

Full Length Research Paper

Cloning and semi-quantitative expression of endochitinase (*ech42*) gene from *Trichoderma* spp.

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Accepted 8 May, 2012

Species of *Trichoderma* such as *T. harzianum*, *T. viride* and *T. atroviride* are some of the most potent antagonistic fungi, and have been used as plant growth promoters in developed countries. Endochitinase (*ech42*) gene which is involved in mycoparasitism, was isolated from *Trichoderma* spp. taken from hot-arid soils of Rajasthan, cloned, sequenced and its expression profiling was carried by reverse transcription-polymerase chain reaction (RT-PCR) technique. The cloned DNA sequence was 1,476 base pairs. Gene encoding endochitinase was ligated in pGEMT cloning vector. The plasmids were transformed in DH5 α *Escherichia coli* competent cells and clones were confirmed through sequencing and restriction analysis. Endochitinase gene expression was then observed for different *Trichoderma* isolates viz., *T. harzianum* (T14 and T12) and *T. atroviride* (T5). Among the three, higher expression of endochitinase was observed in T14 and T12, whereas T5 showed lesser expression with respect to T14 and T12 strain. The *Trichoderma* chitinase enzyme activity was monitored for all isolates under study. The highest chitinase activity was observed in T14 and T11 viz., 17.21 (1 enzyme μ g/ml) and 13.11 enzyme μ g/ml, respectively.

Key words: Endochitinase, cloning, expression, *Trichoderma atroviride*, *Trichoderma harzianum*, *Trichoderma viride*.

INTRODUCTION

Trichoderma species are potentially used as biocontrol agents against soil-inhabiting plant pathogenic fungi. Species of *Trichoderma* have been shown to excrete hydrolytic enzymes such as chitinases, β -glucanases and proteinases (Cruz et al., 1992). These chitinase enzymes induce interactions between *Trichoderma* spp., and cell wall materials of phytopathogenic fungi. The extracellular chitinolytic activity of the *Trichoderma* species has been suggested to be one of the major factors involved in their mycoparasitism (Elad et al., 1982). Chitinolytic enzymes secreted by *Trichoderma* spp. have received much attention because of their lytic and inhibitory activity towards many phytopathogenic fungi (Baek et al., 1999). Various chitinases have been purified and characterized

from several isolates of *T. harzianum* (Cruz et al., 1992; Harman et al., 1993; Schickler et al., 1998). Chitinase-encoding genes from *Trichoderma* are being used to improve plant defense against fungal pathogens. In recent years, fungal endochitinase have attracted world-wide scientific interests due to their great potential in industrial application in the preparation of chito-oligo-saccharide (Shaikh and Deshpande, 1993). However, the yield of this compound is low as per the requirement in industrial application and so there is a need for the usage of recombinant DNA technology.

Keeping this in view, the present study was aimed to isolate *Trichoderma* spp. from different soil samples of Rajasthan and Delhi, screen them for chitinolytic activity and then clone and characterize one of the endochitinase genes (*ech42*) from different *Trichoderma* spp. to check the varying levels of these genes based on pathogenic behavior. Secondary protein structure of the cloned endochitinase gene was predicted and the expression

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Table 1. *Trichoderma* spp. isolates with their place of origin.

Isolate code	Organism	Place of origin	District	State
T1	<i>Trichoderma atroviride</i>	Takarada	Jaipur	Rajasthan
T2	<i>Trichoderma atroviride</i>	Shauli	Kota	Rajasthan
T3	<i>Trichoderma atroviride</i>	Kasampura	Kota	Rajasthan
T4	<i>Trichoderma viride</i>	Vijaysinghpura	Jaipur	Rajasthan
T5	<i>Trichoderma atroviride</i>	Sultanpura	Jaipur	Rajasthan
T6	<i>Trichoderma atroviride</i>	Singodh	Jaipur	Rajasthan
T7	<i>Trichoderma viride</i>	Khejroli	Jaipur	Rajasthan
T8	<i>Trichoderma harzianum</i>	Digodh	Kota	Rajasthan
T9	<i>Trichoderma viride</i>	Devpura	Kota	Rajasthan
T10	<i>Trichoderma viride</i>	Dagara	Kota	Rajasthan
T11	<i>Trichoderma harzianum</i>	Hanutia	Jaipur	Rajasthan
T12	<i>Trichoderma harzianum</i>	Samode	Jaipur	Rajasthan
T13	<i>Trichoderma harzianum</i>	Chaksu	Jaipur	Rajasthan
T14	<i>Trichoderma harzianum</i>	Biocontrol Lab, IARI	New Delhi	New Delhi
T15	<i>Trichoderma viride</i>	Biocontrol Lab, IARI	New Delhi	New Delhi

profiling of the endochitinase gene was checked in three representatives *Trichoderma* isolates viz., *T. harzianum*, *T. atroviride* and *T. viride* in order to check the level of expression in these three different strains of *Trichoderma* spp.

MATERIALS AND METHODS

Collection of isolates

15 different *Trichoderma* isolates (Table 1) were collected from rhizospheric soil of different vegetable crops throughout Jaipur and Kota districts of Rajasthan having different types of soils, vegetation and irrigation system. In Jaipur district, the fertile soil sustains mixed xerophytic and mesophytic vegetation, while Kota has black, fertile and fully irrigated soil. After serial dilution, plating of the collected soil samples were done on potato dextrose agar (PDA, Difco, USA) and *Trichoderma* specific medium (TSM) plates. Distinct *Trichoderma* colonies that appeared on the plates were selected, purified by single spore technique and maintained on PDA slants at 4°C and morphologically identified.

Chitinase activity of the selected *Trichoderma* spp.

The selected *Trichoderma* isolates were cultured at 28°C on a synthetic medium (SM) (Okon et al., 1973) containing (g per liter of distilled water): glucose, 15; MgSO₄·7H₂O, 0.2; KH₂PO₄, 0.9; KCl, 0.2; NH₄NO₃, 1.0; Fe²⁺, 0.002; Zn²⁺, 0.002; agar, 20 (in solid medium). Flasks containing 50 ml of liquid SM medium were inoculated with (5 mm) of mycelia discs cut from the actively growing cultures of the selected *Trichoderma* isolates based on biocontrol activity. Cultures were incubated at 28°C in a rotary shaker at 120 rpm for four days, and then centrifuged at 15,000 × g at 4°C for 10 min. The supernatant was assayed for chitinase activity.

Chitinase assay

The culture filtrates of the selected 15 *Trichoderma* isolates grown

on colloidal chitin amended broth for seven days were used for the assay of endochitinase activity. The assay mixture contained 0.5 ml of 0.2% colloidal chitin, 0.5 ml of enzyme solution (culture filtrate) and 0.5 ml of 0.1 M citrate buffer (pH 5.1). The reaction mixture was incubated for 4 to 6 h at 37°C in a water bath, and the reaction was stopped by centrifugation (5000 rpm for 10 min). Subsequently, 0.5 ml of the reaction mixture was taken, to which 0.1 ml of 0.2 M potassium tetra borate buffer (pH9.2) was added, followed by boiling in a water bath for 3 min. The reaction mixture was then cooled and 5 ml of p-dimethylaminebenzaldehyde (DMAB) solution was added to it. This was incubated in a water bath at 37°C, for 20 min and cooled. The absorbance was measured spectrophotometrically and recorded at 585 nm (Anand and Jayarama, 2009).

Characterization of the endochitinase gene from different *Trichoderma* spp.

Fungal DNA extraction

Fungal cultures were grown at room temperature in PDB for 2 to 4 days. Hyphae were harvested on cheesecloth in a Buchner funnel and washed with 25mM Ethylene Diamine Tetra Acetic acid (EDTA) followed by distilled water. The samples were frozen in liquid nitrogen and lyophilized. The extraction was based on the Cetyl Trimethyl Ammonium Bromide (CTAB) mini extraction method with slight modification DNA extraction employing CTAB (Voigt et al., 1999). The frozen Pestle using liquid nitrogen. The finely ground mycelium was transferred to sterile centrifuge tube and pre-warmed at 65°C. Afterward, DNA extraction buffer [100 mM Tris HCl (pH 8.0), 1.4 M NaCl, 50 mM EDTA (pH 8.0) and 2% CTAB] was added to the mixture, mixed well and incubated in a water bath at 65°C with gentle shaking for 1 h. After incubation, an equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added gently to denature proteins and centrifuged at 10000 rpm at 25°C for 10 min. The aqueous phase was transferred to a new sterile tube and DNA was precipitated with 0.6 volume of cold isopropanol and 0.1 volume of 3 M sodium acetate (pH 5.2) at 15000 rpm for 25 min at 25°C. The supernatant was poured off and the pellet was washed twice with cold 70% ethanol, air-dried at room temperature and suspended in TE buffer [10 mM Tris HCl (pH 8.0), 1 mM EDTA (pH 8.0)].

DNA purification

All samples were treated with RNase A (20 mg/ml) at a concentration of 40 ng/ml of DNA and were incubated for 1 h at 37°C. This was followed by proteinase K (10 mg/ml) treatment for 60 min at 37°C. Equal volume of saturated phenol: chloroform: isoamyl alcohol (25:24:1) was added to the DNA solution and mixed by swirling for 5 min. After centrifuging at 10,000 rpm for 5 min, supernatant was collected in a fresh tube. This was followed by extractions with chloroform: isoamyl alcohol (24:1). The purified DNA was precipitated by adding 1/10 volume of 3 M sodium acetate (pH 5.6) 2.5 times (v/v) along chilled ethanol (95%). Extra salts were removed by further washing with 70% ethanol and DNA was pelleted and dried under vacuum. The quality and quantity of DNA was analyzed both spectrophotometrically and in 1% agarose gel. The pellet was dissolved in a minimum volume of TE (10:1) buffer at room temperature and stored at -20°C.

Amplification of endochitinase gene by polymerase chain reaction

The endochitinase gene were amplified from the DNA samples of the *Trichoderma* isolates under study using the primer set F: 5'-ACG CAA ACG CCG TCT ACT TCA CCA A-3' and R: 5'-GCA TCC CAG AAC ATG CTG CCT CCC A-3' (Baek et al., 1999). Full length chitinase gene from fungus *Trichoderma* spp. was amplified using Bioer GenePro thermal cycler. Amplification reactions were carried out in a total volume of 15 µL containing 20 ng per ml of template DNA. The PCR amplification mixture consisted approximately 100 ng of genomic DNA, 20 pmol of each primer, 2.0 mM of MgCl₂, 0.225 mM of each deoxynucleotide triphosphate (dNTP), 1 U *Taq* DNA polymerase (Geneaid) and 10X PCR buffer. The amplification process was accomplished in thermal cycler (Bioneer MyGenie 32 Thermal Block, Global Genomics Partner). The PCR cycle was as follows: 1 min at 95°C, 30 s at 57°C, and 2 min at 72°C for 35 rounds. The extension period was 10 min at 72°C. Aliquots (4 µL) of the amplified products were analyzed by electrophoresis in 1.3% (wt/vol) agarose gel in 1X TAE buffer [40 mM Tris, 20 mM acetic acid, 1 mM EDTA (pH8)], stained with ethidium bromide (1 µg/ml) and electrophoresis was carried out at 60 V for 3 h in TAE buffer. The molecular marker was 1 Kb and 100 bp ladder (MBI, Fermentas). The gel was observed on a UV trans-illuminator and photographed on gel documentation instrument.

Gel elution of amplified PCR product

The desired bands were cut from the gel with minimum quantity of gel portion using clean sterile scalpel blade. The excised gel piece was weighed in an Eppendorf tube and 3 volume of buffer QIAGEN gel extraction kit (QG) was added, followed by incubation at 50°C for 10 min with intermittent mixing. The gel slice dissolved completely, resulting in yellow colour of the mixture. One gel volume of isopropanol was added to sample and mixed well, and then 800 µl of mixture was added to QIA quick spin column placed on 2 ml collection tube followed by centrifugation for 1 min. The flow-through was discarded and 0.5 ml of buffer QG was added to QIA quick spin column and centrifuged again for 1 min. The QIA quick column was washed by addition of 0.75 ml of buffer PE and incubated for 2 to 5 min, followed by centrifugation for another 1 min to remove any remaining residual buffers. The QIA quick was placed on a clean 1.5 ml Eppendorf tube and 30 µl of buffer EB (10 mM Tris-HCl, pH 8.5) was added to the centre of the column for elution. After centrifugation, eluted DNA was stored at -20°C for further use.

Transformation of the endochitinase gene

The purified PCR products were cloned into the vector pGEMT easy (Promega, Madison, WI, USA) according to the manufacturer's instruction and used to transform competent *Escherichia coli* cells strain DH5α. To ensure that the DNA insert carried by selected bacterial clones corresponded to the putative correct endochitinase gene, screening of blue-white colonies were done on Luria broth (LB) agar plate.

Plasmid isolation

The endochitinase gene insert present within the transformed white colonies were inoculated into 10 ml LB with ampicillin (100 µg/ml) and incubated over night at 37°C under shaking condition at 200 rpm. Subsequently, 1.5 ml of the incubated bacterial culture was centrifuged and pellets were collected and the plasmid DNA was isolated using Qiagen plasmid DNA isolation kit. A comparative restriction digestion analysis was performed for the confirmation of the cloned products by checking the size of the gene insert using the restriction enzyme *EcoRI*. The reaction mixture for restriction digestion consists of 2 µL of 10X *EcoRI* buffer, 3 µg of plasmid DNA, 1 U *EcoRI* enzyme and 14 µL distilled water. Incubation of the reaction mixture was done at 37°C for 3 h, after which 5 µL of the digested product were checked by electrophoresis using 1.3% agarose gel.

Sequencing of cloned endochitinase gene and *in silico* analysis

Cloned endochitinase gene inserts in the pGEMT vector were sequenced using M13 primers by employing primer walking technique at the DNA sequencing facility at Chromos biotech Pvt. Ltd., Bangalore, India. Removal of vector sequences and restriction analysis were done using GENE TOOL and VecScreen service of NCBI. Homology search was done using BLAST search available at <http://www.ncbi.nlm.nih.gov> (Altschul et al., 1997). *In silico* translation was done using NCBI BLAST by selecting the CDS feature and pair wise alignment in BLAST option. Multiple alignments for homology search were performed using the Clustal W algorithm in BioEdit (Hall, 1999) and phylogenetic analysis was done using MEGA 5 software. The endochitinase sequences were submitted to GenBank. Sequences used for comparison were obtained from NCBI database (<http://www.ncbi.nlm.nih.gov>).

Gene expression analysis

Fungal RNA extraction

For endochitinase gene expression studies, three representative isolates from *Trichoderma* spp. namely *T. atroviride* (T5), *T. harzianum* (T12 and T14) were taken and cultured for three days on PDB medium. Mycelium was extracted by filtration, dried and stored at -80°C. Harvested mycelial mats were ground in liquid nitrogen. The total RNA was extracted through Invisorb plant RNA easy extraction kit, and the integrity of RNA was confirmed by running samples in 1% agarose gel.

cDNA synthesis

The cDNA templates for the endochitinase gene were obtained from total RNA using Biomatik first strand cDNA synthesis kit, M-MuLV. cDNA synthesis was carried out using 5X VILO reaction mix (4 µL), 10X SuperScript enzyme mix (2 µL), RNA sample (3 µL), diethyl pyrocarbonate (DEPEC) treated water (11 µL), and the total reaction mixture was made up to 20 µL.

Endochitinase gene expression

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed by using the cDNA as the template and the same primer set F1: TCA GTG AAT CAT AGA ATCT T and R1: TAA TGG ATG CTA GAC CTT TG, following the same PCR reaction as in conventional PCR method for amplification of the endochitinase gene. ITS 1 and ITS 4 (White et al., 1990) was used to amplify the internal transcribed sequence region using cDNA of the three *Trichoderma* isolates, which is used as control for comparing the expression. The expression levels of endochitinase genes of the three fungal isolates *T. harzianum* (T14 and T12) and *T. atroviride* (T5) were observed on 1.5% agarose gel with respect to the control internal transcribed spacer (ITS) sequences.

RESULTS

Collection of isolates

About 100 *Trichoderma* isolates were collected by serial dilution of soil samples collected from different fields of Rajasthan and Delhi. Among the three major species, *T. harzianum*, *T. viride* and *T. atroviride* were found to be the maximum in the soil samples. In the present study, we collected 15 isolates of *Trichoderma* representing the three major spp. (Table.1) as *T. atroviride* (T1, T2, T3, T5 and T6), *T. viride* (T4, T7, T9, T10 and T15) and *T. harzianum* (T8, T11, T12, T13, and T14), respectively.

Chitinase assay

Before the molecular characterization of the endochitinase gene, it was necessary to check the biochemical nature of the chitinolytic activity of all the *Trichoderma* isolates. All the *Trichoderma* isolates showed detectable chitinase activities, with the maximum being observed in *T. harzianum* (T14) and (T11) isolate. The chitinase activity in *T. harzianum* (T14) was 17.21 µg/ml and T11 was 13.11 µg/ml, respectively (Figure 1).

Characterization of the endochitinase (*ech42*) gene from different *Trichoderma* spp.

Cloning of gene encoding endochitinase in *Trichoderma* spp.

PCR amplification of the genomic DNA from *T. harzianum*, *T. viride* and *T. atroviride* isolates with the chitinase primer set yielded a 1.1 kb product in all *Trichoderma* isolates, except in two *T. viride* isolates (T7 and T9) where around 2 kb product was amplified (Figure 2). All the PCR products were eluted from the gel and the eluted fragments were ligated to pGEMT cloning vector, and were transferred to *E. coli* DH5α separately. This recombinant *E. coli* DH5α was maintained on the Luria agar having ampicillin as a selection pressure.

Confirmation of clones

The transformed cells were picked up, streaked on Luria agar supplemented with ampicillin (100 ppm) containing X-GAL and isopropyl-D-thiogalactopyranoside (IPTG) for clonal selection. Recombinant cells were selected based on blue/white colony assay. Blue colonies represent ampicillin-resistant bacteria that contain pGEM-T Vector and express a functional alpha fragment from an intact Lac Z alpha coding sequence. White colonies represent ampicillin-resistant bacteria that contain insert and do not produce Lac Z alpha fragment. Limon et al. (1995) reported difference in the gene expression of the 42-kDa and 33-kDa chitinase in *T. harzianum*, suggesting independent regulation of each of these chitinase. The confirmation of the clones was done by restriction digestion and PCR amplification. The endochitinase genes gave the 1.1 kb amplicon using the specific primer mentioned earlier for amplification of endochitinase gene, and restriction analysis was done using the *EcoR* I which released the 1.1 kb fragment insert from the pGEMT vector of ~3 kb (Figure 3).

Sequencing and in silico analysis of the clones

The cloned endochitinase genes of about 1.1 kb were sequenced using M13 primers, employing primer walking technique. The nucleotide sequences of endochitinase gene were separated from the sequences of the cloned product after removing vector sequence through GENE TOOL and VecScreen service of the NCBI website. The available sequence information from cloned fragments was subjected to analysis using BLAST algorithm available at <http://www.ncbi.nlm.nih.gov>.

Phylogenetic analysis of the chitinase gene from *Trichoderma*

The various species of *Trichoderma* were taken for the analysis of phylogeny. The sequences of *T. atroviride* (T1, T2) and *T. harzianum* (T11, T5 and T12) are also used to know the phylogenetic position of these three species with respect to chitinase gene. Multiple sequence alignment was done by using MEGA5 algorithm and phylogenetic tree. Figure 4 shows the relation between *ech42* gene of five *Trichoderma* isolates (T1, T2, T11, T5, T12) and the three *Trichoderma* species viz. *H. lixii* (HM179247), *H. koningii* (HM179240) and *T. viride* (HM179242) taken from NCBI database.

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 3.99892169 is shown. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were

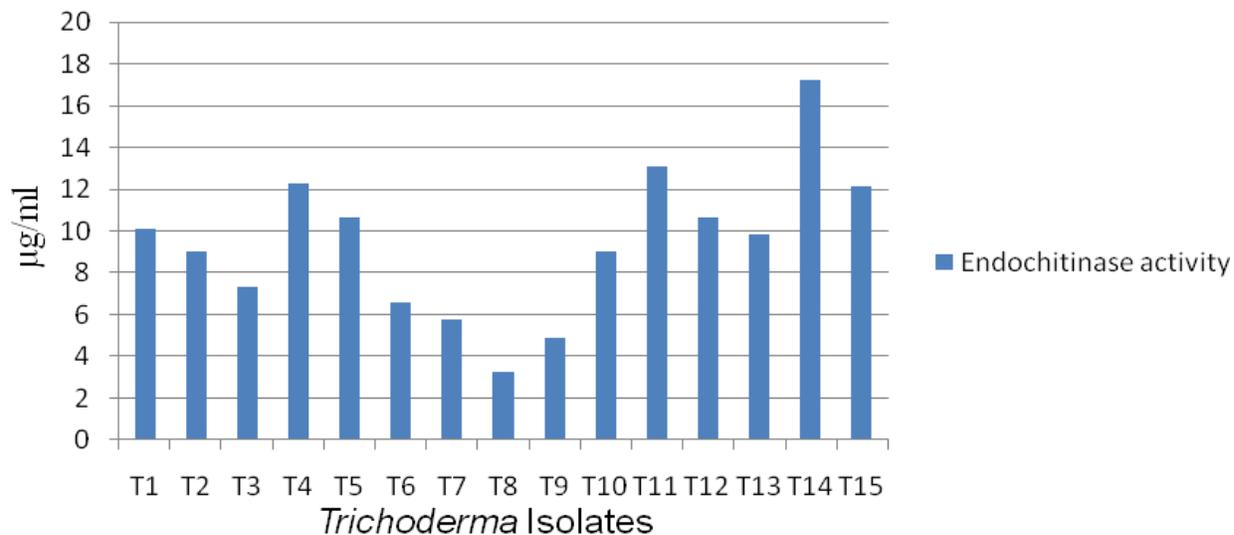


Figure 1. Chitinase activities of different *Trichoderma* isolates.

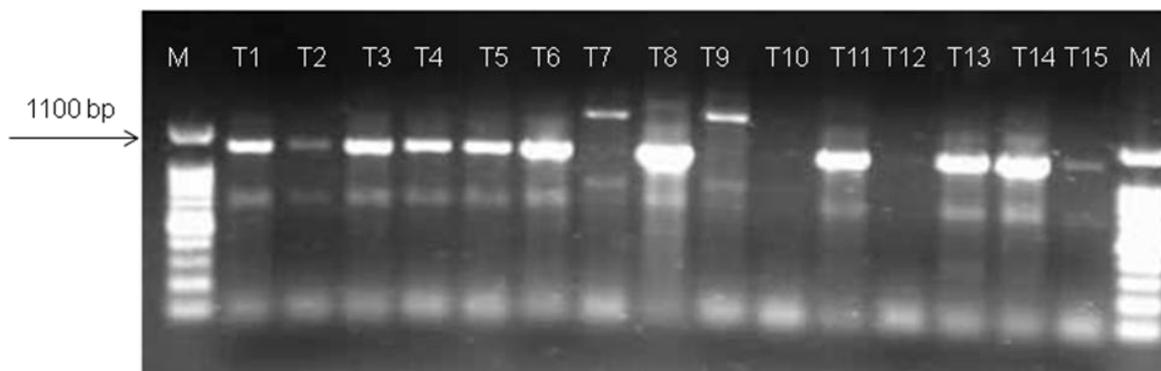


Figure 2. PCR amplification of endochitinase (*ech42*) from different *Trichoderma* isolates using 1.3% agarose gel stained with ethidium bromide. Lanes 1 and 17 contain the Genedirex 1 Kb molecular ladder denoted by M; lanes 2 to 16 represent the *Trichoderma* isolates (T1 to T15 as described in Table 1).



Figure 3. Agarose gel (1.3%) stained with ethidium bromide, showing restriction analysis of the cloned endochitinase gene from different *Trichoderma* isolates (T1 to T15) digested with *EcoR*I. Lane 1 and 17 M, marker 1 Kb; lanes 2 to 16, *Trichoderma* isolates (T1 to T15).

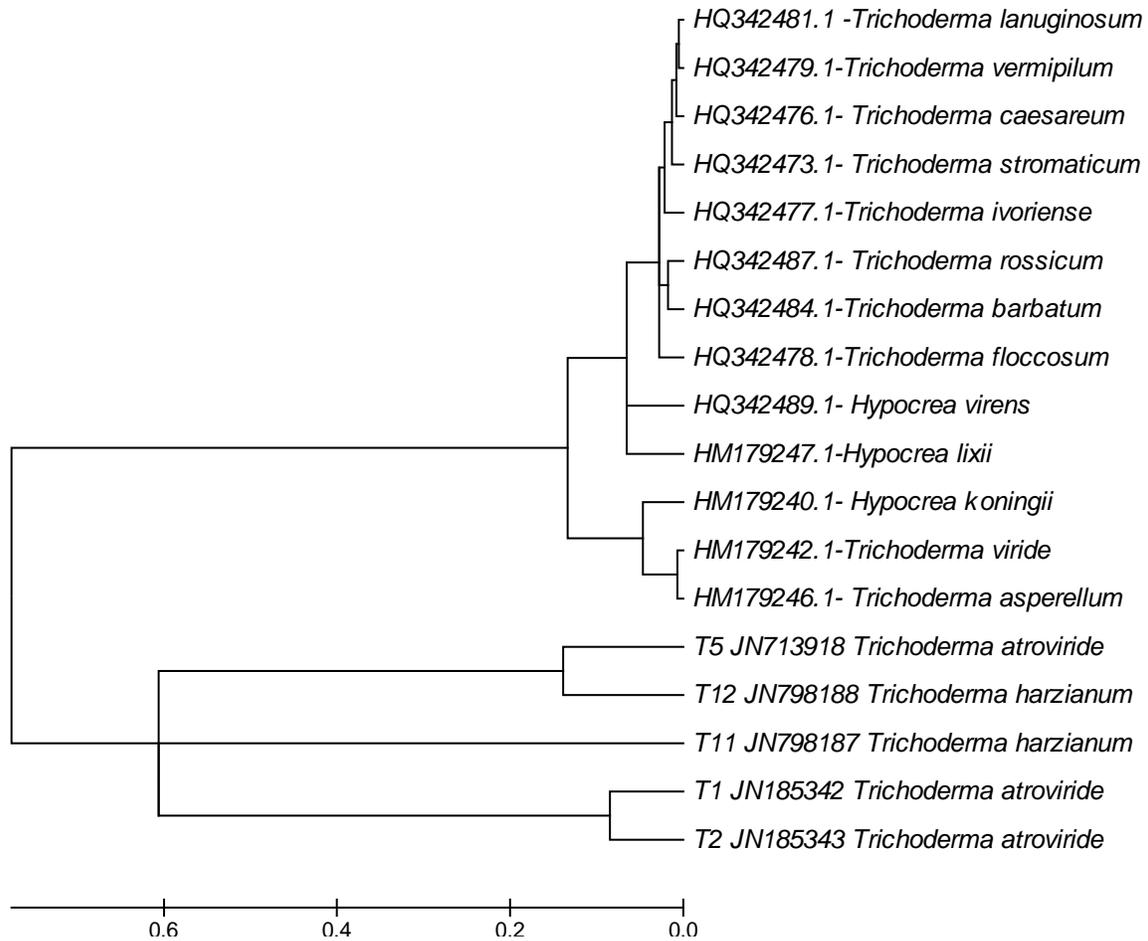


Figure 4. Phylogenetic tree based on nucleotide sequences of cloned endochitinase from different *Trichoderma* species, showing relation between the *ech42* gene of 5 *Trichoderma* isolates (T1, T2, T11, T5, T12) and the three *Trichoderma* species viz. *H. lixii* (HM179247), *H. koningii* (HM179240) and *T. viride* (HM179242) taken from NCBI database.

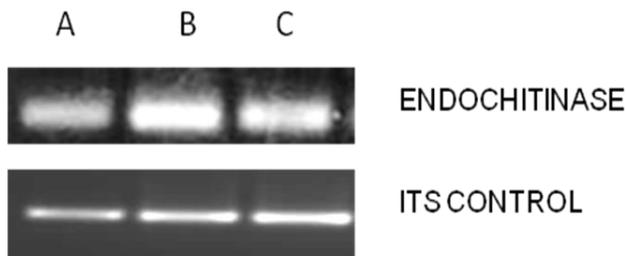


Figure 5. Semi-quantitative gene expression of (*ech42*) of *Trichoderma* isolates. A, *Trichoderma atroviride*; B, and C, *Trichoderma harzianum* in comparison to internal transcribed spacer (ITS) sequence.

computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The analysis involved 18 nucleotide sequences. Codon positions included were 1st + 2nd +

3rd + noncoding. There were a total of 353 positions in the final dataset.

Evolutionary analyses were conducted in MEGA5.

Gene expression analysis

The RNA was extracted from the fungal mycelium and the cDNA to be used as a template in the RT-PCR was amplified using the extracted RNA. Based on the field performance, two representatives *Trichoderma* isolates viz. *T. atroviride* (T5), and *T. harzianum* (T12 and T14) isolates were taken for the expression analysis. Expression profiling of cDNA amplification of *Trichoderma* isolates showed higher expression of endochitinase in T12 and T14 (*T. harzianum*) and a marked lesser expression was observed in T5 (*T. atroviride*) (Figure 5) with respect to other two isolates and internal transcribed spacer (ITS) sequence as control. Future research should focus on the development of endochitinase gene

expression in transgenic plants. Transgenic plants produce highly efficient synergistic combinations of these enzymes which are used for better disease control.

DISCUSSION

With the development of various genetic engineering techniques, genes encoding hydrolytic enzymes were cloned, characterized and transferred to plants to impart resistance against plant pathogens (Lorito et al., 1998; Liu et al., 2004; Jyoti et al., 2000; Shah et al., 2005; Upendra, 2006). The hydrolytic enzymes (Chitin) and compounds (6-pentyl- α -pyrone) produced by *Trichoderma* spp. play an important role in controlling the soil borne plant pathogens (Marco et al., 2003; Sharma and Dureja, 2004). Since the different species of *Trichoderma* and isolates of same species differ in their biocontrol potential and chitinase activity, isolation and characterization of *Trichoderma* from different places is important.

In the present study, 100 isolates from more than 500 soil samples were collected from different fields of Rajasthan and Delhi. Of these, 15 isolates were taken as representative of three major species viz. *T. atroviride* (T1, T2, T3, T5 and T6), *T. viride* (T4, T7, T9, T10 and T15) and *Trichoderma harzianum* (T8, T11, T12, T13, and T14). Differences were observed among different isolates with respect to chitinolytic activity. The measurement of chitinase activities indicates that these activities were maximum in *T. harzianum* (T11 and T14) than the other isolates. Similar differences among the *Trichoderma* isolates for chitinolytic activity have been reported by others (Kovacs et al., 2004; Aishwarya, 2004; Umamaheswari and Sankaralingam, 2005). The activity of chitinase towards *Sclerotinia sclerotiorum* has previously been reported in *T. harzianum*, *T. atroviride* and *Trichoderma longibrachiatum* (Matroudi et al., 2009; Elad, 2000). All these data collectively suggest that *T. harzianum* (T14) are highly effective among all the isolates. These chitinase enzymes induce interactions between *Trichoderma* spp. and cell wall materials of phytopathogenic fungi. Several endo and exo-chitinases (Cruz et al., 1992; Ulhoa and Peberdy 1992; Harman et al., 1993, 2004) and *N*-acetyl- β -D-glucosaminidases (Ulhoa and Peberdy 1991; Lorito et al., 1994) have been purified and characterized from *Trichoderma* spp. The endochitinase has also been purified from *T. virens* (Di Pietro et al., 1993).

Further, the endochitinase gene from representative *T. atroviride* (T1, T2, T3, T5 and T6), *T. viride* (T4, T7, T9, T10 and T15) and *T. harzianum* (T8, T11, T12, T13 and T14) was amplified using a pair of specific primers designed for *ech42* gene (Baek et al., 1999). The resulting amplicon (1.1 kb) from them were separately cloned into pGEMT. Again, the ORF of all the three *Trichoderma* species viz. *T. atroviride* (T1), *T. viride* (T15)

and *T. harzianum* (T14) showed conserved domain of the GH18 (glycosyl hydrolases, family 18) type II chitinases hydrolyzing chitin. Similar observations were recorded by Aishwarya (2004) and Bhat (2007). In reference to this study, the *T. harzianum ech 42* gene encoding a 42-kDa endochitinase was found to be induced in chitin-supplemented medium which was suppressed by carbon source mainly by glucose or sucrose (Carsolio et al., 1994). Moreover, all the genes showed genetic diversity with *T. harzianum* endochitinase gene, though they are cloned from three different species; one from *T. atroviride* and from *T. harzianum*. This may be due to use of endochitinase specific primer for *T. harzianum*. Similar observations were made by Latha et al. (2002), where *T. koningii* was exactly identical to the *Trichoderma hamatum* isolates present in Indian type culture collection when tested through rapid amplified polymorphic DNA (RAPD) primers. Chitinase gene from *T. harzianum* was also characterized (Hayes et al., 1994).

When phylogenetic analysis of 5 *Trichoderma* isolates (T1, T2, T5, T11, and T12) and the three *Trichoderma* species viz. *H. lixii* (HM179247), *H. koningii* (HM179240) and *T. viride* (HM179242) taken from NCBI database were done, diversity of relationship among the isolates were found but when the same 5 *Trichoderma* isolates were evolutionarily cross checked with some other *Trichoderma* species accessions taken from NCBI database, there was a distinct difference among the isolates (Figure 4). The endochitinase gene studies were essential for studying the function of the endochitinase gene and may be useful in developing improved strains of this fungus for enhanced biocontrol activity and also exploiting it for industrial purpose. It is therefore necessary to check the expression of these cloned endochitinase genes.

We predicted the secondary protein structure in which the percentage of alpha helix was found to be 37.44% and the beta turn percentage was 6.51%, the percentage of extension strand was 14.65%. This will help in expression of the gene in other organism. Before that, a semi-quantitative expression analysis of *ech42* of three *Trichoderma* spp. viz. T5 (*T. atroviride*) and T12 and T14 (*T. harzianum*) were performed which showed variable expression pattern of gene. Expression was found to be highest in T12 and T14 isolate in comparison to T5. Similarly, a novel chitinase gene was overexpressed and characterized from *T. atroviride* (Hoell et al., 2005) and *T. harzianum* (Bolar et al., 2000, 2001). Rice plant was transformed with the gene encoding endochitinase (*ech42*) from the biocontrol fungus *T. atroviride* (Liu et al., 2004). The transformed plants showed increased resistance to sheath blight caused by *Rhizoctonia solani* and rice blast caused by *Magnaporthe grisea*. However, the endochitinase had negative effect on the growth of the plant (Jyoti et al., 2000). A 42 kDa endochitinase was also transferred from *T. virens* to cotton (Chandrakanth et al., 2003) and transgenic plants showed resistance to the

pathogens *Alternaria alternata* and *R. solani*.

Recently, (Shah et al., 2005) transformed tobacco plants with *ech42* gene cloned from an Indian isolate of *T. virens* confirmed the integration of endochitinase into tobacco genome by PCR amplification using specific primers. In addition, study on the expression of *T. harzianum* endochitinase gene in yeast cell was tried by Bhat (2007), but it needs to be transferred to a model plant for validation and to generate transgenic in the target crop plant. The detailed study of isolation, cloning and characterization of major biocontrol genes like endochitinase will solve the future problems in plant pathology related to disease resistance in plant.

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