Int.J.Curr.Microbiol.App.Sci (2017) *6*(6): 3132-3149

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 6 (2017) pp. 3132-3149**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2017.606.370

Molecular and Biochemical Characterization of Potential Isolates of *Trichoderma* Species Effective against Soil-Borne Pathogens

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Keywords

Biochemical, Molecular, Hydrolytic enzymes, *Trichoderma harzianum*, *Trichoderma virens*, Soil-borne pathogens.

Article Info

Accepted:
29 May 2017
Available Online:
10 June 2017

A B S T R A C T

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 *Trichoderma* species, based on internal transcribed spacer (ITS 1 & 4), translation elongation factor(*tef*-1) gene region and hydrolytic enzymes. In this study*tef*-1 was found to be better than ITS, to distinguish the *Trichoderma* isolates into two different species *viz.*, *Trichoderma virens* and *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 *Trichoderma*, tested against three different pathogens such as *Fusarium oxysporum*, *Rhizoctonia solani* and *Sclerotiumrolfsii*. It was also found that cultivation of *Trichoderma* isolates with soil borne pathogen (during interaction) produced high hydrolytic enzymes compared to *Trichoderma* species alone. Among the potential isolates tested for enzyme assay, three isolates *viz.*, V-7, V-19 and V-21 of *T. virens* and three isolates such as H-10, H-12 and H-21 of *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.

Introduction

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 *Trichoderma* (Druzhinina *et al*., 2005). Only morphological attributes are not enough to define the species of *Trichoderma* used against plant pathogens. The authentic identification of *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 *tef*-1 (gene) of the five *Trichoderma virens* isolates were analyzed (Chaverri *et al*., 2001). Hermosa *et al.*, 2004, attempted to analyze the genetic variability within bio-control isolates of *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 wallencompass 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 10^8cfu/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).

ß-1, 3 glucanase (E.C. 3.2.1.58)

ß-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).

ß-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-salicyclic 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 Mcllvaine'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 100^0 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 37^0 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 4^0 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 amplication 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, ß-1, 3-glucanase, ß-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), 18 (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

(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 *Sclerotiumrolfsii*, 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-21of *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-1* 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 didnot 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	ITS-1: 5'- TCCGTAGGTGAACCTGCGG-3' ITS-4: 5'-TCCTCCGCTTATTGATATGC-3'	(2)
Intron b/w 5thand6th exon of *tef* -1 region	*tef-1*fw: 5'-GTGAGCGTGGTA-TCACCA-3' *tef-1*rev: 5'GCCATCCTTGGAGACCAGC-3'	(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	Uttarakhand	KF144623	KF668105	*T. virens*
V-9	MTCC-1373	Soil	Pantnagar	Uttarakhand	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	Uttarakhand	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	Rhizosphere 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	Chikpearhizosphere 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	AndhraPradesh	KF144662	KF668144	*T. harzianum*
H-26	ITCC-7348	Soil	Bapatla	AndhraPradesh	KF144664	KF668146	*T. harzianum*
H-28	ITCC-7350	Soil	Bapatla	AndhraPradesh	KF144666	KF668148	*T. harzianum*

Table.3 Specific activity of hydrolytic enzymes produced by the *Trichoderma* isolates without interaction

	Specific activity IU mg^{-1}				
Isolates	**Cellulase**	**ß-1-3 glucanase**	**ß-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**	**0.79**
CD (p=0.05)	**3.10**	**2.23**	**1.86**	**1.42**	**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**	**1.01**
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	Specific activity IU mg^{-1}														
	Cellulase			ß-1,3 Glucanase			ß-1,4 Glucanase			Chitinase			Protease		
	F. oxysporum	*R. solani*	*S. rolfsii*	*F. oxyspoum*	*R. solani*	*S. rolfsii*	*F. oxysporum*	*R. solani*	*S. rolfsii*	*F. oxyspoum*	*R. solani*	*S. rolfsii*	*F. oxyspoum*	*R. solani*	*S. rolfsii*
V-7	**34.88**	**42.11**	10.71	12.21	**16.20**	14.70	**18.22**	10.46	16.32	20.56	**33.29**	20.31	**18.94**	**18.29**	**18.50**
V-8	10.65	22.84	15.28	**13.45**	8.27	6.31	14.44	7.29	10.58	18.56	13.30	18.14	8.65	8.23	6.29
V-9	15.06	11.71	12.93	10.48	9.67	6.92	9.36	7.53	8.30	10.27	14.02	19.60	7.70	11.10	8.14
V-17	9.58	15.69	15.69	11.89	9.75	8.92	13.71	**11.89**	14.78	**30.13**	**38.73**	14.98	12.06	15.87	12.05
V-19	22.94	**31.40**	**30.31**	**19.56**	**12.29**	**19.01**	**19.28**	**19.28**	**16.79**	20.31	22.58	**24.21**	**19.44**	18.17	17.13
V-21	**26.91**	15.17	**16.75**	10.02	10.82	**16.46**	17.52	10.68	**19.43**	24.33	18.70	**22.71**	17.11	**18.48**	**18.20**
V-22	9.21	12.72	10.31	9.71	8.61	6.31	8.21	9.21	8.76	17.24	13.58	12.24	8.30	9.06	6.43
V-23	13.73	16.62	11.48	9.97	10.19	7.83	9.22	4.83	9.60	**24.37**	14.22	19.56	7.63	8.37	6.83
V-4	8.41	8.83	7.71	6.03	4.06	4.06	7.57	6.73	9.46	8.57	12.16	14.99	2.60	4.19	3.29
V-18	7.55	10.38	8.49	7.41	7.14	6.20	7.28	5.93	7.89	9.89	11.28	11.81	6.21	7.44	4.87
SEm±	**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**
H-2	11.42	16.91	15.15	12.96	8.78	9.66	12.74	12.08	13.72	17.00	**51.72**	18.57	**16.32**	8.77	9.61
H-3	10.86	24.27	**26.31**	10.18	12.39	12.90	11.54	9.50	14.85	**49.29**	23.37	31.99	12.49	7.47	12.32
H-7	**18.88**	**28.82**	13.99	12.81	10.62	11.63	12.14	**13.32**	13.74	22.33	23.21	22.84	8.83	8.08	8.10
H-9	13.23	24.12	15.85	10.06	9.78	11.44	13.23	8.82	12.75	26.12	28.24	23.73	10.04	13.42	11.25
H-10	17.54	19.19	18.09	10.41	**15.90**	16.44	10.41	13.16	17.81	**83.78**	**62.63**	**88.80**	13.44	**31.37**	**23.88**
H-11	13.27	14.12	13.44	9.53	9.02	13.10	12.25	9.19	16.25	17.51	21.34	15.43	9.67	17.01	10.35
H-12	**20.83**	**52.07**	23.57	10.96	**16.44**	**18.09**	**15.35**	**13.70**	**19.59**	39.65	23.54	**43.56**	13.14	**21.90**	**23.88**
H-16	13.33	14.30	14.30	9.30	8.47	9.58	9.02	11.94	12.29	27.02	24.92	27.30	10.89	14.24	16.06
H-18	17.60	15.10	22.90	**13.90**	9.50	14.90	8.80	9.20	9.75	16.91	19.58	18.03	13.19	14.03	15.10
H-21	15.02	18.33	**29.22**	**13.03**	13.92	**18.78**	12.37	11.93	**22.42**	43.89	25.46	29.26	**14.20**	10.26	14.63
H-26	12.48	17.62	23.06	10.77	9.06	17.82	10.67	9.77	15.25	29.34	31.92	25.95	12.95	10.31	16.17
H-28	13.24	14.74	20.36	9.63	10.33	9.33	**13.34**	9.53	9.98	28.81	29.95	29.53	13.60	15.26	14.23
H-6	7.99	9.39	8.74	7.91	6.50	6.17	6.42	7.71	8.62	10.84	18.87	12.74	9.84	4.84	7.78
H-24	9.15	5.23	7.44	9.25	8.39	8.67	8.20	7.01	8.31	15.37	16.93	14.99	8.17	6.27	9.45
SEm±	**2.14**	**5.02**	**1.19**	**0.12**	**0.13**	**0.01**	**0.53**	**0.52**	**0.30**	**0.13**	**0.10**	**0.13**	**0.01**	**0.01**	**0.01**
CD (p=0.05)	**5.84**	**0.89**	**4.35**	**1.43**	**1.44**	**1.01**	**2.90**	**2.88**	**2.19**	**1.46**	**1.26**	**1.42**	**0.54**	**0.26**	**0.40**

Table.5 Grouping of *Trichoderma virens* and *Trichoderma harzianum* isolates based on specific enzymatic activity without and during interaction with soil-borne pathogens

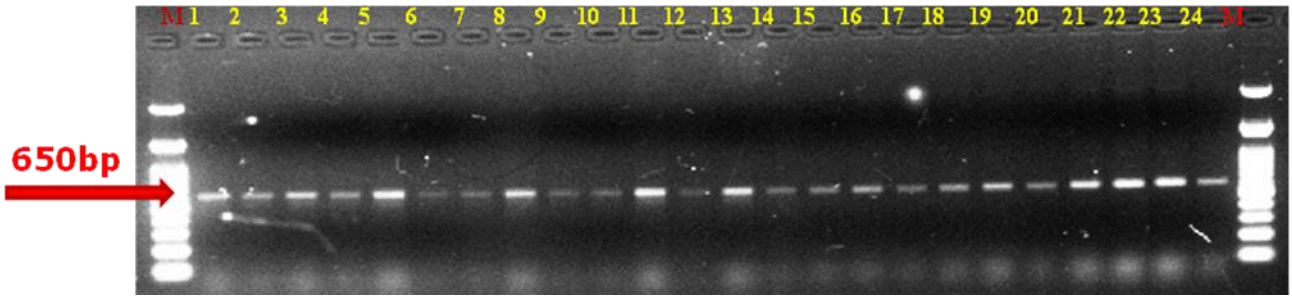
Interaction	Specific activity of enzymes	Groups	Name of the species	Cellulase	ß-1,3 Glucanase	ß-1,4 Glucanase	Chitinase	Protease
Trichoderma without interaction	High	Group-1 (>20 IU/mg) Specific activity	*T. virens*	V-19	None	None	None	None
			T. harzianum	None	None	None	H-7, H-10, H-16, H-21, H-26, 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-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-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
			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
Trichoderma species with *Fusarium oxysporum*	High	Group-1 (>20 IU/mg) Specific activity	*T. virens*	V-7, V-19, V-21	None	None	V-7, V-17, V-19, V-21, V-23	None
			T. harzianum	H-12	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-12, H-18, H-21, H-26	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

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

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

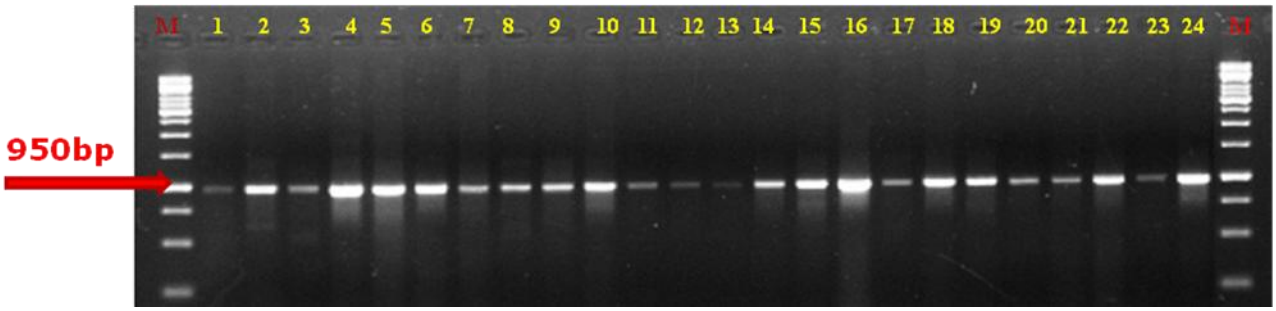
Name of the species	High potential	Low potential
Trichoderma virens	V-7, V-19, V-21 (03 isolates)	V-4 (01 isolates)
Trichodermaharzianum	H-10, H-12, H-21 (03 isolates)	H-6 (01 isolate)

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



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



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

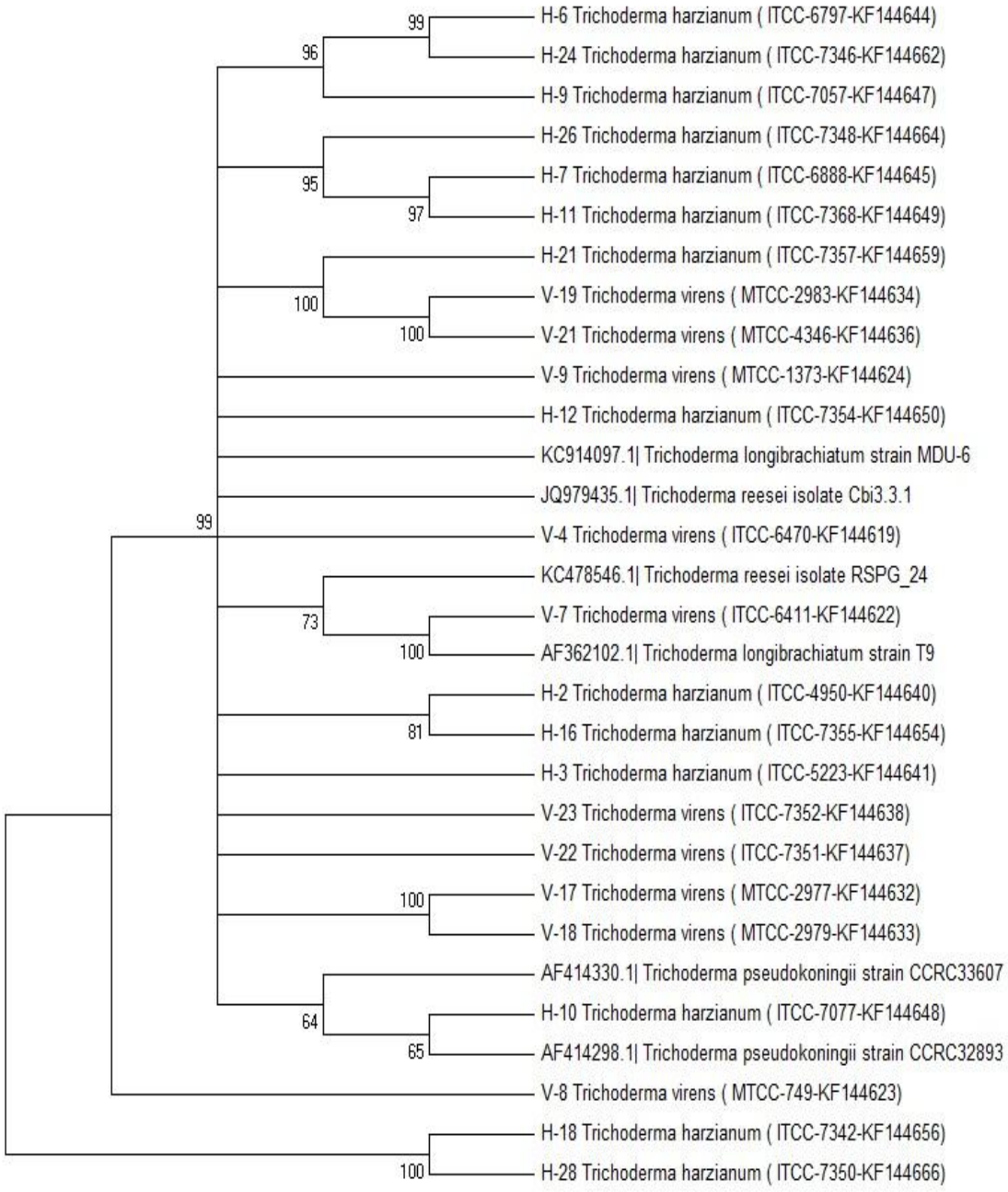
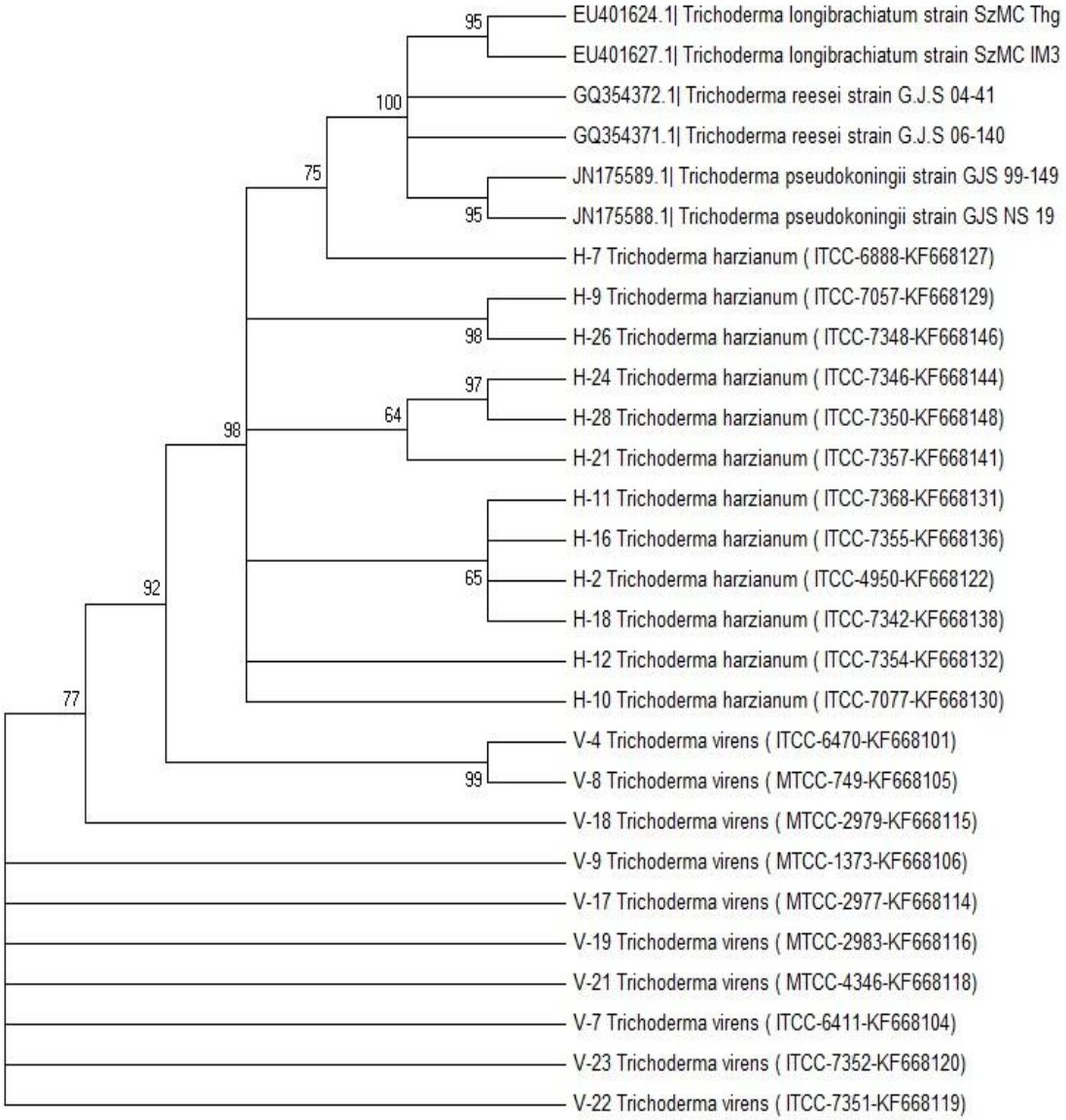


Fig.4 Phylogenetic relationship of twenty four isolates of *Trichoderma* species based on tef-1 region of nucleotide sequence aligned using software MEGA 5.2 v. The tree was generated by the Maximum parsimony analysis method



The highest hydrolytic enzymes production *viz.*, cellulase, ß-1, 3-glucanase, ß-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-4considered as a low potential based on the specific activity of the enzymes. Similarly, a total of14 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, ß-13, 3-glucanase, ß-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. virensviz.,*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.rolsfii*.

Acknowledgements: The authors are thankful to the ICAR–Indian Agriculture Research Institute for providing the facilities to carry out this study and UGC for financial support.

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How to cite this article:

Srinivasa, N., Deeba Kamil, Chandu Singh, Avinash Singode and Deeksha Gupta. 2017. Molecular and Biochemical Characterization of Potential Isolates of *Trichoderma* Species Effective against Soil-Borne Pathogens. *Int.J.Curr.Microbiol.App.Sci.* 6(6): 3132-3149.
doi: https://doi.org/10.20546/ijcmas.2017.606.370