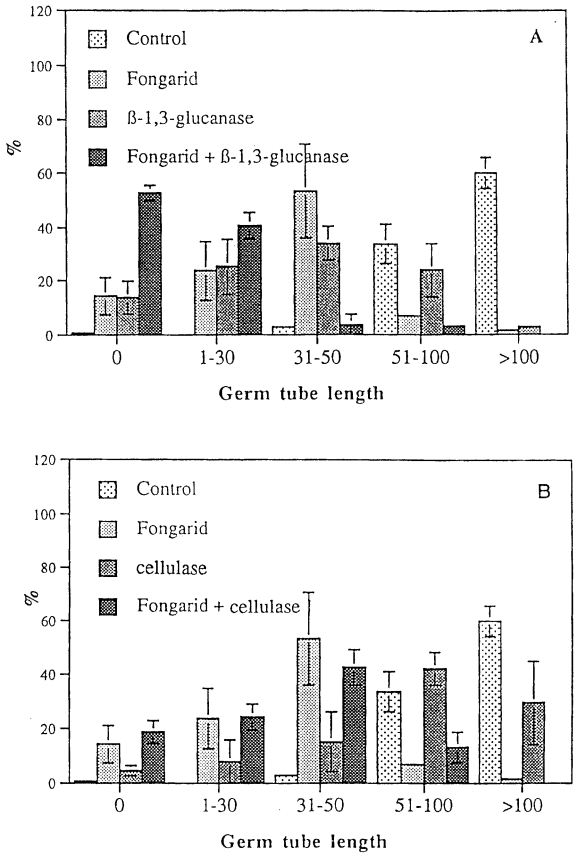

Figure 6. Bioassay of the effect of the fungicide Fongarid (10 ppm) in combination with the *Trichoderma harzianum* isolate T3 purified enzymes and H_20 (control) on the germination rate of encysted zoospores and growth of germ tubes of *Pythium* P11. Values on the Y-axis are the percentage of spores that are ungerminated (shown as 0 on the X-axis), or germinated with germ tube lengths of 1–30 μm, 31–50 μm, 51–100 μm, and more than 100 μm. (A) endo-1,3-β-glucanase: 60 μg ml^{-1}. (B) cellulase: 14 μg ml^{-1}. Error bars indicate standard deviations.

β-glucanase, resulting in a large number of ungerminated cysts. The protruding from the cyst is made up of only 1,3-β-glucan (Bartnicki-Garcia and Wang, 1983). The fungus would thus be more sensitive to the fungicide, which has its target site in the cytoplasm, after the hyphal tip had been attacked by enzyme.

Synergism between *T. harzianum* purified enzymes and fungicides or peptaibol antibiotics are also reported by Lorito et al. (1994) and Schirmböck et al. (1994) from their *in vitro* assays of germination of spores from plant pathogenic fungi containing chitin as major components of their cell wall structure. Schirmböck et al. (1994) showed that *T. harzianum* cell wall degrading enzymes and peptaibol antibiotics were co-produced in liquid culture, indicative of the likely importance of synergism between hydrolytic enzymes and toxic compounds *in situ*. Although the chitinase from *T. virens* had little biological activity (see above) a synergistic effect was also found by combining this enzyme with gliotoxin (Di Pietro et al., 1993). Despite the strong evidence of the importance of gliotoxin production in the biological control of *Pythium* spp. by *T. virens*, Jones and Hancock (1988) have argued that the effect of gliotoxin *in situ* is likely to be enhanced by the presence of enzymes that change the chemical composition and architecture of the cell wall.

Our results show that the activity of *Trichoderma* is high on the root surface of cucumber seedlings for at least four weeks. Conidia of *T. harzianum* germi-

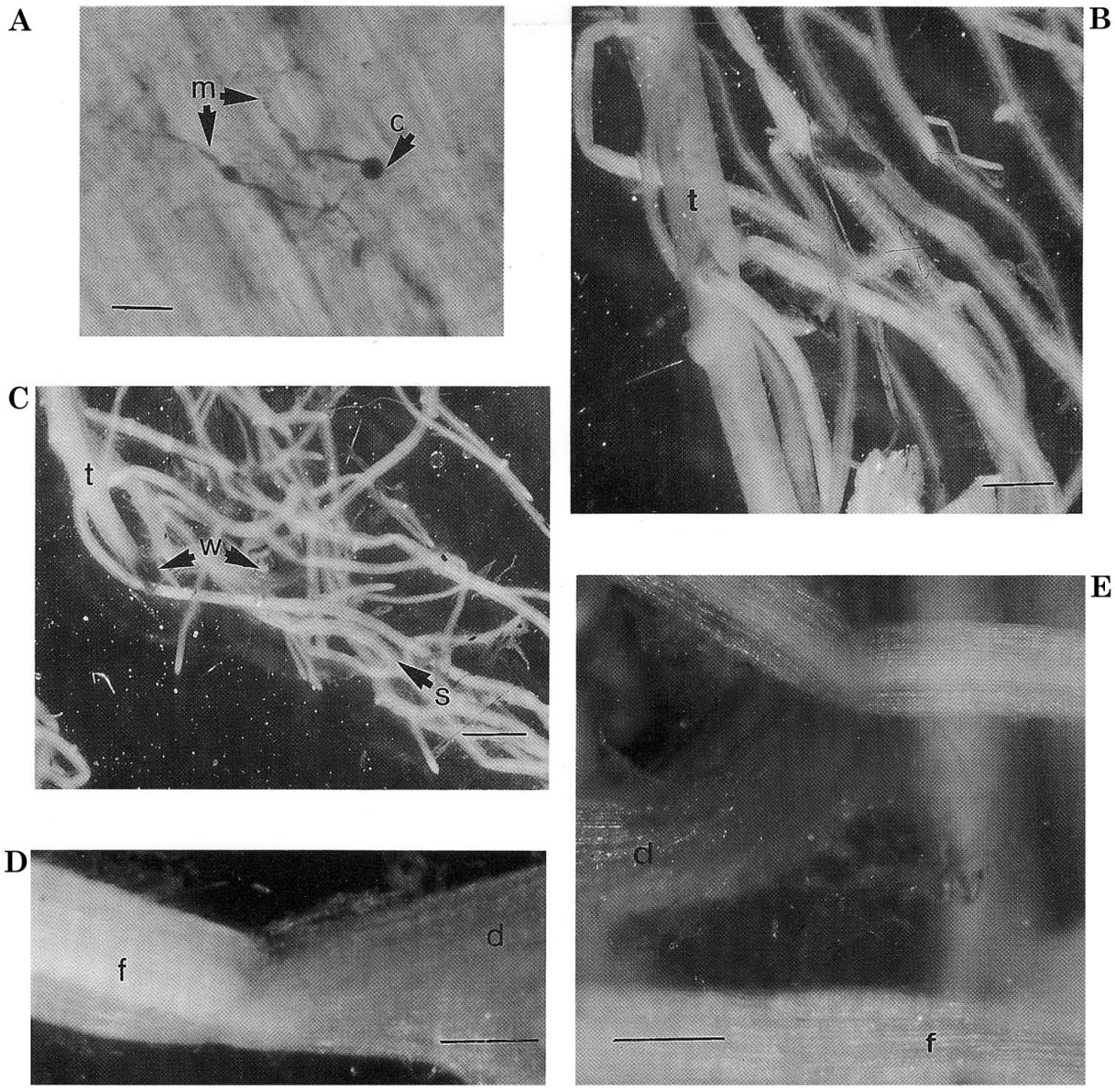

Figure 7. Colonization of cucumber seedlings by *Trichoderma harzianum* isolate T3a (GUS-transformant). The stained hyphae of the fungus can be distinquished from the plant tissue by its greenish-blue colour. (A) Germinating conidia on the tap root (7 days). c = conidium. m = mycelium. Light microscope. Bar = 10 μm. (B) Tap root and adventive roots colonized by actively growing *T. harzianum* isolate T3a (28 days). t = tap root. Stereo microscope. Bar = 5 mm. (C) The whole root system (28 days). Wounded areas are heavily colonized whereas other healthy areas only are colonized superficially by *T. harzianum* isolate T3a. s = superficially colonized areas of the root and rootlets. t = tap root. w = wounded areas of the root heavily colonized. Stereo microscope. Bar = 1 cm. (D) Heavy staining of the distal part of the wounded root and lighter staining of the healthy root (28 days). d = distal part of wounded root heavily colonized. f = healthy part of root superficially colonized. Stereo microscope. Bar = 2 mm. (E) Broken root. Strong staining indicative of heavy colonization on the distal part of the broken root (28 days). d = distal part of broken root. f = healthy part of root superficially colonized. Stereo microscope. Bar = 2 mm.

nate quickly in peat, however, in bulk soil the turnover number of *T. harzianum* cfu is very low after one week (Heiberg et al., 1996), and in the rhizosphere, the mycelia cease growing actively four days after conidia are added (Green and Jensen, 1995). Jeffries and Young (1994) suggested that spore dormancy in bulk soil can be broken by the addition of nutrients or by the presence of the pathogen. Our spore germination assay in fact showed that the presence of the pathogen did increase the germination rate of the *T. harzianum* conidia. Preliminary results from a pot experiment showed an increased number of *T. harzianum* cfu as well as increased extractable endo-1,3-β-glucanase activity when *Pythium* and *T. harzianum* isolate T3 were inoculated simultaneously (H. Green, N. Heiberg, C. Thrane, and D.F. Jensen, unpublished).

The shown data together with some preliminary results (C. Thrane, A. Tronsmo, D. F. Jensen, unpubl.) do not refute the findings of Ahmad and Baker (1987) who showed a positive correlation between the potential cellulolytic activity and the rhizosphere competence of *Trichoderma* isolates. High root colonization ability of *T. harzianum* isolates has been found to correlate with better prevention of root-rot and wilt pathogens (Sivan and Harman, 1991). It is likely that the benomyl-resistant mutant of *T. harzianum*, which has increased competitive saprophytic ability, cellulase production, and rhizosphere competence (Ahmad and Baker, 1987; Ahmad and Baker 1988), is a carbon catabolite repression mutant like the RUT-C30 mutant of *T. reesei* (Ilmén et al., 1995), and might be better able to establish on roots even in the presence of easy fermentable carbon sources as plant root exudates. However, a tight repression of the expression of cellulose degrading enzymes by the presence of easy fermentable carbon sources amongst saprophytic *Trichoderma* spp., might be one reason why these isolates, in general, not are pathogenic to plants.

Pythium spp. readily infect juvenile or succulent tissues (Howell, 1991) and mucilaginous layers of root hairs, root tips, and emerging lateral roots (Foster et al., 1983; Wester et al., 1991). In our study, the mucilage of these areas were colonized by *T. harzianum* isolate T3. Light and stereo microscope observations showed that isolate T3 does not grow in living plant cells (Green and Jensen, 1995). Colonization seemed to be restricted to the outer epidermal layers of the cortex on the healthy parts of the roots, whereas on the distal parts of wounded or broken roots colonization by *T. harzianum* isolate T3 was intensive. Plant defence mechanisms can be induced by the presence of antagonistic bacteria in intercellular spaces on plant roots as shown by Liu et al. (1995). This far, there has been no studies of this possible mechanism of antagonism by *T. harzianum* isolate T3.

Knowledge of specific mechanisms involved in antagonism can help us to optimize the effect of biocontrol agents.

Acknowledgements

We thank Linda Hjeljord for useful comments on the manuscript, and Karin Olesen and Marianne Krause Pedersen for very good technical assistance. This work was supported by the Danish Agricultural and Veterinary Research Council (SJVF), grant no 13-4591. Charlotte Thrane's work in Arne Tronsmo's laboratory at the Agricultural University of Norway, was financed by the Nordic Academy for Advanced Study (NorFa) Network on Transformation of Fungi.

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