Molecular and Biochemical Characterization of Potential Isolates of \textit{Trichoderma} Species Effective against Soil-Borne Pathogens

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\textbf{A B S T R A C T}

\textit{Trichoderma} species is one of the key potential bio-control agents against soil-borne pathogens. In this study molecular and biochemical characterization were done using twenty four potential isolates of \textit{Trichoderma} species, based on internal transcribed spacer (ITS 1 & 4), translation elongation factor(\textit{tef}-1) gene region and hydrolytic enzymes. In this study \textit{tef}-1 was found to be better than ITS, to distinguish the \textit{Trichoderma} isolates into two different species \textit{viz.} \textit{Trichoderma virens} and \textit{Trichoderma harzianum}, on the basis of maximum parsimony sequence analysis. The specific activity of the hydrolytic enzymes showed the significance difference between both the species of \textit{Trichoderma}, tested against three different pathogens such as \textit{Fusarium oxysporum}, \textit{Rhizoctonia solani} and \textit{Sclerotium rolfsii}. It was also found that cultivation of \textit{Trichoderma} isolates with soil borne pathogen (during interaction) produced high hydrolytic enzymes compared to \textit{Trichoderma} species alone. Among the potential isolates tested for enzyme assay, three isolates \textit{viz.}, V-7, V-19 and V-21 of \textit{T. virens} and three isolates such as H-10, H-12 and H-21 of \textit{T. harzianum} were found as high potential isolates based on its specific activity of the hydrolytic enzymes. Therefore, the identified isolates could be effectively used as potential bio-control agents against soil-borne plant pathogens.

\textbf{Introduction}

\textit{Trichoderma} spp. Is one of the widespread saprophytic fungi in rhizosphere, which have received considerable attention as potential bio-control agents against most of plant pathogens as well as high utility towards medical and industrial sciences. The advent of molecular era could be judiciously utilized for investigations in fungal taxonomy prompted research in the mid-nineties to re-assess the morphology based taxonomy in \textit{Trichoderma} (Druzhinina \textit{et al.}, 2005). Only morphological attributes are not enough to define the species of \textit{Trichoderma} used against plant pathogens.

The authentic identification of \textit{Trichoderma} facilitates the researchers for definitive taxonomy.

The internal transcribed spacer (ITS-1) and internal transcribed spacer (ITS-2) region of 5.8Sr DNA and \textit{tef}-1 (gene) of the five \textit{Trichoderma virens} isolates were analyzed (Chaverri \textit{et al.}, 2001). Hermosa \textit{et al.}, 2004, attempted to analyze the genetic variability within bio-control isolates of \textit{Trichoderma} using sequence data obtained from the ITS
region of the nuclear rDNA and a fragment of translation elongation factor gene (tef -1 alpha). There are various mechanisms encompass in Trichoderma antagonism, such as competition, mycoparasitism and antibiosis etc., whereby the antagonistic fungus shows production of antibiotics. In case of mycoparasitism, Trichoderma directly attack the plant pathogens by excreting various lytic enzymes such as cellulase, chitinase, β-1,3 glucanases, proteases, poly-galacturanase (PG), pectin esterase, depolymerase, endoxylanase (1,4 β-D-xylanxylano-hydrolase) etc, these enzymes involved in the degradation of cell wall which leads tolysis of hyphae of the pathogen. The skeleton of pathogenic fungi cell wall encompass chitin, glucan, pectin, xylan and cellulose enzymes that are hydrolyse these components have to be present in the successful antagonists in order to play a significant role in cell wall lysis of the pathogen (Chernin et al., 2002; Kubicek et al., 2001; Viterbo et al., 2002).

The present investigation was an attempt for the effective utilization of the molecular and biochemical methods based on hydrolytic enzymes, to select potential isolates against soil-borne pathogens. This can help in the improvement and enhancement of bio-control strain and comprehend their mechanism of protection against soil-borne pathogens.

Materials and Methods

Molecular confirmation based on ITS and tef-1 regions

Twenty-four isolates of Trichoderma (Table 1) were molecularly characterized and analyzed for their hydrolytic enzymes production. The molecular characterization based on DNA sequencing of two unlinked loci, the ribosomal ITS region and the tef-1 gene (White et al., 1990). The tef-1 fragment was amplified by PCR using the specific primers (Geiser et al., 2004; Hermosa et al., 2004) (Table 1). The DNA was extracted using modified C-TAB method and PCR product was performed and analyzed through 1.2 agarose gel electrophoresis. Purified PCR products were sequenced separately in an automated ABI 3100 Genetic Analyser (Applied Biosystem, USA) by Bangalore Genei (Bangalore, India). Homologies to known sequences were searched in gene bank database using the Basic Alignment Search Tool (BLAST) available online from the National Centre for Biotechnology Information (NCBI). Phylogenetic analyses were performed using MEGA5 (Tamura et al., 2011) and a parsimony analysis tree was constructed using the Kimura-2- parameter distance model (Kimura, 1980).

Biochemical characterization of Trichoderma isolates based on hydrolytic enzymes

For biochemical characterization a total of twenty four isolates of Trichoderma (Table 3) (without interaction and during the interaction with F. oxysporum, R. solani and S. rolfsii) were used, to study various hydrolytic enzymes (cellulase, β-1,3glucanase, β-1,4 glucanase, chitinase and protease). All the Trichoderma isolates were grown in a minimal synthetic medium (MSM), (11) supplemented with different substrates as sole carbon sources. The 50 ml medium was inoculated with Trichoderma isolates with pathogens (2 X 10⁸cfu/ml), in interaction studies and no pathogens were inoculated in, without interaction studies. Enzyme activity was expressed in specific activity as IU/ mg protein. The protein estimation in culture supernatants of each treatment was followed by the method of Bradford (1976).

Enzyme assay

Cellulase (E.C. 3.2.1.4)

The assay mixture contained 1 ml of 0.5% cellulose (Sigma Co.) suspended in 50Mm
(0.05 M) citrate phosphate buffer (pH 4.8) and 1 ml of culture filtrates of various *Trichoderma* strains in 15 ml test tubes. The reaction mixture was incubated for 30 minute at 50°C. The blanks were made using distilled water in place of culture filtrate. The absorbance was measured at 540 nm and the amount of reducing sugar released was calculated with standard curve of glucose (Miller, 1959).

**β-1, 3 glucanase (E.C. 3.2.1.58)**

β-1, 3 glucanase was assayed similarly by incubating 1 ml 0.2% laminarin (w/v) in 50 Mm sodium acetate buffer (pH 4.8) with 1 ml enzyme solution at 40°C for 1 hr and by determining the reducing sugars with DNS (Nelson, 1944).

**β-1, 4 glucanase (E.C.3.2.1.91)**

(exoglucanase)

A mixture of 1 ml of 1.0% carboxymethyl cellulose, 2.0 ml of 0.05M citrate buffer (pH 4.8) and 1.0 ml culture filtrate, incubated at 55°C for 30 minute in water bath with periodical shaking. The reaction was stopped by boiling and adding of 4.0 ml of dinitro-salicylic acid reagent and the said enzyme activity was estimated (Thrane et al., 2000).

**Chitinase (E.C. 3.2.1.14)**

The reaction mixture prepared with 0.5 ml suspension of colloidal chitin (0.5%), 1.0 ml McIlvaine’s buffer (pH 4.0) and 0.5 ml culture filtrate (enzyme source), this was mixed thoroughly and incubated at 37°C for 20 minute in water bath with periodical shaking.

The reaction was stopped by boiling the mixture for 3 minute in boiling water bath. 3.0 ml potassium ferric cyanide reagent was added and warmed in boiling water bath for 15 minute. The amount of N-acetyl glucosamine (NAG) released was calculated from the absorbance of reaction mixture at 420 nm. The activity of chitinase was expressed as IU/mg (Sahai et al., 1993).

**Protease (Tyrosinase-E.C.1.14.18.1)**

The substrate used (1% casein in 50Mm phosphate buffer, pH 7.0) was denatured at 100°C for 15 minute in water bath and cooled at room temperature. The reaction-mixture containing 1 ml of substrate and 1 ml of enzyme solution was incubated at 37°C for 20 minute and the reaction was stopped with adding 3 ml of 10% tri-chloro acetic acid (TCA). The tubes were allowed to stand for 1 hour at 4°C to allow undigested protein to precipitate. The absorbance of liberated tyrosine in the filtrate was measured at 280 nm (Yang et al., 1994).

**Grouping of Trichoderma virens and Trichodermaharzianum isolates on the basis of specific activity of enzymes against soil-borne pathogens**

Twenty four isolates of *Trichoderma* were evaluated for their potentiality to produce various extracellular enzymes. The isolates were categorized into three groups based on their specific activity of enzymes viz., Group-1: (>20 IU/mg) high, Group-2: (10-20 IU/mg) moderate and Group-3: (0-10 IU/mg) low specific activity of potential isolates respectively.

**Statistical analysis**

The data were analyzed using pair-t test to differentiate the significance of results of enzyme activities.

**Results and Discussion**

**Molecular identification of Trichoderma isolates based on ITS 1 & 4 and tef-1 regions:** A total of twenty four isolates of *Trichoderma* species were used for the
molecular confirmation based on their ITS and tef-1 nucleotide sequences (Table 2).

**PCR amplification and sequencing**

Successful PCR amplifications were done using ITS 1 & 4 and tef-1 primers in twenty four isolates of *Trichoderma* species. A PCR product size was obtained as 600-650 bp for ITS 1 & 4 and 900-950 bp for tef-1 based on sequence analysis (Figs.1 and 2). All the distance values were calculated using the Kimura 2-parameter distance algorithm (Mega-5 software) and the obtained sequences were submitted to NCBI database.

**Molecular phylogenetic analysis**

To elucidate the genetic closeness of the twenty four isolates of *Trichoderma* phylogenetic tree was constructed based on sequence analysis of ITS 1 & 4 and tef-1 regions using the maximum parsimony analysis method using Mega 5.2 v.

A random sequence of other species of *Trichoderma* was used in the present study for out-group as to demonstrate the situation of the root and to comparison with *Trichoderma virens* and *Trichoderma harzianum* isolates. Phylogenetic analysis of ITS region revealed that there are three major clusters present, but this region could not differentiate the *Trichoderma* isolates in different groups with the bootstrap value ranging from 64-100% (Fig.3). But, the phylogenetic analysis based on tef-1 sequences revealed that there are three major clusters.

The cluster I contained all the isolates of *T. harzianum* (14 isolates) was supported with a bootstrap value higher than 65%along with other species such as *T. longibrachiatum* (2 isolates), *T. pseudokoningii* (2 isolates) and *T. reesei* (2 isolates). The cluster II and III comprised the *Trichoderma virens* (10 isolates) is supported with a bootstrap value of 92% and 77%, respectively (Fig.4).

**Biochemical characterization of *Trichoderma* isolates**

The investigation was focused on biochemical characterization of *Trichoderma* isolates by production of hydrolytic enzymes such as cellulase, β-1, 3-glucanase, β-1, 4-glucanase, chitinase and protease (Table 3). These enzymes specifically involved for degradation of cell wall of the pathogen, which intern helps in understanding the mechanism of biological control activity and selecting of potential isolates of *Trichoderma* species against soil-borne pathogens. The perusal of entire results revealed that the 08 potential isolates of *T. virens* and 12 potential isolates of *T. harzianum* significantly produced various hydrolytic enzymes without any interaction with soil borne pathogen.

However, among the *T. virens* isolates inoculated with sole carbon source without any interaction with soil-borne pathogens, the isolates V-19 (21.85 IU/mg) / V-17 (14.02 IU/mg), V-19 (18.19 IU/mg) / V-21 (18.00 IU/mg), V-7 (18.85 IU/mg) / V-19 (17.10 IU/mg), V-7 (19.68 IU/mg) / V-17 (18.01 IU/mg) and V-19 (16.01 IU/mg) / V-21 (15.27 IU/mg) showed highest production of hydrolytic enzymes activity viz., cellulose, β-1,3 glucanase, β-1,4 glucanase, chitinase and protease respectively whereas, the isolates, V-4 (6.17 IU/mg) / V-18 (6.80 IU/mg), V-4 (4.08 IU/mg) / V-18 (5.86 IU/mg), I8 (5.05 IU/mg) / V-22 (6.15 IU/mg), V-4 (9.16 IU/mg) / V-18 (9.25 IU/mg) and V-4 (3.88 IU/mg) and V-18 (4.26 IU/mg) showed lowest production of hydrolytic enzymes activity viz., cellulose, β-1,3 glucanase, β-1,4 glucanase, chitinase and protease respectively. Similarly, among the *T. harzianum*, the isolates H-10/ H-12 (18.64 IU/mg) / H-21 (16.35 IU/mg), H-10 (13.16 IU/mg) / H-12 (10.41 IU/mg), H-12 (17.95 IU/mg) / H-10 (12.06 IU/mg), H-10 (34.63 IU/mg) / H-26 (25.34 IU/mg) and H-21
(18.56 IU/mg) / H-10 (18.05 IU/mg) showed highest production of hydrolytic enzymes activity viz., cellulose, β-1,3 glucanase, β-1,4 glucanase, chitinase and protease respectively whereas, the isolates, H-24 (7.43 IU/mg) / H-6 (8.33 IU/mg), H-24 (4.33 IU/mg) / H-6 (5.42 IU/mg), H-6 (4.16 IU/mg) / H-24 (5.73 IU/mg), H-6 (5.91 IU/mg) / H-2 (8.82 IU/mg) and H-6 (4.92 IU/mg) / H-24 (6.03 IU/mg) showed lowest production of hydrolytic enzymes activity viz., cellulose, β-1,3 glucanase, β-1,4 glucanase, chitinase and protease respectively (Table 4).

Further, it was also observed that interaction between *Trichoderma* with soil-borne pathogens (*F. oxysporum*, *R. solani* and *S. rolfsii*) were also produced various hydrolytic enzymes. When the *T. virens* and *T. harzianum* isolates interacted with soil-borne pathogens, during their interaction all the isolates showed increased production of the hydrolytic enzymes (Table 5).

The isolates of *T. virens* during antagonism with *Fusarium oxysporum* interactions showed significant production in all the enzymes. The isolate V-7 (34.88 IU/mg) / V-21 (26.91 IU/mg), V-19 (19.56 IU/mg) / V-8 (13.45 IU/mg), V-19 (19.28 IU/mg) / V-7 (18.22 IU/mg), V-17 (30.13 IU/mg) / V-23 (24.37 IU/mg) and V-19 (19.44 IU/mg) / V-7 (18.94 IU/mg) showed highest production of hydrolytic enzymes activity viz., cellulose, β-1,3 glucanase, β-1,4 glucanase, chitinase and protease respectively whereas, the isolates, V-18 (7.55 IU/mg) / V-4 (8.41 IU/mg), V-4 (6.03 IU/mg) / V-18 (7.41 IU/mg), V-18 (7.28 IU/mg) / V-4 (7.57 IU/mg), V-4 (8.57 IU/mg) / V-18 (9.89 IU/mg) and V-4 (2.60 IU/mg) / V-18 (6.21 IU/mg) showed lowest production of hydrolytic enzymes activity viz., cellulose, β-1,3 glucanase, β-1,4 glucanase, chitinase and protease respectively.

During antagonism with *Rhizoctonia solani*, isolate V-7 (42.11 IU/mg), V-19 (31.40 IU/mg) / V-7 (16.20 IU/mg), V-19 (12.29 IU/mg) / V-19 (19.28 IU/mg), V-17 (11.89 IU/mg) / V-17 (38.73 IU/mg), V-7 (33.29 IU/mg) and V-21 (18.48 IU/mg), V-7 (18.29 IU/mg) showed highest production of hydrolytic enzymes activity viz., cellulose, β-1,3 glucanase, β-1,4 glucanase, chitinase and protease respectively whereas, the isolates, V-4 (8.83 IU/mg), V-18 (10.38 IU/mg) / V-4 (4.06 IU/mg), V-18 (7.14 IU/mg) / V-23 (4.83 IU/mg), V-18 (5.93 IU/mg) / V-18 (11.28 IU/mg), V-4 (12.16 IU/mg) / V-4 (4.19 IU/mg) and V-18 (7.44 IU/mg) showed lowest production of hydrolytic enzymes activity viz., cellulose, β-1,3 glucanase, β-1,4 glucanase, chitinase and protease respectively.

Similarly, with *Sclerotium rolfsii* the isolates, V-19 (30.31 IU/mg), V-21 (16.75 IU/mg) / V-19 (19.01 IU/mg), V-21 (16.46 IU/mg) / V-21 (19.43 IU/mg), V-19 (16.79 IU/mg) / V-19 (24.21 IU/mg), V-21 (22.71 IU/mg) / V-7 (18.50 IU/mg), V-21 (18.20 IU/mg) showed highest production of hydrolytic enzymes activity viz., cellulose, β-1,3 glucanase, β-1,4 glucanase, chitinase and protease respectively whereas, the isolates, V-4 (7.71 IU/mg), V-18 (8.49 IU/mg) / V-4 (4.06 IU/mg), V-18 (6.20 IU/mg) / V-18 (7.89 IU/mg), V-9 (8.30 IU/mg) / V-18 (11.81 IU/mg), V-22 (12.24 IU/mg) and V-4 (3.29 IU/mg), V-18 (4.87 IU/mg) showed lowest production of hydrolytic enzymes activity viz., cellulose, β-1,3 glucanase, β-1,4 glucanase, chitinase and protease respectively.

However, among the *T. harzianum* isolates inoculated with sole carbon source with *F. oxysporum* interaction showed significant production in all the enzymes. The isolates, H-12 (20.83 IU/mg), H-7 (18.88 IU/mg) / H-18 (13.90 IU/mg), H-21 (13.03 IU/mg) / H-12 (15.35 IU/mg), H-28 (13.34 IU/mg) / H-10

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(83.78 IU/mg), H-3 (49.29 IU/mg) / H-2 (16.32 IU/mg), H-21 (14.20 IU/mg) showed highest production of hydrolytic enzymes activity viz., cellulose, β-1,3 glucanase, β-1,4 glucanase, chitinase and protease respectively whereas, the isolates, H-6 (7.99 IU/mg), H-24 (9.15 IU/mg) / H-6 (7.91 IU/mg), H-24 (9.25 IU/mg) / H-6 (6.42 IU/mg), H-24 (8.20 IU/mg) / H-6 (10.84 IU/mg), H-24 (15.37 IU/mg) and H-24 (8.17 IU/mg), H-7 (8.83 IU/mg) showed lowest production of hydrolytic enzymes activity viz., cellulose, β-1,3 glucanase, β-1,4 glucanase, chitinase and protease respectively.

During antagonism with *Rhizoctonia solani* interaction showed the isolates, H-12 (52.07 IU/mg), H-7 (28.82 IU/mg) / H-12 (16.44 IU/mg), H-10 (15.90 IU/mg) / H-12 (13.70 IU/mg), H-7 (13.32 IU/mg) / H-10 (62.63 IU/mg), H-2 (51.72 IU/mg) and H-10 (31.37 IU/mg), H-12 (21.90 IU/mg) showed highest production of hydrolytic enzymes activity viz., cellulose, β-1,3 glucanase, β-1,4 glucanase, chitinase and protease respectively whereas, the isolates, H-24 (5.23 IU/mg), H-6 (9.39 IU/mg) / H-6 (6.50 IU/mg), H-24 (8.39 IU/mg) / H-24 (7.01 IU/mg), H-6 (7.71 IU/mg) / H-24 (16.93 IU/mg), H-6 (18.87 IU/mg) and H-6 (4.84 IU/mg), H-7 (6.27 IU/mg) showed lowest production of hydrolytic enzymes activity viz., cellulose, β-1,3 glucanase, β-1,4 glucanase, chitinase and protease respectively.

Similarly with *Sclerotium rolfsii*, the isolate H-18 (29.22 IU/mg), H-3 (26.31 IU/mg) / H-21 (18.78 IU/mg), H-12 (18.09 IU/mg) / H-21 (22.42 IU/mg), H-12 (19.59 IU/mg) / H-10 (88.80 IU/mg), H-12 (43.56 IU/mg) and H-10 and H-12 (23.88 IU/mg), H-26 (16.17 IU/mg) showed highest production of hydrolytic enzymes activity viz., cellulose, β-1,3 glucanase, β-1,4 glucanase, chitinase and protease respectively whereas, the isolates, H-24 (7.44 IU/mg), H-6 (8.74 IU/mg) / H-6 (6.17 IU/mg), H-24 (8.67 IU/mg) / H-24 (8.31 IU/mg), H-18 (8.62 IU/mg) / H-6 (12.74 IU/mg), H-24 (14.99 IU/mg) and H-6 (7.78 IU/mg), H-24 (9.45 IU/mg) showed lowest production of hydrolytic enzymes activity viz., cellulose, β-1,3 glucanase, β-1,4 glucanase, chitinase and protease respectively.

**Grouping of Trichoderma virens and Trichoderma harzianum isolates on the basis of specific activity of enzymes against soil-borne pathogens**

Twenty four isolates of *Trichoderma* were evaluated for their potentiality to produce various extracellular enzymes against three soil-borne plant pathogens. All the isolates were categorized into different groups based on their enzymes activity as Group-1: (>20 IU/mg)-High, Group-2: (10-20 IU/mg)-Moderate and Group-3: (0-10 IU/mg)-Low potential. It was also inferred that the most of isolates appeared under moderate as well as low potential groups and very few isolates appeared under high potential in both with and without interaction with the pathogens (Table 6).

With the above investigation it was found that, V-7, V-19 and V-21 of *T. virens* have high potential isolates and V-4 was considered as low potential isolate. Similarly, the isolates H-10, H-12 and H-21 of *T. harzianum* have high potential and the isolate H-6 was considered as low potential.

The advent of molecular technology would help in molecular characterization of potential *Trichoderma* strains and could help for taxonomic identification. For molecular characterization, there is a need of precise molecular data resulting from DNA sequencing (Samuels, 2006). The internal transcribed spacer (ITS) and tef-I regions of the ribosomal DNA (rDNA) are the most
reliable targets to identify a strain at the species level (19). In this way, combination of both (ITS and tef-1) region, allow most identifications at the species level. Use of two unlinked loci (ITS and tef-1), further helped in molecular identification, where it was difficult to conclude with the ITS region alone. It can be concluded that the combined approach of morphological and molecular techniques are necessary for authentic identification of Trichoderma strains.

A total of twenty four isolates of Trichoderma spp. were used in present investigation to analyze various hydrolytic enzyme activities as well as molecular characterization based on their ITS and tef-1 nucleotide sequences of T. virens and T. harzianum. The Phylogenetic tree, based on ITS did not clearly separated the species but tef-1 gene analysis showed separation of Trichoderma isolates into T. virens and T. harzianum. Therefore, the tef-1 region could be a better tool for differentiation of both the species. The findings are matching with the observations made by Samuels, 2006. It was reported that Trichoderma secretes hydrolytic enzymes at a constitutive level and detects the presence of another fungus by sensing the molecules released from the host with enzymatic degradation (Lorito et al., 2006). The antifungal arsenals of Trichoderma spp. encompass a great variety of lytic enzymes (Lorito et al., 1993, 1996, 1998) and most of enzymes play key role in bio-control (Harman et al., 1998; Baek et al., 1999; Carsolio et al., 1999; Woo et al., 1999; Zeilinger et al., 1999; Kulling et al., 2000; Vinale et al., 2008).

In the present investigation, twenty four isolates of Trichoderma species were evaluated for their potentiality to produce various extracellular enzymes against three soil-borne plant pathogens, viz., F. oxysporum, R. solani and S. rolfsii and based on high potentiality of isolates was utilized for subsequent studies. Present findings are consistent with the earlier findings (Mach et al., 1999; El-Katatny et al., 2001, 2004) where they were reported that the addition of some carbon sources in growth medium with and without interaction of soil-borne pathogens significantly improved the secretion of certain cell wall degrading enzymes. In the present investigation, 10 isolates of T. virens and 14 isolates of T. harzianum produced different hydrolytic enzymes (cellulase, β-1,3 glucanase, β-1,4 glucanase, chitinase and protease) when the basal medium (minimal synthetic media) was supplemented with different carbon sources and soil-borne pathogens (F. oxysporum, R. solani and S. rolfsii). The extracellular enzymes activity was observed in all the isolates and they were categorized into different groups based on their specific enzyme activity.

| Table 1 | Primers used for amplification of ITS 1 & 4 and tef-1 gene regions |
|---------|---------------------------------------------------------------|
| **Region** | **Primer sequence** | **Reference** |
| ITS1-5.8S-ITS2 region of rDNA | 5’- TCCGTAGGTGAACCTGCGG-3’ | (2) |
| | ITS-4: 5’TCTCCGCTTATGATGC-3’ | |
| Intron b/w 5th and 6th exon of tef-1 region | tef-1fw: 5’-GTGAGCGTGTA-TCACCA-3’ | (3) |
| | tef-irev: 5’GCCATCTTGGAGACCAGC-3’ | |
Table 2 Molecular confirmation of *Trichoderma* isolates by using ITS and tef-1 region

| Name of the isolates/Strain No. | Accession No. | Sources | Origin | NCBI GeneBank accession numbers | Morphological/Molecular/Definitive identification |
|---------------------------------|---------------|---------|--------|---------------------------------|-----------------------------------------------|
|                                 |               |         | Place  | State              | ITS     | tef-1       |
| V-4 ITCC-6470                   | Soil          | Pusa    | Bihar  | KF144619            | KF668101 | *T. virens* |
| V-7 ITCC-6411                   | Soil          | Barrackpur | West Bengal | KF144622 | KF668104 | *T. virens* |
| V-8 MTCC-749                    | Soil          | Pantnagar | Uttarakhhand | KF144623 | KF668105 | *T. virens* |
| V-9 MTCC-1373                   | Soil          | Pantnagar | Uttarakhhand | KF144624 | KF668106 | *T. virens* |
| V-17 MTCC-2977                  | Soil          | Kolkata  | West Bengal | KF144632 | KF668114 | *T. virens* |
| V-18 MTCC-2979                  | Soil          | Kolkata  | West Bengal | KF144633 | KF668115 | *T. virens* |
| V-19 MTCC-2983                  | Soil          | Kolkata  | West Bengal | KF144634 | KF668116 | *T. virens* |
| V-21 MTCC-4346                  | Soil          | Almora   | Uttarakhhand | KF144636 | KF668118 | *T. virens* |
| V-22 ITCC-7351                  | Soil          | Kozhikode | Kerala  | KF144637 | KF668119 | *T. virens* |
| V-23 ITCC-7352                  | Soil          | Kozhikode | Kerala  | KF144638 | KF668120 | *T. virens* |
| H-2 ITCC-4950                   | Soil          | New Delhi | Delhi   | KF144640 | KF668122 | *T. harzianum* |
| H-3 ITCC-5223                   | Compost       | New Delhi | Delhi   | KF144641 | KF668123 | *T. harzianum* |
| H-6 ITCC-6797                   | Soil          | Bengaluru | Karnataka | KF144644 | KF668126 | *T. harzianum* |
| H-7 ITCC-6888                   | Soil          | Navasari  | Gujarat  | KF144645 | KF668127 | *T. harzianum* |
| H-9 ITCC-7057                   | Compost       | New Delhi | Delhi   | KF144647 | KF668129 | *T. harzianum* |
| H-10 ITCC-7077                  | Sugarcane soil | Navasari  | Gujarat  | KF144648 | KF668130 | *T. harzianum* |
| H-11 ITCC-7368                  | Chikpearl rhizosphere soil | New Delhi | Delhi   | KF144649 | KF668131 | *T. harzianum* |
| H-12 ITCC-7354                  | Soil          | Navasari  | Gujarat  | KF144650 | KF668132 | *T. harzianum* |
| H-16 ITCC-7355                  | Compost       | Jammu    | J& K    | KF144654 | KF668136 | *T. harzianum* |
| H-18 ITCC-7342                  | Soil          | New Delhi | Delhi   | KF144656 | KF668138 | *T. harzianum* |
| H-21 ITCC-7357                  | Soil          | Shalimar  | J& K    | KF144659 | KF668141 | *T. harzianum* |
| H-24 ITCC-7346                  | Soil          | Bapatla   | Andhra Pradesh | KF144662 | KF668144 | *T. harzianum* |
| H-26 ITCC-7348                  | Soil          | Bapatla   | Andhra Pradesh | KF144664 | KF668146 | *T. harzianum* |
| H-28 ITCC-7350                  | Soil          | Bapatla   | Andhra Pradesh | KF144666 | KF668148 | *T. harzianum* |
Table 3 Specific activity of hydrolytic enzymes produced by the *Trichoderma* isolates without interaction

| Isolates | Cellulase | β-1-3 glucanase | β-1-4 glucanase | Chitinase | Protease |
|----------|-----------|-----------------|-----------------|-----------|----------|
| V-7      | 10.46     | 11.21           | **18.85**       | 19.68     | 15.19    |
| V-8      | 13.00     | 7.75            | 7.13            | 12.78     | 5.60     |
| V-9      | 9.71      | 8.50            | 7.60            | 9.70      | 6.00     |
| V-17     | **14.00** | 7.93            | 9.42            | **18.01** | 6.86     |
| V-19     | **21.85** | **18.19**       | **17.10**       | 10.29     | **16.01** |
| V-21     | 10.02     | **18.00**       | 17.04           | 14.11     | **15.27** |
| V-22     | 9.00      | 7.75            | 6.15            | 10.25     | 6.25     |
| V-23     | 9.65      | 7.30            | 6.56            | 16.56     | 5.65     |
| V-4      | 6.17      | 4.08            | 6.87            | 9.16      | 3.88     |
| V-18     | 6.80      | 5.86            | 5.05            | 9.25      | 4.26     |
|          | **SEm±**  | **0.61**        | **0.31**        | **0.22**  | **1.29**  |
|          | **CD (p=0.05)** |     |     |     | **3.53** |
| H-2      | 8.56      | 8.78            | 10.54           | 10.74     | 8.51     |
| H-3      | 8.83      | 9.42            | 9.84            | 13.83     | 8.12     |
| H-7      | 10.96     | 9.44            | 10.45           | 20.95     | 7.53     |
| H-9      | 13.23     | 8.20            | 9.58            | 19.66     | 9.10     |
| H-10     | **18.64** | **13.16**       | **12.06**       | **34.63** | **18.05** |
| H-11     | 10.55     | 8.59            | 11.57           | 10.23     | 9.15     |
| H-12     | **18.64** | **10.41**       | **17.95**       | 19.55     | 11.00    |
| H-16     | 12.77     | 7.71            | 9.86            | 22.14     | 8.95     |
| H-18     | 10.70     | 8.50            | 9.20            | 15.74     | 10.36    |
| H-21     | 16.35     | 10.05           | 10.60           | 24.20     | **18.56** |
| H-26     | 10.87     | 8.46            | 9.21            | **25.34** | 8.83     |
| H-28     | 12.63     | 8.32            | 8.52            | 24.62     | 10.50    |
| H-6      | 8.33      | 5.42            | 4.16            | 5.91      | 4.92     |
| H-24     | 7.43      | 4.33            | 5.73            | 8.82      | 6.03     |
|          | **SEm±**  | **1.35**        | **0.27**        | **0.31**  | **0.86**  |
|          | **CD (p=0.05)** | **4.63** | **2.08** | **2.22** | **1.17** | **4.01** |
Table 4 Specific activity of hydrolytic enzymes produced by the *Trichoderma* isolates during the interaction

| Isolates   | Cellulase (F. oxysporum) | β-1,3 Glucanase (R. solani) | β-1,4 Glucanase (S. rolfsii) | Chitinase (F. oxysporum) | Protease (R. solani) |
|------------|--------------------------|-----------------------------|-----------------------------|-------------------------|---------------------|
| V-7        | 34.88                    | 42.11                       | 10.71                       | 12.21                   | 16.20               |
| V-8        | 10.65                    | 22.84                       | 15.28                       | 13.45                   | 8.27                |
| V-9        | 15.06                    | 11.71                       | 12.93                       | 10.48                   | 9.67                |
| V-17       | 9.58                     | 15.69                       | 15.69                       | 11.89                   | 9.75                |
| V-19       | 22.94                    | 31.40                       | 30.31                       | 19.56                   | 12.29               |
| V-21       | 26.91                    | 15.17                       | 16.75                       | 10.02                   | 10.82               |
| V-22       | 9.21                     | 12.72                       | 10.31                       | 9.71                    | 8.61                |
| V-23       | 13.73                    | 16.62                       | 11.48                       | 9.97                    | 10.19               |
| V-4        | 8.41                     | 8.83                        | 7.71                        | 6.03                    | 4.06                |
| V-18       | 7.55                     | 10.38                       | 8.49                        | 7.41                    | 7.14                |
| H-2        | 11.42                    | 16.91                       | 15.15                       | 12.96                   | 8.78                |
| H-3        | 10.86                    | 24.27                       | 26.31                       | 10.18                   | 12.39               |
| H-7        | 18.88                    | 28.82                       | 13.99                       | 12.81                   | 10.62               |
| H-9        | 13.23                    | 24.12                       | 15.85                       | 10.06                   | 9.78                |
| H-10       | 17.54                    | 19.19                       | 18.09                       | 10.41                   | 15.90               |
| H-11       | 13.27                    | 14.12                       | 13.44                       | 9.53                    | 9.02                |
| H-12       | 20.83                    | 52.07                       | 23.57                       | 10.96                   | 16.44               |
| H-16       | 13.33                    | 14.30                       | 14.30                       | 9.30                    | 8.47                |
| H-18       | 17.60                    | 15.10                       | 22.90                       | 13.90                   | 9.50                |
| H-21       | 15.02                    | 18.33                       | 29.22                       | 13.03                   | 13.92               |
| H-26       | 12.48                    | 17.62                       | 23.06                       | 10.77                   | 9.06                |
| H-28       | 13.24                    | 14.74                       | 20.36                       | 9.63                    | 10.33               |
| H-6        | 7.99                     | 9.39                        | 8.74                        | 7.91                    | 6.50                |
| H-24       | 9.15                     | 5.23                        | 7.44                        | 9.25                    | 8.39                |

**SEm± (p=0.05)**: 0.86, 0.48, 0.48, 0.05, 0.05, 0.01, 0.21, 0.21, 0.12, 0.05, 0.04, 0.05, 0.01, 0.01, 0.01

**CD (p=0.05)**: 3.68, 2.76, 2.76, 0.92, 0.92, 0.01, 1.84, 1.84, 1.38, 0.93, 0.80, 0.93, 0.34, 0.17, 0.25
**Table 5** Grouping of *Trichoderma virens* and *Trichoderma harzianum* isolates based on specific enzymatic activity without and during interaction with soil-borne pathogens

| Interaction | Specific activity of enzymes | Groups | Name of the species | Cellulase | β-1,3 Glucanase | β-1,4 Glucanase | Chitinase | Protease |
|-------------|-----------------------------|--------|---------------------|-----------|----------------|----------------|-----------|---------|
| High        | Group-1 (>20 IU/mg) Specific activity | *T. virens* | V-19                | None      | None           | None           | None      | None    |
|             |                              | *T. harzianum* | None               | None      | None           | None           | H-7, H-10, H-16, H-21, H-26, H-28 | None    |
| Moderate    | Group-2 (10-20 IU/mg) Specific activity | *T. virens* | V-7, V-8, V-17, V-21 | V-7, V-19, V-21 | V-7, V-19, V-21 | V-7, V-8, V-17, V-19, V-21, V-22, V-23 | V-7, V-19, V-21 |
|             |                              | *T. harzianum* | H-7, H-9, H-10, H-11, H-12, H-16, H-18, H-21, H-26, H-28 | H-10, H-12, H-21 | H-2, H-7, H-10, H-11, H-12, H-21 | H-2, H-3, H-9, H-11, H-12, H-18, H-21, H-28 | H-10, H-12, H-18, H-21, H-28 |
| Low         | Group-3 (0-10 IU/mg) Specific activity | *T. virens* | V-4, V-9, V-18, V-22, V-23 | V-4, V-8, V-9, V-17, V-18, V-22, V-23 | V-4, V-9, V-18 | V-4, V-8, V-9, V-17, V-18, V-22, V-23 | V-4, V-8, V-9, V-17, V-18, V-22, V-23 |
|             |                              | *T. harzianum* | H-2, H-3, H-6, H-24 | H-2, H-3, H-6, H-7, H-9, H-11, H-16, H-18, H-24, H-26, H-28 | H-3, H-6, H-9, H-16, H-18, H-24, H-26, H-28 | H-6, H-24 | H-2, H-3, H-6, H-7, H-9, H-11, H-16, H-24, H-26 |
| High        | Group-1 (>20 IU/mg) Specific activity | *T. virens* | V-7, V-19, V-21     | None      | None           | None           | V-7, V-17, V-19, V-21 | None    |
|             |                              | *T. harzianum* | H-12               | None      | None           | None           | H-3, H-7, H-9, H-10, H-12, H-16, H-21, H-26, H-28 | None    |
| Moderate    | Group-2 (10-20 IU/mg) Specific activity | *T. virens* | V-8, V-9, V-23     | V-7, V-8, V-9, V-17, V-19, V-21 | V-7, V-8, V-17, V-19, V-21 | V-8, V-9, V-22 | V-7, V-17, V-19, V-21 |
|             |                              | *T. harzianum* | H-2, H-3, H-7, H-9, H-10, H-11, H-16, H-18, H-21, H-26, H-28 | H-2, H-3, H-7, H-9, H-10, H-11, H-12, H-21, H-26, H-28 | H-2, H-6, H-11, H-18, H-24 | H-2, H-3, H-9, H-10, H-12, H-16, H-18, H-21, H-26, H-28 |
| Low         | Group-3 (0-10 IU/mg) Specific activity | *T. virens* | V-4, V-17, V-18, V-22 | V-4, V-18, V-22, V-23 | V-4, V-9, V-18, V-22, V-23 | V-4, V-18 | V-4, V-8, V-9, V-18, V-22, V-23 |
|             |                              | *T. harzianum* | H-6, H-24 | H-6, H-11, H-16, H-24, H-28 | H-6, H-16, H-18, H-24 | None | H-6, H-7, H-11, H-24 |
|             | High               | Moderate               | Low                |
|-------------|--------------------|------------------------|--------------------|
| **Trichoderma species with Rhizoctonia solani** |                    |                        |                    |
| **Group-1** | (>20 IU/mg)       | Specific activity      |                    |
| **Group-2** | (10-20 IU/mg)     | Specific activity      |                    |
| **Group-3** | (0-10 IU/mg)      | Specific activity      |                    |
| **T. virens** | V-7, V-8, V-19    | None                   |                    |
| **T. harzianum** | H-3, H-7, H-9, H-12 | None                   | H-3, H-7, H-9, H-10, H-11, H-12, H-16, H-21, H-26, H-28 |
| **T. virens** | V-9, V-17, V-18, V-21, V-22, V-23 | V-7, V-19, V-21 | V-7, V-8, V-9, V-18, V-21, V-22, V-23 |
| **T. harzianum** | H-2, H-10, H-11, H-16, H-18, H-21, H-26, H-28 | H-3, H-7, H-10, H-12, H-21, H-28 | H-2, H-7, H-10, H-12, H-16, H-21, H-28 |
| **T. virens** | V-4, V-8, V-17, V-18, V-22 | None | V-4, V-8, V-9, V-18, V-21, V-22, V-23 |
| **T. harzianum** | H-6, H-24 | H-2, H-6, H-9, H-11, H-16, H-18, H-24, H-26, H-28 | H-3, H-6, H-9, H-11, H-18, H-24, H-26, H-28 |

| **Trichoderma species with Sclerotium rolfsii** |                    |                        |                    |
| **Group-1** | (>20 IU/mg)       | Specific activity      |                    |
| **Group-2** | (10-20 IU/mg)     | Specific activity      |                    |
| **Group-3** | (0-10 IU/mg)      | Specific activity      |                    |
| **T. virens** | V-19               | None                   | V-7, V-19, V-21    |
| **T. harzianum** | H-3, H-12, H-18, H-21, H-26, H-28 | None | H-3, H-7, H-9, H-10, H-12, H-16, H-21, H-26, H-28 |
| **T. virens** | V-7, V-8, V-17, V-18, V-22, V-23 | V-7, V-19, V-21 | V-7, V-8, V-9, V-17, V-18, V-22, V-23 |
| **T. harzianum** | H-2, H-7, H-9, H-10, H-11, H-16, H-26 | H-3, H-7, H-9, H-10, H-11, H-12, H-16, H-26 | H-2, H-3, H-7, H-9, H-10, H-11, H-12, H-16, H-26 |
| **T. virens** | V-4, V-8, V-17, V-18, V-22, V-23 | None | V-4, V-8, V-9, V-18, V-22, V-23 |
| **T. harzianum** | H-6, H-24 | H-2, H-6, H-16, H-24, H-28 | H-6, H-18, H-24, H-28 |

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**Table 6** High and low potential isolates of *T. virens* and *T. harzianum* selected on the basis of enzyme activity studies

| Name of the species       | High potential | Low potential |
|---------------------------|----------------|---------------|
| *Trichoderma virens*     | V-7, V-19, V-21 (03 isolates) | V-4 (01 isolates) |
| *Trichoderma harzianum*  | H-10, H-12, H-21 (03 isolates) | H-6 (01 isolate) |

**Fig 1** A representative gel picture showing amplification profile of twenty four isolates of *T. virens* (V) and *T. harzianum* (H) using ITS 1 & 4 region nucleotide sequence (Table 1) for molecular conformation of isolates

![Image](image1.png)

M: 1 Kb DNA Ladder (both the side). Lane 1-24 isolates: V-4, V-7, V-8, V-9, V-17, V-18, V-19, V-21, V-22, V-23, H-2, H-3, H-6, H-7, H-9, H-10, H-11, H-12, H-16, H-18, H-21, H-24, H-26 and H-28.

**Fig 2** A representative gel picture showing amplification profile of twenty four isolates of *T. virens* (V) and *T. harzianum* (H) using tef-1 region nucleotide sequence (Table 1) for molecular conformation of isolates

![Image](image2.png)

M: 1 Kb DNA Ladder (both the side). Lane 1-22 isolates: V-4, V-7, V-8, V-9, V-17, V-18, V-19, V-21, V-22, V-23, H-2, H-3, H-6, H-7, H-9, H-10, H-11, H-12, H-16, H-18, H-21, H-24, H-26 and H-28.
Fig.3 Phylogenetic relationship of twenty four isolates of *Trichoderma* species based on ITS 1 & 4 region of nucleotide sequence aligned using software MEGA 5.2 v. the tree was generated by the Maximum parsimony analysis method.

```
+--- H-6 Trichoderma harzianum (ITCC-6797-KF144684)
+---------- H-24 Trichoderma harzianum (ITCC-7346-KF144662)
+---------- H-9 Trichoderma harzianum (ITCC-7057-KF144647)
+---------- H-26 Trichoderma harzianum (ITCC-7348-KF144654)
|          +--- H-7 Trichoderma harzianum (ITCC-5888-KF144654)
|          +--- H-11 Trichoderma harzianum (ITCC-7368-KF144649)
|          +--- H-21 Trichoderma harzianum (ITCC-7357-KF144659)
|          +--- V-19 Trichoderma virens (MTCC-2983-KF144634)
|          +--- V-21 Trichoderma virens (MTCC-4346-KF144636)
|          +--- V-9 Trichoderma virens (MTCC-1373-KF144624)
|          +--- H-12 Trichoderma harzianum (ITCC-7354-KF144650)
|          +--- KC914097.1 Trichoderma longibrachiatum strain MDU-6
|          +--- JQ979345.1 Trichoderma reesei isolate Cb03.3.1
|          +--- V-4 Trichoderma virens (ITCC-6470-KF144619)
|          +--- KC478546.1 Trichoderma reesei isolate RSPG_24
|          +--- V-7 Trichoderma virens (ITCC-6411-KF144622)
|          +--- AF362102.1 Trichoderma longibrachiatum strain T9
|          +--- H-2 Trichoderma harzianum (ITCC-4950-KF144640)
|          +--- H-16 Trichoderma harzianum (ITCC-7356-KF144654)
|          +--- H-3 Trichoderma harzianum (ITCC-5223-KF144641)
|          +--- V-23 Trichoderma virens (ITCC-7352-KF144638)
|          +--- V-22 Trichoderma virens (ITCC-7351-KF144637)
|          +--- V-17 Trichoderma virens (MTCC-2977-KF144632)
|          +--- V-18 Trichoderma virens (MTCC-2975-KF144633)
|          +--- AF414330.1 Trichoderma pseudokoningii strain CCRC33607
|          +--- H-10 Trichoderma harzianum (ITCC-7077-KF144646)
|          +--- AF414298.1 Trichoderma pseudokoningii strain CCRC32893
|          +--- V-8 Trichoderma virens (MTCC-749-KF144623)
|          +--- H-18 Trichoderma harzianum (ITCC-7342-KF144656)
|          +--- H-20 Trichoderma harzianum (ITCC-7350-KF144666)
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Fig. 4 Phylogenetic relationship of twenty four isolates of *Trichoderma* species based on tef-1 region of nucleotide sequence aligned using software MEGA 5.2 v. The tree was generated by the Maximum parsimony analysis method.

The highest hydrolytic enzymes production viz., cellulase, β-1, 3-glucanase, β-1, 4-glucanase, chitinase and protease were observed in *T. harzianum* in both without and also during antagonism studies comparable with *T. virens* isolates. Further, it was also found that cultivation of *Trichoderma* isolates with soil borne pathogen (during interaction) produce high hydrolytic enzymes compared to cultivation of *Trichoderma* species alone. The enzymatic production were categorized into >20 IU/mg as high, 10-20 IU/mg as moderate and 0-10 IU/mg as low potential.

A total of 10 isolates of *T.virens* were tested, only three isolates viz., V-7, V-19 and V-21 were considered as high potential isolates and the isolate V-4 considered as a low potential based on the specific activity of the enzymes. Similarly, a total of 14 isolates of
T. harzianum were tested, only three isolates such as H-10, H-12 and H-21 were considered as high potential and the isolate H-6 was considered as low potential based on the specific activity of the enzymes. There are many reports demonstrating that cellulase, β-1,3glucanase, β-1,4glucanase, chitinase and proteases are effective features associated with the ability of Trichoderma to control plant pathogens (Brimner et al., 2003; Haran et al., 1996; Wang et al., 2003; Lorito et al., 1994).

In conclusion, present investigation was carryout to investigate molecular taxonomy (based on ITS 1 & 4 and tef-1 sequences analysis) and biochemical characterization (based on hydrolytic enzymes such as cellulase, β-13, 3-glucanase, β-1, 4-glucanase, chitinase and protease) of selected (through bio-efficacy tests) isolates of Trichoderma which intern helps in understanding the mechanism of biological control activity. The twenty four isolates of Trichoderma were molecularly analyzed for the confirmation of its species with their morphology using ITS 1 & 4 and tef-1 regions. tef-1 region was found better to separate the T. virens and T. harzianum in the present study. Three isolates of T. virensvitsiz. V-7, V-19 and V-21 and another three isolates of T. harzinaum such as H-10, 12 and H-21 were selected as potential based on their high specific enzymatic activity (>20 IU/mg) and identified isolates could be used as bio-control agents against F. oxysporum, R. solani and S. rolfsii.

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