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Overexpression of an Endochitinase Gene (*ThEn-42*) in *Trichoderma atroviride* for Increased Production of Antifungal Enzymes and Enhanced Antagonist Action Against Pathogenic Fungi

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Abstract *Trichoderma* is one of the most promising biocontrol agents against plant fungal diseases. In this study, a transgenic strain of *Trichoderma atroviride* was characterized. The transgenic strain contains an endochitinase gene (*ThEn-42*) driven by the cellulase promoter *cbh1* of *T. reesei* for overexpression of *ThEn-42*. The culture filtrates of the transformant and the parental strain grown in eight different media were evaluated for chitinase and antifungal enzyme production based on activity gels, protein profiles, and antifungal activities. Results demonstrated that chitinases are important components and synergistic interactions play a key role in the antagonistic action of *T. atroviride*. Moreover, altering medium nutrient concentration and composition led to enhanced production of antifungal enzymes, a potential strategy for mass production. Two of the culture filtrates contained almost pure endochitinase, and could be excellent commercial sources for this enzyme. Several culture filtrates were highly antifungal. Two filtrates were so effective in biocontrol of a fungal pathogen, *Penicillium digitatum*, that they not only inhibited spore germination but destroyed the spores completely when 20 μl of culture filtrate (corresponding to approx-

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imately 104 μg of total protein) was applied in a total volume of 150 μl (approximately 0.7 mg protein ml^{-1}).

Keywords Biocontrol · Chitinase · Gene expression · Synergistic interaction · *Trichoderma* spp

Abbreviations

PAGE	Polyacrylamide gel electrophoresis
SSC	Sodium chloride–sodium citrate
SDS	sodium dodecyl sulfate
PDB	potato dextrose broth

Introduction

Fungi in the genus *Trichoderma* are among the most promising biocontrol agents against plant-pathogenic fungi [1–4]. The mechanisms by which they control plant-pathogenic fungi include antibiosis, competition, and mycoparasitism [1, 5]. Parasitism by *Trichoderma* spp. is destructive, causing death of the host fungus [1].

The complex process of mycoparasitism requires production of enzymes that digest the fungal cell wall. *Trichoderma* spp. are efficient producers of cell-wall-degrading enzymes, including chitinases, cellulases, polysaccharide lyases, proteases, and lipases [3, 4, 6–8]. Purified proteins of the three classes of chitinolytic enzymes produced by *Trichoderma*, including *N*-acetyl-*β*-D-glucosaminidase (EC 3.2.1.30), chitobiosidase, and endochitinase (EC 3.2.1.14) [7, 9, 10] were strongly antifungal to different pathogenic fungi [4, 10, 11]. Mixtures of the chitinolytic enzymes exhibited synergistic effects in the inhibition of spore germination and hyphal elongation of plant pathogens [3, 4, 11–15].

The biocontrol activities of *Trichoderma* spp. can be enhanced through modifying the growth conditions for enhanced induction, or overexpressing the enzymes of interest by gene manipulation [16, 17]. Margolles-Clark et al. [18] demonstrated that the endochitinase gene (*ThEn-42*) from *Trichoderma. atroviride* can be overexpressed by about 20-fold in *Trichoderma reesei* with the *cbh1* promoter of *T. reesei*. Unfortunately, the endochitinase showed little antifungal activity. The lack of antifungal capability of endochitinase produced by this transgenic strain was attributed, in part, to posttranslational modification of the enzyme produced by *T. reesei* and to reduced synergistic interactions of this enzyme with other cell-wall-degrading enzymes from *T. atroviride* strain P1. Thus, attempts were made to overexpress this endochitinase gene in its parental strain. A fivefold increase in the production of this enzyme was observed when this gene was expressed in *T. atroviride* strain P1 with the *cbh1* promoter of *T. reesei* (strain VTT-D-95467) [19]. Characterization of strain 95467 would provide more information on the mechanisms involved in the antagonistic action of *Trichoderma* and on factors affecting this complex biological process. Stable antifungal enzymes may be useful in many agricultural systems, including both pre- and postharvest applications. Such strains may be useful in developing a strategy for mass production of antifungal enzymes for biocontrol purposes.

Therefore, the objectives of this study were: (1) to characterize strain 95467, a transformant derived from *T. atroviride* P1 with altered chitinase genes; (2) to examine responses of the transgenic and parental strains to different growth media in the induction and production of chitinolytic and other antifungal enzymes; and (3) to evaluate the

antifungal activities against *Penicillium digitatum* by culture filtrates of the transgenic and parental strains growing in eight different media.

Materials and Methods

Materials

Trichoderma atroviride Rifai strain P1 (ATCC 74058) was derived from a strain isolated from wood chips [20] and possesses biocontrol ability against *Botrytis cinerea* Pers:Fr, *Penicillium digitatum* (Pers:Fr.) Sacc., and other plant-pathogenic fungi in vivo [4, 11, 20].

Transgenic strain 95467 of *T. atroviride* Rifai strain P1 carries an endochitinase gene driven by a cellulase promoter *cbh1* of *T. reesei* for overexpression of the endochitinase gene *ThEn-42*. It was prepared as described by Margolles-Clark et al. [19].

Preparation of Culture Filtrates

The strains of interest were grown in eight different media developed in our laboratory in an effort to identify an enzyme mixture with high levels of antifungal activity. These media, designated A, B, C, D, E, F, G, and H, vary in mineral nutrients, trace elements, or carbon sources.

- Medium A contained 10 g of KNO_3, 5 g of KH_2PO_4, 1.3 g of $MgSO_4$, 20 mg of $FeCl_3$, 3.5 mg of $ZnSO_4.7H_2O$, 0.4 mg of $CuSO_4.5H_2O$, 0.31 mg of $MnSO_4$, 0.13 mg of $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.5 g sucrose, and 10 g crab shell chitin (Sigma) in 1 l of water.
- Medium B contained the same composition of mineral nutrients as in medium A, but 10 g of cellulose (Sigma) was used instead of chitin.
- Medium C contained 5 g of lactose, 1 g of glucose, 5 g of $(NH_4)_2SO_4$, 15 g of KH_2PO_4, 0.6 g of $MgSO_4$, 0.6 g of $CaCl_2$, 5 mg of $FeSO_2.7H_2O$, 1.6 mg of $MnSO_4$, 1.4 mg of $ZnSO_4.7H_2O$, 2 mg of $CoCl_2$, and 10 g crab shell chitin in 1 l of water.
- Medium D contained the same nutrients as in medium C, but 10 g of cellulose was used instead of 10 g of chitin.
- Medium E contained the same nutrients as in medium A, but 5 g of crab shell chitin and 5 g of cellulose were used instead of 10 g of chitin.
- Medium F contained the same nutrients as medium C, but 5 g of crab shell chitin and 5 g of cellulose was used instead of 10 g of chitin.
- Medium G contained 12.5 g of KH_2PO_4, 5 g of KNO_3, 2.5 g of $(NH_4)_2SO_4$, 1.6 g of $MgSO_4$, 0.3 g of $CaCl_2$, 20 mg of $FeCl_3$, 2.5 mg of $FeSO_4.7H_2O$, 2.5 mg of $ZnSO_4.7H_2O$, 0.2 mg of $CuSO_4.5H_2O$, 1 mg of $MnSO_4$, 0.06 mg of $(NH_4)_6Mo_7O_{24}.4H_2O$, 1 mg of $CoCl_2$, 2.5 g of lactose, 0.5 g of glucose, 0.25 g of sucrose, and 10 g of crab shell chitin in 1 l of water.
- Medium H contained the same nutrients as in medium G, but 10 g of cellulose was used instead of 10 g chitin in 1 l of water.

The media were adjusted to pH 6.0 and sterilized by autoclaving in Erlenmeyer flasks (100 ml of medium in a 250-ml flask). Trace elements, especially $FeCl_3$ and $FeSO_4$, were filter-sterilized separately and added immediately before use because some trace elements precipitated with time when all components in the media were mixed (data not shown). Crab shell chitin or cellulose was added separately into each flask before autoclaving. Each

flask was inoculated with a spore suspension that contained approximately 5×10^6 conidia ml^{-1}. The inoculated flasks were then placed on a rotary shaker at 150 rpm for incubation at 25°C for 5 to 6 days. The culture medium containing the enzymes of interest was separated from biomass by centrifugation at 6,000×*g* for 10 min. Residual particulates were removed by filtration through a glass fiber filter (type A/E, Gelman Sciences). The culture filtrates were then dialyzed against deionized water for 3 days at 4°C using 12,000 m.w. cutoff standard cellulose dialysis tubing (Spectra molecularporus membrane tubing, Spectrum Laboratories, Inc., USA). During dialysis, the deionized water was replaced with prechilled deionized water every 4 h. For the enzyme solutions with high cellulase activities, the dialysis tubes were changed daily to avoid leakage caused by the action of cellulase on the cellulose membrane. After dialysis, the enzyme solutions were filter-sterilized and kept at 4°C until use.

Enzyme Assays

Glucosaminidase and chitobiosidase were assayed using modified procedures described by Tronsmo and Harman [21], Ohtakara [22], and Roberts and Selitrennikoff [23]. Briefly, the release of *p*-nitrophenol from *p*-nitrophenol-*β*-D-*N*-acetylglucosaminide or from *p*-nitrophenol-*β*-D-*N,N′*-diacetylchitobiose (both from Sigma) was quantified by measuring the yellow color intensity at 410 nm in a microtiter plate reader. Activity of the enzymes was expressed as nanomoles of *p*-nitrophenol released per second per milliliter of culture filtrate. Endochitinase activity was measured by reduction in turbidity (absorbance reading at 510 nm) of a colloidal chitin suspension [21] after incubation of colloidal chitin and the enzyme solution (1:1 ratio) for 24 h at 30°C. Colloidal chitin suspensions contained 1% (*w*/*v*) purified colloidal chitin [24] in 100 mM of acetate buffer at pH 5.0 and were sterilized by autoclaving. Chitinase activity was calculated as the percentage reduction in turbidity relative to a similar suspension that contained water rather than enzyme solution. One enzyme unit was defined as the amount of enzyme required to reduce the turbidity of a chitin suspension by 5% [21].

PAGE, Chitinase Activity, and Protein Determination

Nondenaturing polyacrylamide gel electrophoresis (PAGE) was used to assess the relative amounts and banding patterns of the three chitinases. The gels contained 12% acrylamide (11.64% acrylamide, 0.36% bisacrylamide) and 25 mM of Tris (pH 8.8) in the resolving phase and 5% acrylamide (4.85% acrylamide, 0.15% bisacrylamide) and 25 mM of Tris (pH 6.8) in the stacking phase. The running buffer contained 25 mM Tris and 192 mM glycine, pH 8.5. Enzyme solutions were mixed 3:1 (vol/vol) with a loading dye (2 mg of xylene cyanol in 5 ml of 40% glycerol), loaded to the gel and electrophoresed for about 12 h at a constant 70 V. Gels were removed from the glass plates and washed twice with acetate buffer (100 mM, pH 5.0) for 15 min for each wash. Gels were then stained for chitinase activity by overlaying with 1% low melting (≤35°C gelation temperature) agarose that contained 1 mM of methylumbelliferyl substrate in 100 mM acetate buffer (pH 5.0). Substrates used were 4-methylumbelliferyl-*N*-acetyl-*β*-D-glucosaminide, 4-methylumbelliferyl-*N*-acetyl-*β*-D-*N,N′*-diacetylchitobioside, and 4-methylumbelliferyl-*N*-acetyl-*β*-D-*N,N′, N″*-triacetylchitotriose (Sigma) for *N*-acetyl-*β*-glucosaminidase, chitobiosidase, and endochitinase, respectively. The substrates were dissolved in acetate buffer (100 mM, pH 5.0) with 1% low melting agarose in a microwave oven and kept in a water bath at 37°C before application. The activity bands were observed in less than 10 min at room temperature

under UV light. After taking photo image of chitinase activity banding patterns, gels were rinsed in deionized water and stained for protein profiles by silver staining using procedures described by Blum et al. [25]. Protein concentrations in enzyme solutions were quantified using a BioRad protein assay kit (BioRad Laboratories, Hercules, CA, USA).

PCR Reaction

A double-stranded PCR product of the endochitinase gene *ThEn-42* was generated using genomic DNA of *T. atroviride* as a template and the primers NCO-S (5′-GTCGTACCA TGGTGGGCTTCCTCGGAAAATC-3′) and BEND (5′-GGGGGGATCCTCTAGTTGA-GACCGCTTC-3′). The glucosaminidase gene *nag1* was amplified using genomic DNA of *T. atroviride* as a template and the primers JNAG95F (5′-GCTCTAGACCGCCTCG-GTCGTCCATCAT-3′) and JNAG1989R (5′-CGGGATCCTTATGCGAACAAGGTG CAAGCCGTAGC-3′). Thermocycling parameters were 35 cycles consisting of 1 min denaturation at 94°C, 2 min primer annealing at 65°C, and 3 min DNA extension at 72°C.

Southern Blot Analysis

Five micrograms of genomic DNA from the transformant or its parental strain were separated in a 1% agarose gel and then transferred to a nylon membrane by capillary transfer in the presence of 10× sodium chloride–sodium citrate (SSC). After prewashing the membrane in a solution containing 5× SSC, 0.5% sodium dodecyl sulfate (SDS) and 1 mM EDTA for 30 min at 50°C with gentle agitation, the membrane was prehybridized with hybridization buffer that contained 0.5 M sodium phosphate, 2 mM ethylenediaminetetra-acetic acid (EDTA), 7% SDS, and 0.1% sodium pyrophosphate, pH 7.1 for 2 h at 65°C. The membrane was then hybridized overnight at 65°C with the *ThEn-42* or *nag1* probe in gentle agitation. Nonradioactive DIG-labeled probes were prepared by PCR using primers that are specific for *ThEn-42* or *nag1* as described above, and a PCR probe synthesis kit from Roche Molecular Biochemicals (Mannheim, Germany). Hybridization between the labeled probes and fungal DNA was detected by color development.

Antifungal Activity

Antifungal assays were conducted in microplate wells. Each well contained 50 μl of PDB (potato dextrose broth, Difco), 50 μl of a conidial suspension (10^5–10^6 conidia ml^{-1}) of the test pathogen *P. digitatum*, and 50 μl of an enzyme solution (culture filtrates prepared as described above). Sterile water was added in the place of enzyme solutions for the control, or to bring the enzyme solution to a final volume of 50 μl when less than 50 μl of enzyme solution was used for the assay. The microplates were incubated for 12–14 h at 25°C. Observations after incubation were taken directly from the microplate wells under a Nikon Diaphot brightfield microscope using a Pulnix TM-745 CCD video camera (Nikon Inc., Melville, NY, USA). The image analyzer used was an Image1/AT from Universal Imaging (Molecular Devices Corporation, Sunnyvale, CA, USA). The percentage of conidia germinated was determined based on a screen focused to the center of the well. Microscope slides were also prepared for observation at higher levels of magnification. Abnormal mycelial growth and morphological anomalies such as branching, bursting, appearance of necrotic zones, and lysis of the hyphal tips were recorded and photographed. Three treatment replicates were performed and each experiment was repeated independently on two separate days.

Results

Characterization of Chitinolytic Enzymes Produced by the Transgenic and Native Strains

Four major chitinase activity bands were observed on a nondenaturing PAGE gel (Fig. 1). Based on published studies [2, 6] and activity banding patterns shown with specific substrates (data not shown), bands closest to the dye front (higher electrophoretic mobility) were caused by activities of chitobiosidase. Bands of lowest electrophoretic mobility corresponded with *N*-acetyl-β-glucosaminidases. Proteins of 72 and 118 kDa have been reported for this enzyme [2, 21]. Bands of medium electrophoretic mobility corresponded to endochitinase activities (42 kDa) [26, 27].

The intensity and pattern of activity bands varied depending on the strain and the growth medium (Fig. 1). In media B and F, and to a certain extent, in medium E, strong endochitinase activity was shown for strain 95467, but little was detected for the wild-type P1. In other tested media, endochitinase activity banding patterns were similar for the two

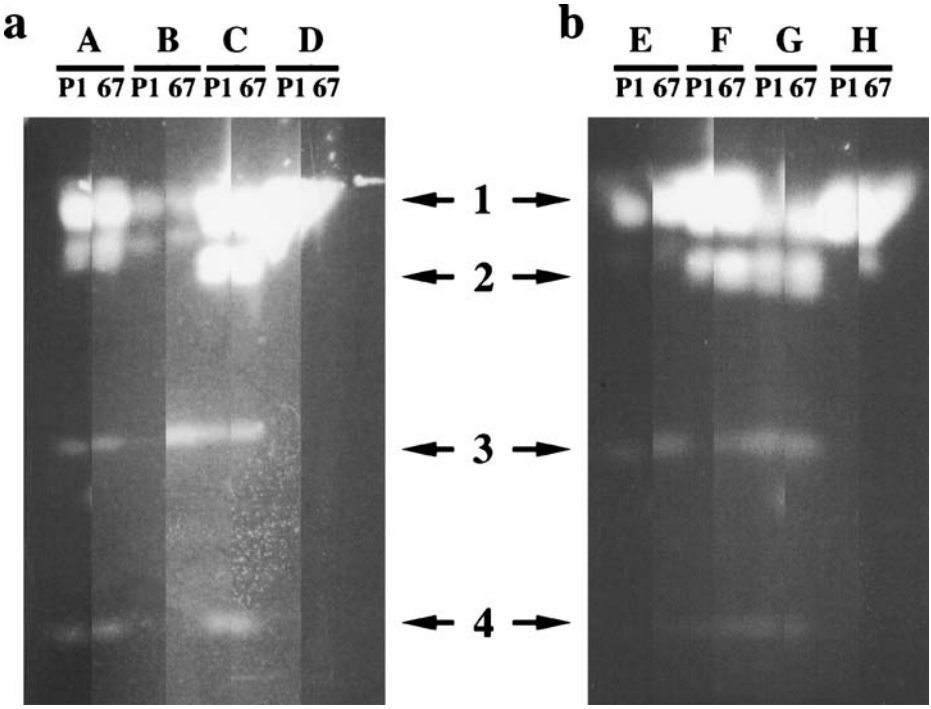

Fig. 1 Comparison of chitinase activity banding patterns of culture filtrates obtained from strains P1 and 95467 grown in eight different media compositions, including media A, B, C, D, E, F, G, and H. As discussed in the "Materials and Methods" section, polyacrylamide gels (containing 12% acrylamide) were removed from the glass plates after electrophoresis, washed with acetate buffer (100 mM, pH 5.0) twice for 15 min each time, and stained for chitinase activities using 1 mM of methylumbelliferyl substrates that were dissolved in 100 mM acetate buffer (pH 5.0) containing 1% low-melting agarose. The activity bands were observed in less than 10 min at room temperature under UV light. The bands designated by 1 correspond to the large molecular *N*-acetylglucosaminidase, the bands designated by 2 correspond to the 70 kDa *N*-acetylglucosaminidase, whereas bands at position 3 correspond to endochitinase and at position 4 to chitiobiosidase

strains. However, the levels of expression of specific enzymes were media-dependent. There were considerable differences in protein compositions between strains 95467 and P1. In media B and E, numerous protein bands were shown for the wild-type P1, but a relatively strong and almost pure protein band at the location of endochitinase was shown for strain 95467 (Fig. 2). Of the media tested, only medium G generated relatively strong protein bands at all four locations corresponding to the three classes of chitinase for both tested strains. Chitinases were the predominant proteins in the enzyme solutions produced by both P1 and 95467 grown in medium G. Although both tested strains produced relatively high activities of all four chitinases in medium C (Fig. 1), there were only two predominant proteins in each of these two enzyme solutions (Fig. 2).

Genetic Characterization of the Transgenic Strains

The differences in banding patterns of chitinase activity and protein staining between the transgenic strain and the wild-type P1 could be caused by mutation of the genes encoding for endochitinase (*ThEn-42*) or to alteration of its regulatory genes. PCR amplification of the entire *ThEn-42* gene in the transgenic and parental strains was performed using primers designed specifically for this gene based on sequence information provided by Hayes et al. [8]. A band of the expected size of *ThEn-42* was obtained from strain 95467 (data not shown).

Further information on the nature of the changes induced by the transformations of this strain was deduced by Southern analysis using a probe prepared with the *ThEn-42* gene.

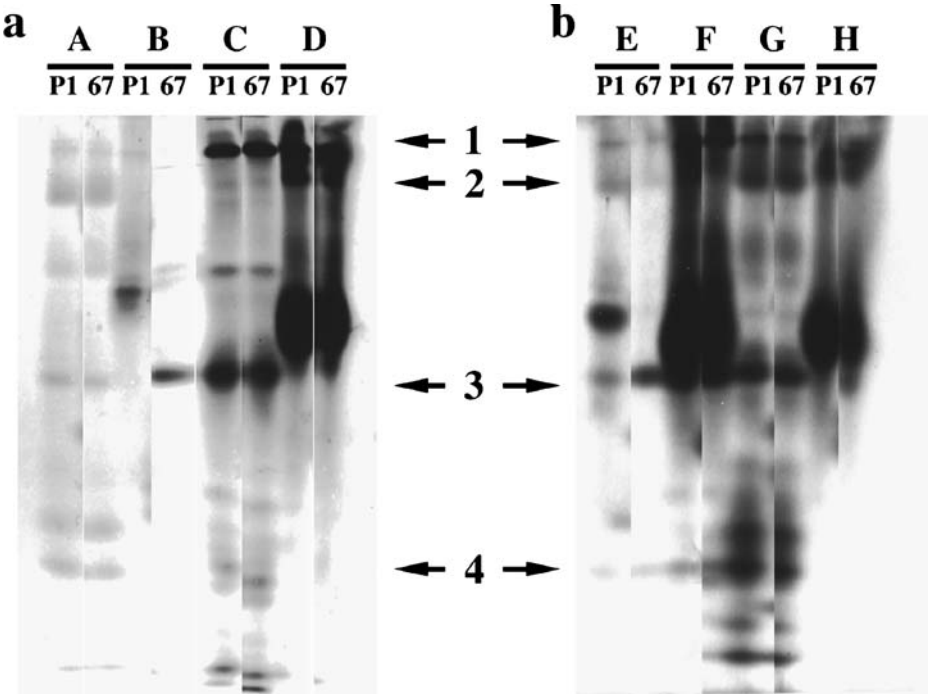

Fig. 2 Silver staining of gels shown in Fig. 1 to reveal protein profiles of culture filtrates. The lane assignments and band numbers are the same as described for Fig. 1

Previous studies suggested that *Eco*RV does not digest *ThEn-42* [8]. Hybridization with the probe that was specific for *ThEn-42* gave an expected single band in strain P1, but yielded numerous additional bands larger than the expected size of *ThEn-42* in strain 95467 (Fig. 3a). After restriction with *Hin*dIII, which cuts *ThEn-42* once, strain P1 showed the expected two bands, whereas strain 95467 gave these two bands and numerous other bands of various sizes (Fig. 3a).

Southern analyses also were performed using a probe prepared for the glucosaminidase *nag1* gene. After digestion with *Eco*RV, which cuts this gene once, strains P1 and 95467 both gave the expected two bands (Fig. 3b). After restriction with *Hin*dIII, which does not cut the native *nag1* in strain P1 [28], a single band was seen in strain 95467 (Fig. 3b). These results suggest that *nag1* in the transgenic strain is intact and the intended transformation was successful.

Antifungal Activities of Culture Filtrate Against *Penicillium digitatum*

Sixteen different culture filtrates were obtained by growing the two tested strains in eight different media. The protein concentrations in these culture filtrates ranged from 1.0 to 7.3 mg ml^{-1} (Table 1). Previous research findings [3] suggested that the antifungal activity detected from a specific culture filtrate came from a combined action of chitinases and other cell-wall-degrading enzymes and these enzymes acted synergistically. It is, therefore, not

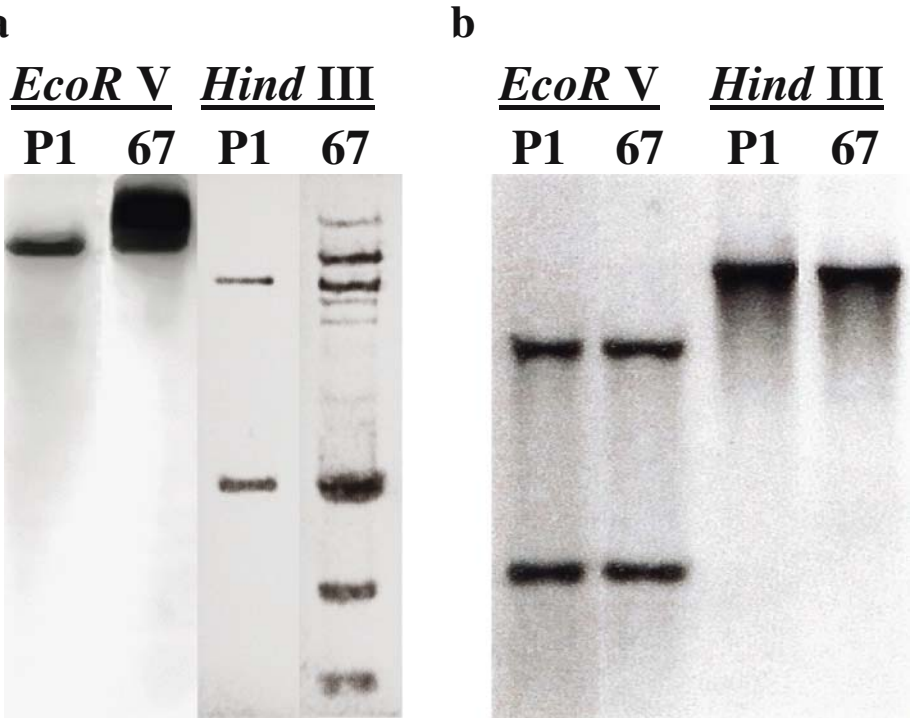

Fig. 3 Southern blot analysis. The genomic DNA of strains P1 or 95467 was digested with *Eco*RV or *Hin*dIII overnight at 37°C. Five micrograms of the digested DNA was separated on a 1% agarose gel. (a) Probed with the endochitinase gene *ThEn-42*. (b) Probed with the *N*-acetyl-β-D-glucosaminidase gene *nag1*

Table 1 Antifungal activities of different culture filtrates against *Penicillium digitatum.*

Growth medium	Strain	Protein concentration[a] (mg ml^{-1})	Percent germination[b] (%)
A	P1	1.6	0
	67	1.6	0
B	P1	2.2	0
	67	1.0	0
C	P1	2.8	95–100
	67	2.3	95–100
D	P1	3.4	95–100
	67	30.	95–100
E	P1	2.4	1–3
	67	2.3	1–3
F	P1	7.3	1–5
	67	7.2	5–10
G	P1	5.2	0 (no spores)
	67	5.8	0 (no spores)
H	P1	5.5	30–40
67	67	5.8	25–35

[a] Protein concentration in dialyzed culture filtrates

[b] Antifungal effect as indicated by reduction in *Penicillium digitatum* spore germination when 50 μl of an enzyme solution (culture filtrates prepared as described in the text) was assayed in a medium containing 50 μl of PDB (potato dextrose broth, Difco) and 50 μl of a *P. digitatum* conidial suspension.

appropriate to quantify antifungal action using chitinase activity alone or using protein concentration without defining the protein composition of the solution. Thus, the antifungal assays were performed using the same amount of culture filtrates regardless of their chitinase activities or protein concentrations. Results from these assays would provide information on biocontrol capabilities of antifungal enzymes produced from different strains under the inoculation and growth conditions specified. When 50 μl of culture filtrates (corresponding to 50 to 360 μg total protein) were used for the antifungal assay, P1 and 95467 grown in three out of the eight media, including media A, B, and G, showed 100% inhibition of *P. digitatum* spore germination (Table 1). In particular, culture filtrates in medium G resulted not only in inhibition of spore germination but in total destruction of the spores. Antifungal activities were also shown in culture filtrates of P1 and 95467 grown in media E, F, and H. However, these enzyme mixtures were less effective in inhibiting germination of *P. digitatum* spores when compared with those from medium G, although total protein concentrations in these culture filtrates were similar. No detectable antifungal activities were observed from culture filtrates obtained by growing P1 or 95467 in media C or D.

Investigations were pursued to demonstrate the antifungal activities at different levels of protein concentration. The wild-type P1 was of particular interest for this test because of the potential benefit in field application. Strong antifungal activity was detected in culture filtrates from P1 grown in medium G. Antifungal activity increased with increasing amounts of the culture filtrates added (Fig. 4). Compared to the control, aberrant spider-like germination and spore fusion caused by weakened cell walls were evident. Spore germination was reduced to approximately 25% or less when 5 μl of this enzyme mixture (corresponding to approximately 26 μg of protein) was applied in a final volume of 150 μl (Fig. 4a, b). As shown in Fig. 4c, 100% inhibition in spore germination was observed when

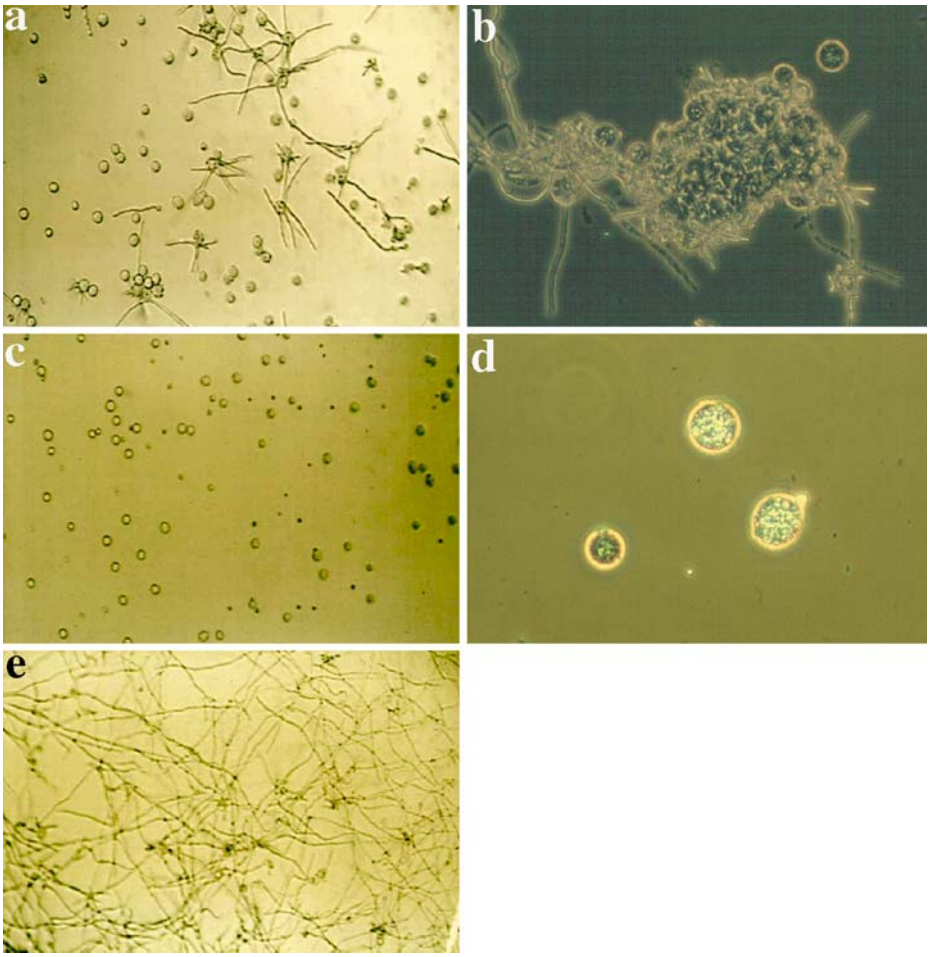

Fig. 4 Microscopic photographs showing the antagonistic effects of enzymes on germination of *P. digitatum* spores. The swelling, weakening, and spider-like germination of the spores are shown. Culture filtrates obtained from P1 grown in medium G were used to generate these photographs. Each large spore structure in Fig. 5a–d is 5–10 μm in diameter. (a) Five microliters of culture filtrate was added to 45 μl of sterile water, 50 μl of PDB (potato dextrose broth, Difco), and 50 μl of *P. digitatum* spore solution (mixture 1). (b) Higher magnification using germination mixture 1 showing fusion of the spores and lysing of cell walls. (c) Ten microliters of culture filtrate was added to 40 μl of sterile water, 50 μl of PDB, and 50 μl of *P. digitatum* spore solution (mixture 2). (d) Higher magnification using mixture 2 showing swelling and weakening of cell walls which appear ready to burst. (e) Control; the germination mixture contained 50 μl of sterile water, 50 μl of PDB, and 50 μl of *P. digitatum* spore solution

10 μl of enzyme solution was added. In this germination mixture, spores were swollen, the cell walls were weakened, and the cells appeared ready to burst (Fig. 4d). Spores of *P. digitatum* were completely destroyed when 20 μl of this culture filtrate was applied.

Inhibition of spore germination caused by the enzyme mixtures could be partially reversed by the addition of NaCl (Fig. 5). Although 100% inhibition of spore germination was observed in dialyzed culture filtrates of P1 or 95467 grown in medium B, the percentage of spore germination increased from 0 to 90 % with increasing concentration of

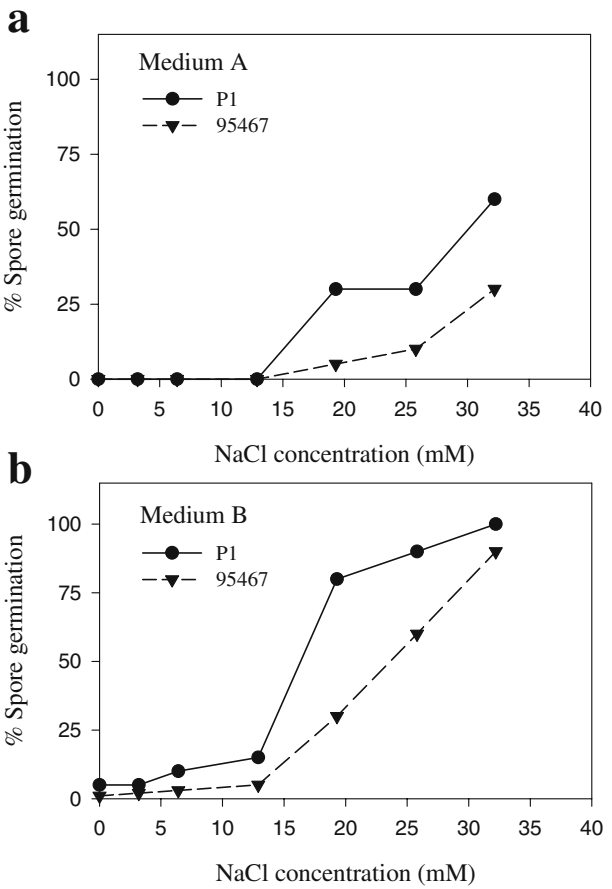

Fig. 5 Effect of NaCl concentration on antifungal activities of culture filtrates obtained from strains P1 or 95467 grown in medium A (a) or medium B (b). The antifungal activities of culture filtrates were tested against spore germination of *Penicillium digitatum*. Assays were conducted in microplate wells. Each well contained 50 μl of PDB (potato dextrose broth, Difco), 50 μl of a conidial suspension (10^5–10^6 conidia ml^{-1}) of the test fungus *P. digitatum*, and 50 μl of an enzyme solution (culture filtrates prepared as described in the text)

NaCl from 0 to 30 mM (Fig. 5b). However, the germ tubes that resulted from addition of NaCl were abnormal. In general, they were short, curly and stunted, and were morphologically similar to those affected by low dose applications of enzyme mixtures (data not shown).

Discussion

Southern analyses indicated that strain 95467 contains the native endochitinase gene as well as multiple copies of the introduced endochitinase gene that were under the control of the *cbh1* promoter as expected [19]. Therefore, the transgenic and parental strains differed in their reactions to different media in chitinase and protein production. However, both strains produced multiple proteins showing *N*-acetylglucosaminidase activity; supporting findings that *T. atroviride* produces as many as five separate *N*-acetylglucosaminidases [29]. These enzymes play crucial roles in the antagonist action against plant pathogens by *T. atroviride* [3], and many of them are bound to the cell walls of *T. atroviride* [28].

Enzyme production by the evaluated strains was highly responsive to growth medium composition. All the media contained complex carbohydrates (chitin or cellulose)

supplemented with various compositions and concentrations of trace elements. In medium B, and to a limited extent medium E, endochitinase was the predominant protein produced. Because these two enzyme solutions were also highly antifungal against *P. digitatum*, this supports the previous findings that endochitinase is a crucial component of the antagonist action against plant-pathogenic fungi by the genus *Trichoderma* [1–4]. The relatively strong activities of chitinases, but low antifungal activities against *P. digitatum* in enzyme solutions generated by the two tested strains in medium C suggested that synergistic interactions played a crucial role in the biological control of plant pathogens by *T. atrovidide* [17]. The synergistic interactions in the antagonist action were further evidenced by the fact that the strongest antifungal activity was shown in enzyme solutions that were composed of multiple proteins, such as those obtained by growing P1 or 95467 in medium E.

The different enzyme and protein compositions were obtained by altering carbon sources and trace elements, which often serve as inducers of enzyme production by living organisms. The difference between media A and B was that medium A contained chitin rather than cellulose as the carbon source. Chitin gave rise to diverse proteins for strain 95467. Chitin is known to be an inducer of numerous proteins in *Trichoderma* that are associated with mycoparasitism [16]. Cellulose apparently is an efficient elicitor of endochitinase, leading to production of almost pure endochitinase by the transgenic strain in medium B. Nevertheless, enzyme solutions produced by both strains growing in either medium A or B were highly antifungal against spore germination of *P. digitatum*. Protein composition of a solution played a key role in the antagonist action of an enzyme solution. The mere presence or absence of inductive complex carbohydrates was not the only determinant of the protein profiles in these fungi. Trace elements also played a crucial role in determining protein and enzyme productions by these fungi. Little antifungal activities were detected in the enzyme solutions generated by growing P1 and 95467 in medium C or D. Yet media C and D differed from media A and B in trace element composition and concentrations. When trace element composition in the eight tested media were compared, Cu and Mo were present in media A, B, E, G, and H, but absent from media C, D, and F. With the exceptions of media H and F, enzyme solutions generated in the presence of Cu and Mo were highly antifungal, whereas little antifungal activities were detected when Cu and Mo were absent in the growth media. These results suggested that Cu and Mo were critical in the production of highly antifungal solutions by *T. atroviride*. Although these two elements were present in medium H, antifungal activity in enzyme solutions generated by growing the two tested strains in this medium was markedly lower than media A, B, E, or G, suggesting the complex nature of synergistic interactions in the mode of antagonist action by *T. atroviride*.

The toxicity of the enzymes to *P. digitatum* spores was partially reversed by the addition of NaCl, in part, because of stabilization of protoplasts (see Fig. 4) by increased osmotic potential of the enzyme reaction mixture. Salts, sugars, and other materials have been demonstrated to stabilize protoplasts and to prevent damage caused by osmotic stress [31]. However, addition of cell-membrane-active compounds that have synergistic interactions with cell-wall-degrading enzymes [14] will destabilize membrane-bound structures as shown in Fig. 4.

Findings from this study indicated that the transgenic strain 95467 is useful in producing relatively pure endochitinase that is strongly antifungal. However, efficient large-scale production of strongly antifungal enzyme preparations is possible using either the transgenic or parental *T. atroviride* strain P1. The strong antifungal activity of relatively pure endochitinase solution further supports reports that endochitinase is one of the active

antifungal enzymes as suggested in earlier studies [32]. However, the medium used to produce endochitinase has a profound influence on the protein profile in the enzyme solution. Response surface modeling procedures [33] and medium optimization studies are likely to enhance endochitinase production and provide commercially useful methods to produce this enzyme.

The obtained results indicated that the native strain P1 could also be used to produce strongly antifungal enzymes, providing that medium composition is optimized. One potential advantage of using P1 for the preparation is that multiple enzymes are produced, which promotes synergistic interactions between chitinolytic enzymes [32]. In addition, field application of the native strain P1 is also more desirable in the effort to minimize environmental and ecosystem impacts by genetically modified organisms. Results from this study are in strong contrast to our earlier studies where only low levels of antifungal enzymes were obtained and a lengthy purification process was required to observe antifungal activity [3, 4, 27]. To this point, production protocols have been limited as only microassays of antifungal activity of these enzymes have been possible. However, we now have the means to conduct meaningful in situ assays of various potential applications of these enzymes. The regulation of cell-wall-degrading enzymes in *Trichoderma* is complex and governed by several factors, including carbon and nitrogen nutrition and pH [29].

Induction of enzymes in both native and the transgenic strain occurs with complex carbohydrates, and is strongly influenced by medium trace element composition, suggesting that there are other regulatory factors controlling the gene expression system. This study demonstrated the potential for large-scale production of endochitinase and antifungal enzyme mixtures from *T. atroviride*.

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