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Genetic relatedness among *Trichoderma harzianum*, *T. asperellum* and *T. erinaceum* and their evaluation for management of Sclerotial blight of *Vigna radiata*

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Ten isolates of *Trichoderma harzianum*, three of *T. asperellum* and four of *T. erinaceum* obtained from forest soil, agricultural field and rhizosphere of plantation crops were characterized by morphological features as well as bright field and scanning electron microscopy. *In vitro* tests of the isolates of *Trichoderma* sp. showed significant inhibition against different fungal pathogens (*Sclerotium rolfsii, Rhizoctonia solani, Fusarium oxysporum, F. solani, F. graminareum* and *Macrophomina phaseolina)*. They also exhibited exo-and endo-chitinase activities. Their diversity was analysed using Denature Gradient Gel Electrophoresis (DGGE). Multiple sequence alignment of rDNA gene of these *Trichoderma* isolates was carried out in BIOEDIT and MEGA-4 software to compare with other ex-types gene sequences and their evolutionary distances. The isolates which were positively identified as *T. harzianum, T. asperellum* and *T. erinaceum* were used as an outgroup in these analyses. The isolates were evaluated for the management of Sclerotial blight of *Vigna radiata* in green house and field conditions. Direct application of these isolates in the rhizosphere of *Vigna radiata* significantly reduced Sclerotial blight incidence by enhancing key defense enzymes such as chitinase, β 1-3 glucanase, peroxidase and phenylalanine ammonia lyase.

Key words : *Trichoderma harzianum, T. asperellum, T. erinaceum, Vigna radiata*

INTRODUCTION

Trichoderma a genus under Ascomycota, Pezizomycotina, Sordariomycetes, Hypocreales, Hypocreaceae has gained adequate significance since last few decades due to its biological control activities against several plant pathogens (Chakraborty and Chakraborty, 2017). Besides, they are of great economic importance as sources of enzymes, antibiotics, plant growth promoters, xenobiotic degraders and most importantly as commercial biofungicides. Taxonomy of these groups of microorganisms is based largely on morphological character such as conidial form, size, colour and ornamentation and special branching pattern. It is at this point the correct identification of the organism is needed since different species show highly different characteristics, as for *e.g.* while most species are beneficial, two species (*T. longibrachiatum* and *T. citrinoviride*) are known to be opportunistic pathogens. Molecular methods have been introduced into *Trichoderma* taxonomy with revision of *Longibrachiatum* and *Trichoderma*, respectively and related teleomorphs. These techniques have been proven to be valuable tools in fungal taxonomy and their application has led to the reconsideration of several genera. The close morphological resemblance that exists between the different species of *Trichoderma* has been resolved clearly without any controversy using molecular and biochemical analysis.

Internal transcribed spacer sequences of Ribosomal DNA (rDNA) analysis and universally primed polymerase chain reaction have been used to categorize the isolates of *Trichoderma.* The Random Amplified Polymorphic DNA (RAPD) procedure that involves simultaneous amplification of several anonymous loci in the genome using primers of arbitrary sequence has been used for genetic, taxonomic and ecological studies of several fungi and this technique has been equally deployed to identify strains of *Trichoderma*.

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Advances in molecular ecology and genomics also indicate that the interactions of *Trichoderma* spp. with plants may have evolved as a result of saprotrophy on fungal biomass (mycotrophy) and various forms of parasitism on other fungi (mycoparasitism), combined with broad environmental opportunism (Druzhinina *et al.* 2011). The purpose of the present study was establishment of the gene sequences of *Trichoderma harzianum. T. asperellum* and *T. erinaceum* from North Bengal based on the sequence analysis of ITS regions of the rDNA gene for rapid identification and for analysis of genetic variability among the isolates, as well as comparison of biocontrol efficacy of the different isolates.

MATERIALS AND METHODS

Isolation of fungi

Soil samples were collected from rhizophere soil of plantation crops and agricultural fields of North Bengal. The location of soil samples were recorded through GIS mapping tool (Garmin). *Trichoderma* selective medium containing (g/l): $Ca(NO_3)_2$-1.0, KNO_3-0.26, $MgSO_4 \cdot 7H_2O$-0.26, KH_2PO_4-0.12,$CaCl_2 \cdot 2H_2O$-1.0, citric acid-0.05, sucrose-2.0, agar-20.0, chlorotetracycline-0.05,captan (50% wettable powder) was used for isolation of *Trichoderma* from soil samples.

Pathogen

Naturally infected plant parts/diseased tissues mostly from the lower portion of the stem and roots of *Vigna radiata* growing in North Bengal were collected and washed thoroughly with distilled water to remove all the soil particles, cut into small pieces (1cm) and surface sterilized with 0.1% of $HgCl_2$. All the pieces were transferred to sterile 100 ml beakers and rinsed 4-5 times with sterile water. Isolation was carried out on PDA slants and plates. Disease plant parts were incubated for 2-3 days at 25°C until the appearance of vegetative mycelium as white mycelial colonies from the infected portion. After that they were subcultured and maintained under optimum condition for future use.

Genomic DNA Extraction and purification

Isolation of fungal genomic DNAwas done from 3-4 d old fungal mycelia. For purification, genomic DNA was resuspended in 200 ìl 1 X TE buffer and incubated at 37°C for 30 min with RNAse. After incubation the sample was re-extracted with PCI (Phenol: Chloroform: Isoamylalcohol 25:24:1) and RNA free DNA was precipitated with chilled ethanol as described earlier. The quality and quantity of DNA was analyzed in 0.8% agarose gel and spectrophotometrically respectively. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA.

PCR amplification of ITS region

All isolates of *Trichoderma* were taken up for ITS-PCR amplification. Genomic DNA was amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase Chain Reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10 X Taq pol buffer, 1µl of 1 U Taq polymerase enzyme, 6 ìl 2 mM dNTPs, 1.5 µl of 100 mM reverse and forward primers and 1 µl of 50 ng template DNA. For amplification of the ITS1–5.8S–ITS2 region of *Trichoderma* isolates, the primer pair T/ITS1 TCTGTAGGTGAACCTGCGG and T/ITS4 TCCTCCGCTTATTGATATGC was used. PCR was programmed with an initial denaturing at 94°C for 5 min. followed by 30 cycles of denaturation at 94^0C for 30 sec, annealing at 59 °C for 30 sec and extension at 70 °C for 2 min and the final extension at 72 °C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 ìl) was mixed with loading buffer (8 m1) containing 0.25% bromophenol blue, 40 % w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1 % ethidium bromide for examining by with horizontal electrophoresis. PCR products were sent for sequencing to Genie, Bangalore.

Denaturing gradient gel electrophoresis (DGGE)

18S rDNA (200 bp with GC clamp) was amplified with the forward primer containing GC clamp at 5' end (F352T: 5'- CGC_CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG TGG C- 3' and 519r: 5'-ACC GCG GCT GCT GGC AC- 3') in 25 µl of reaction mixture containing 1×PCR buffer, 2.5mM $MgCl_2$ (Bangalore Genei, India), 100 ng of the template DNA, 25.0 pmol each of the forward and reverse primers, 250 µM each of dNTPs, and 1 U of *Taq* DNA

polymerase (Bangalore Genei, India). The touchdown PCR program was performed which consisted of an initial denaturation at 95°C for 5 min, followed by 6 cycles of 95°C for 1 min, 65°C for 1 min, and 72°C for 1 min, in which the annealing temperature was reduced by 0.5°C/cycle from the preceding cycle, and then 24 cycles of 95°C. Perpendicular DGGE was performed with "The Decode Universal Mutation Detection System" (Bio-Rad Laboratories, USA). A uniform gradient gel of 0% to 100% denaturant was prepared which was changed several times so as to optimize suitable concentration and finally 20 to 60% denaturant was found optimal for the best result.

Data analysis

Sequences were aligned following the Clustal Walgorithm included in the Megalign module (DNASTAR Inc.).Multiple alignment parameters used were gap penalty = 10 and gap length penalty = 10. Both of these values are aimed to prevent lengthy or excessive numbers of gaps. The default parameters (Ktuple= 2, gap penalty = 5, window = 4, and diagonals saved = 4) were used for the pairwise alignment. The use of Clustal W determines that, once a gap is inserted, it can only be removed by editing. Therefore, final alignment adjustments were made manually in order to remove artificial gaps. Phylogenetic analyses were completed using the MEGA package (version 4.01; Institute of Molecular Evolutionary Genetics, University Park, PA). Neither gaps (due to insertion-deletion events) nor equivocal sites were considered phylogenetically informative. Hence, complete deletion prevented the use of any of these sites in further analyses. Phylogenetic inference was performed by the UPGMA method. Bootstrap tests with 1,000 replications were conducted to examine the reliability of the interior branches and the validity of the trees obtained. An additional standard error test was performed with the data set using the same characters in order to evaluate the statistical confidence of the inferred phylogeny and finally, a principal coordinate analysis was performed in order to highlight the resolving power of the ordination. A two (2-D) and three dimensional (3-D) principal component analysis was constructed to provide another means and testing the relationship among accessions using EIGEN progamme (NTSYS-PC).

18S rDNA sequencing

Sequencing of 18S rDNA was done with forward primer from both the ends with ABI Prism (ABI-3700-96) automated DNA sequencer using Big Dye- Terminator cycle sequencing method. The sequences obtained were compared with nucleotide sequences present in GenBank using the BLASTn program. Sequence of desired genes (18S rDNA) was analyzed using BLASTn program of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Neighbor-joining phylogenetic tree was constructed in ClustalW (version 1.83) and bootstrapping for 1000 interactions was done.

Assay of exocelllulase and endocellulase activity

A combined assay for endo and exo cellulose activity in culture filtrate was carried out by Filter Paper Assay (Zhang *et al*. 2009) to determine endo and exo â-1, 4 glucanase activity and the amount of glucose released in the medium corresponding to the amount of substrate utilized. The substrate used was Whatman No. 1 filter paper which was homogenized in 0.2 M sodium acetate buffer, pH 5.5 (5 mg in 20 ml buffer). An aliquot of culture filtrate (0.5 ml) was added to 2ml of substrate. The mixture was incubated at 35°C for one hour, 2 ml of DNS reagent was added and reaction was terminated. Then it was heated in a boiling water bath for 5 min following which 1 ml of potassium sodium tartarate (40%) was added to the warm tubes. The tubes were allowed to cool and the absorbance was recorded at 540 nm in a U.V. Vis spectrophotometer.

Assay of Chitinase activity

Chitinase activity was assayed using the colorimetric method described by Agrawal and Kotasthane (2009). The assay mixture contained 1 mL of 0.5 % pure chitin (suspended in 50 mM acetate buffer pH=5.2) and 1 mL of enzyme solution. The reaction mixture was incubated for 12 h at 37 °C with shaking and was stopped by centrifugation (5000 g/min) for 10 min and the addition of 1 mL of dinitrosalicylate (DNS) reagent.

In vitro Antagonistic activity

For *in vitro* evaluation of antagonistic activity of fungal isolates following fungal pathogens viz.,

Sclerotium rolfsii, *Rhizoctonia solani* and *Fusarium oxysporum* were used. Isolated microorganisms were tested for their *in vitro* antifungal activity against plant pathogens by dual inoculation technique.

Mass Multiplication and application

For mass multiplication, wheat bran was used. Spore suspension (100 ml) of *Trichoderma* spp containing 10^6 spores/ml was used to inoculate 5 Kg of wheat bran.

Inoculation technique and disease assessment

Pot-grown 2-3 week old plants (*Vigna radiata*) were used for artificial inoculation in the rhizosphere with *Sclerotium rolfsii* grown in sand maize meal (9:1). Disease assessment was done 15 d after inoculation. In order to determine the effects of *Trichoderma* isolates showing *in vitro* antifungal activity on disease reduction, four treatments were taken into consideration. There were four treatments in the experiment: (a) distilled water (control); (b) S. *rolfsii* (c) *S. rolfsii + Trichoderma asperellum* and (d) *S. rolfsii + Trichoderma erinaceum* (e) *S. rolfsii* + *T. harzianum*. Disease intensity was calculated using a 0-6 scale. The experiment was conducted with four replicates per treatment where each pot served as a replicate, and the data was pooled for analysis.

Extraction and Estimation of defense enzymes

Post challenge activity of different defense enzyme were conducted after 12 h, 24 h, 36 h and 48 h after inoculation of the pathogen.

Biochemical assays of the following enzyme activities were conducted as follows:

Chitinase (EC. 3.2.1.39) was extracted and assayed by following the method described by Boller and Mauch (1988) with modifications.

Peroxidase (EC. 1.11.17) was extracted and activity was assayed following Chakraborty et al. (1993).

β-1,3- glucanase (EC.3.2.1.39) extraction and assay of enzyme activity of β-1,3- glucanase was done following the method described by Pan *et al.* (1991).

RESULTS

Isolation and identification of the microorganisms

Ten isolates of *T. harzianum*, three isolates of *T. asperellum* and four isolates of *T. erinaceum* were obtained on TSM medium from soils of higher altitude regions of North Bengal.

Morphological observation

Spore suspensions were prepared from individual culture. Drops of spore suspension were placed on clean grease free glass- slides, mounted with lacto phenol cotton blue, covered with cover slip and sealed. The slides were then observed under the Scanning electron microscopy following which spore characteristics were determined and size of spore measured. These isolates were also taken for determining their spore, conidia, phialide structures. These isolates were deposited to National Agriculturally Important Microbial Culture Collection (NAIMCC), Maunath Bhanjan and their accession numbers have been provided in Table 1.

Denaturing gradient gel electrophoretic (DGGE) analyses of Trichoderma

The isolates of *T. harzianum* (F-01955), *T. asperellum* (F-01967*)* and *T. erinaceum* (F-01960) were used in the present study. For this, 18S rDNA (320 bp with GC clamp) of each isolates of *T. harzianum* were amplified with the forward primer containing GC clamp at NS1 (5'-GTAGTCATATGCTTGTCTC-3') and GCfung (5'-CGCCCGCCGCGCCCCGCGCCC GGCCCG CCGCCCCCGCCCCA TTCCCCGTTAC CCGTTG-3'). In this uniform gradient gel of 0% to 100% and shorter run time could not separate the individual bands so as to optimize in suitable concentration for the running time was changed to 12h at 110V which resulting the a minimum separation of bands in the 40% region of denaturant gel. So, finally 20 to 60% denaturant was found optimal for the best result in 110V for 8h (Fig 1). The profile obtained after 8 hours of run time from 20-60 % gradient showed all the bands have co migrated however the profile obtained 12 hours of run time showed a close variation in presence or absence of dominant bands. The DGGE analysis demonstrated that

Table 1 : *Trichoderma* isolates from Forest, Riverine and Agricultural soil of North Bengal

Organisms	Type of soil	GPS location		NCBI	NAIMCC-
		Latitude	Longitude		
T. harzianum					
FS/C-90	FS	N26 º 47'49.16"	E88º 21.27.75"	GU187914	F-0157
FS/S-455	FS	N26 º 45'11.75"	E88º 23'28.27"	HM107420	F-01958
FS/S-458	FS	N 26 º 48'18.68"	E88º 21.14.61"	HM107421	F-01959
Ag/S471	AS	N 25 º 01'13.13"	E88º 08'98"	GU564469	F-01950
Ag/S479	AS	N 25 º 01'11.13"	E88º 68'88"	GU564470	F-01955
RHS/T- 460	RHS	N26 º 45.11.75"	E88º 23'28.27"	GU564471	F-01952
RHS/T- 477	RHS	N 26 º 48'18.68"	E88º 21'14.61"	HM117840	F-01962
RHS/M 501	RHS	N 27o02'43.17"	E 88º28'04.69"	HQ334993	F-01964
RHS/S 560	RHS	N 27o07'14.40"	E 88º06'18.70"	HQ334995	F-01966
RHS/S 559	RHS	N 27o07'14.40"	E 88º06'18.70"	HQ334997	F-01968
T. asperellum					
RHS/M512	RHS	N26 º 42'42.56"	E 88º 21'.15.47"	HQ265418	F-01963
RHS/M 517	RHS	N 27o07'14.40"	E 88º06'18.70"	HQ334994	F-01965
RHS/S 561	RHS	N 27o07'14.40"	E 88º06'18.70"	HQ334996	F-01967
T. erinaceum					
FS/L-20	FS	N26 º 45'13.08"	E88º23'28.72	HM107419	F-01949
FS/S-474	FS	N 26 º 48'18.68"	E88º 21'14.61"	GU187915	F-01960
FS/S-475	FS	N 26 º 48'18.64"	E88º 21'14.61"	GU191829	F-01953
FS/S-478	FS	N 26 º 48'18.68	E88 º 21'14.61	HM117841	F-01954

FS=Forest soil; AS= agricultural soil; RHS= Rhizosphere soil
NAIMCC= National Agriculturally Important Microbial Culture Collection

all the corresponding three bands on DGGE gels belonged to the isolates of *T. harzianum* (F-01955), *T. asperellum* (F-01967) and *T. erinaceum* (F-01960). A similar type of distinct band was formed for all selected isolates but two separate bands were formed in the gel due to their G+C variation in their ITS region of rDNA. The PCA analysis based on DGGE banding pattern of polymorphisms grouped the three different cluster (Fig 2). A few differences in clustering were

Table 2: Net exocellulase activity of selected fungal isolates

Organisms		Exo and endo cellulase activity (μg reducing sugar produced/ml/h)	Endocellulase activity (μg/ml/h)	Net exo-cellulase activity (μg/ml/h)
T. harzianum				
	F-0157	22.8	10	12.8±1.75
	F-01958	21.5	5	16.5±2.06
	F-01959	23.3	6.7	16.6±2.43
	F-01950	19.9	5.2	14.7±1.12
	F-01955	18.7	11.1	7.6±1.55
	F-01952	19	6.7	12.3±2.98
	F-01962	19.9	5.2	14.7±1.12
	F-01964	12.3	8.1	4.2±1.55
	F-01966	13.2	7.3	5.9±1.63
	F-01968	13.5	7.8	5.7±0.93
T. asperellum				
	F-01963	13.5	8.4	5.1±3.40
	F-01965	13.2	4.9	8.3±1.55
	F-01967	16.3	10.9	5.4±1.46
T. erinaceum				
	F-01949	21.2	11.5	9.7±1.96
	F-01960	19.9	5.2	14.7±1.12
	F-01953	18.7	11.1	7.6±1.55
	F-01954	19	6.7	12.3±2.98

observed with UPGMA clustering and principal coordinate analysis. *Trichoderma harzianum*, *Trichoderma asperellum* and *Trichoderma erinaceum* were grouped separately in PCA.

rDNA sequence analysis

In the present study, we focused on the ITS regions of ribosomal genes for the construction of primers that can be used to identify *Trichoderma* isolates. In case of *Trichoderma* ITS region of rDNA was amplified using genus specific ITS-1 and ITS-4 primers. Amplified products of size in the range of 600 bp were produced . All these PCR products were sequenced that could be aligned and showed satisfactory homology with ex-type strains of *Trichoderma harzianum*, *T. asperellum* and *T. erinaceum* sequences from the NCBI Genbank data base as analysed by BLAST. On the first approach all the conserved regions of 18S rDNA sequences with other ex-type isolate sequences obtained from NCBI Gene bank data base were analyzed using the bioinformatics tool BioEdit. A multiple sequence alignment was carried out that included the ITS region, including gaps and the complete sequences align. There were quite a number of gaps that were introduced in the multiple sequence alignment within the region

Table 3 : Chitinase activities of different isolates of *Trichoderma* spp.

Organisms	Chitinase activity	
	Exo*	Endo*
T. harzianum		
F-0157	4.35±0.55	26.59±0.46
F-01958	4.29±0.55	25.65±0.51
F-01959	4.24±0.63	24.24±0.23
F-01950	4.18±0.46	24.47±0.04
F-01955	4.34±0.46	23.82±0.46
F-01952	4.53±0.55	26.59±0.51
F-01962	4.41±0.55	24.24±0.52
F-01964	5.00±0.46	25.18±0.46
F-01966	4.76±0.04	28.71±0.24
F-01968	4.53±0.44	23.76±0.26
T. asperellum		
F-01963	4.65±0.63	27.8±0.69
F-01965	4.71±0.63	28.94±0.63
F-01967	3.94±0.46	24.47±0.46
T. erinaceum		
F-01949	4.47±0.40	25.41±0.24
F-01960	4.24±0.46	25.88±0.63
F-01953	4.47±0.93	25.65±0.46
F-01954	4.18±0.04	24.47±0.63

*Chitinase activity expressed as mg N-Acetyl glucosamine released/ ml culture filtrate/h (Endo) and mg N-Acetyl glucosamine released/ g mycelium /h (Exo).

that were closely related and similar sequence indicated whether the isolates were closely related (Fig. 3). From the sequence alignment observed between conserved region of isolates the evolutionary history was inferred using the UPGMA method (Fig 4). While analyzing the ITS sequences of *Trichoderma*, the optimal tree with the sum of branch length = 0.48437862 was obtained There were a total of 463 positions in the final dataset. In case of *T. harzianum* the optimal tree with the sum of branch length = 0.12242726 was obtained (Fig 5) where there were a total of 444 positions in the final dataset. The percentage of replicate trees in which the associated thirty four taxa available in the NCBI Database clustered together in the bootstrap test (10000 replicates) are shown next to the branches the tree is 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 computed using the Maximum Composite Likelihood method (Tamura *et al.* 2007) and are in

Table 4: Antagonistic activities against phytopathogens

Antagonists	*Rhizoctinia solani* Colony diam.(mm)		*Fusarium oxysporium* diam.(mm)		*Sclerotium rolfsii* Colony diam.(mm)	
	Pathogen	Inhibition (%)	Pathogen	Inhibition (%)	Pathogen	Inhibition (%)
T. harzianum						
F-0157	20	77.8	13	85.6	22	75.6
F-01958	22	75.6	20	77.8	22	75.6
F-01959	22	75.6	22	75.6	17	81.1
F-01950	23	74.4	17	81.1	14	84.4
F-01955	13	85.6	13	85.6	14	84.4
F-01952	22	75.6	17	81.1	14	84.4
F-01962	20	77.8	20	77.8	20	77.8
F-01964	22	75.6	22	75.6	22	75.6
F-01966	22	75.6	22	75.6	22	75.6
F-01968	12	86.7	13	85.6	11	87.8
CD (0.05) For *Trichoderma harzianum* isolates = 4.7 ; For pathogens = 2.6						
T. asperellum						
F-01963	14	84.4	19	78.9	17	81.1
F-01965	13	85.6	23	74.4	14	84.4
F-01967	13	85.6	14	84.4	19	78.9
CD (0.05) For *Trichoderma asperellum* isolates = 8.9; For pathogens = 4.5						
T. erinaceum						
F-01949	19	78.9	14	84.4	13	85.6
F-01960	14	84.4	14	84.4	14	84.4
F-01953	12	86.7	12	86.7	12	86.7
F-01954	29	67.8	31	65.6	36	60.0
CD (0.05) For *Trichoderma erinaceum* isolates = 6.2 ; For pathogens = 5.4						

Table 5: Activities of defense enzymes in *Vigna radiata* against *Sclerotium rofsii* following application of *Trichoderma* spp.

Treatments		Enzyme activity		
		Glucanase (μg/g/min)	Chitinase mg GlcNAC/g /min	Peroxidase ?OD/g/min
Untreated Healthy		19.7	13.9	63
Treated *T. harzianum*				
	F-0157	31.1	21.4	82.2
	F-01958	30.7	20.9	80.1
	F-01959	32.2	21.5	83.3
	F-01950	30.6	20.6	84.1
	F-01955	33.2	21.7	85.0
	F-01952	29.9	21.5	81.1
	F-01962	31.9[a]	20.5	82.9
	F-01964	32.8	19.6	80.8
	F-01966	32.7	21.9	83.6
	F-01968	31.6	22.5	84.7
T. asperellum				
	F-01963	33.2	23.5	84.1
	F-01965	33.6	22.7	85.7
	F-01967	34.7	24.0	86.0
T. erinaceum				
	F-01949	33.1	20.9	86.8
	F-01960	33.3	23.4	87.0
	F-01953	31.9	21.9	80.8
	F-01954	32.8	22.5	83.6
Inoculated *S. rofsii*		13.5	16.6	61
Treated and inoculated *S. rofsii* + *T. harzianum*				
	F-0157	28.7	18.8	76.8
	F-01958	27.9	17.6	73.0
	F-01959	27.8	17.8[a]	78.8
	F-01950	28.7	18.9	77.6
	F-01955	29.3	20.6	79.0
	F-01952	28.3	18.4	76.1
	F-01962	27.2	17.5	73.3
	F-01964	27.1	17.1	78.1
	F-01966	28.4	18.2	77.2
	F-01968	28.3	18.4	76.1
T. asperellum +*S. rofsii*				
	F-01963	29.1	20.7	81.6
	F-01965	29.4	20.2	83.1
	F-01967	30.1	21.9	84.0
T. erinaceum +*S. rofsii*				
	F-01949	28.2	18.5	79.3
	F-01960	29.1	22.8	81
	F-01953	26.2[a]	19.5	77.3
	F-01954	27.1	18.1[a]	79.1

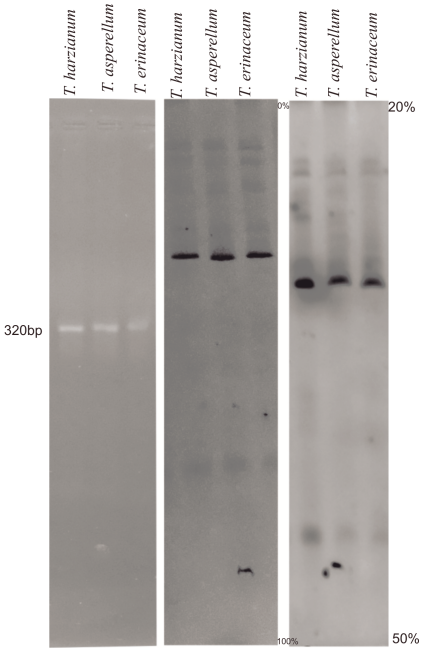

Fig. 1: DGGE analysis of of *Trichoderma harzianum, T, asperellum* and *T. erinaceum*

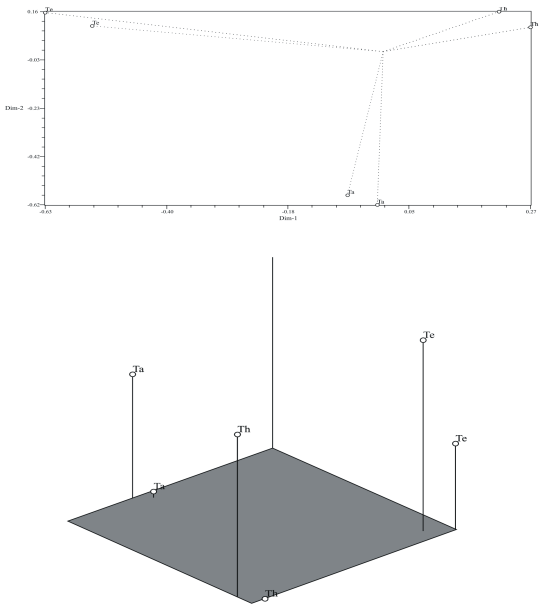

Fig. 2 : PCA of of *Trichoderma harzianum, T, asperellum* and *T. erinaceum*

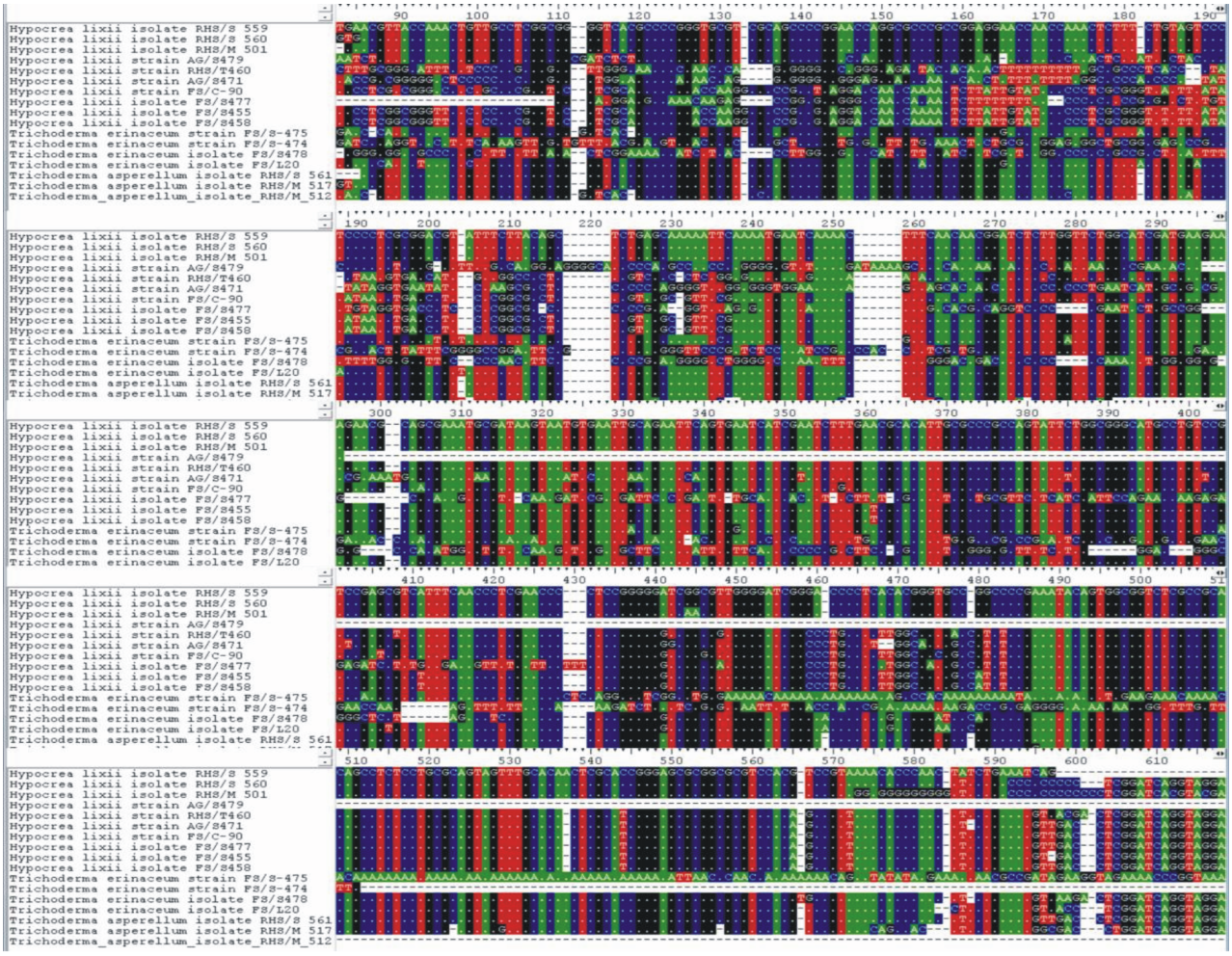

Fig. 3. 18S rDNA sequence alignments of *Trichoderma* isolates. Data for other species were gathered from NCBI.The conserved regions of the gene are demonstrated in different colour.

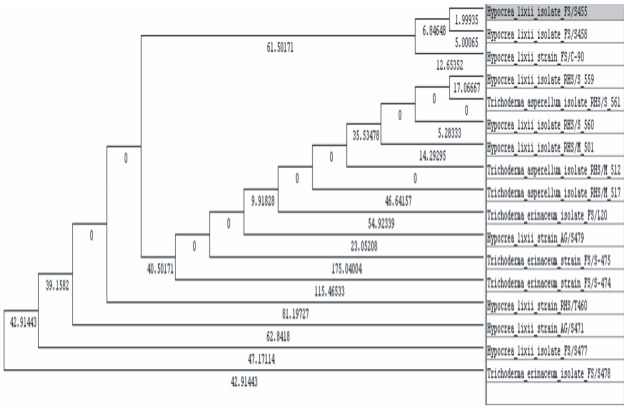

Fig. 4. Sequence alignments conserved regions of the rDNA gene of *Trichoderma* isolates.

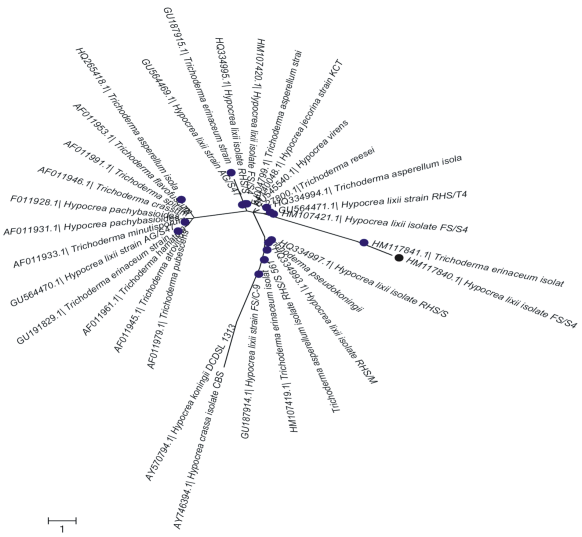

Fig. 5. The phylogenetic analyses conducted using the UPGMA method among the isolated *Trichoderma* spp form North Bengal region with other 33 ex-type isolates of *Trichoderma* spp of NCBI data base by MEGA4.1 software.

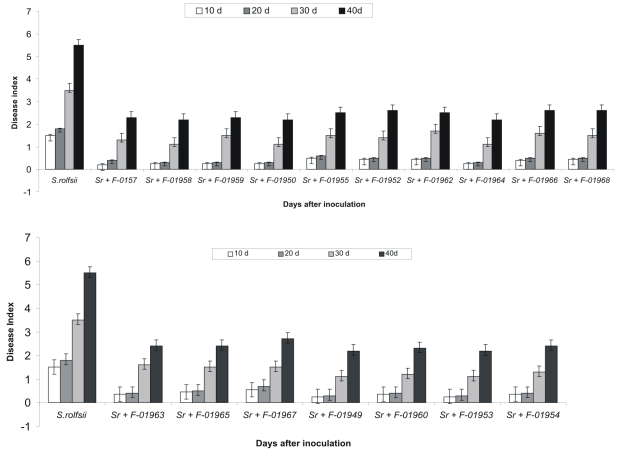

Fig. 6 Disease index of *Vigna radiata* after application *with T. harzianum. T. asperellum* and *T. erinaceum* followed by inoculation with *Sclerotium rolfsii*

the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option).

Cellulase activity of selected fungal isolates showing antifungal activities

The amount of glucose released by endocellulase activity of fungal hyphae during their growth using cellulose as C source was measured, where the culture filtrate was collected from the fermentation media by centrifugation. In the present study, 10 isolates of *T. harzianum*, 3 isolates of *T. asperellum* and 4 isolates of *T. erinaceum* were selected for determination of their cellulase activities. The isolates of *T. erinaceum* showed moderate cellulase activities while isolates of *T. harzianum* and *T. asperellum* showed good cellulase activities (Table 2).

Chitinase activities of Trichoderma isolates showing antifungal activities

Seventeen *Trichoderma* isolates which showed antagonistic activity were selected further for determining the chitinase activities for comparison among the isolates. Assay of both endo and exo chitinase activities of the different isolates revealed that *T. harzianum* (F-01964) had maximum activity while *T. asperellum* (F-01967) had minimum (Table 3).

In vitro test

In vitro interaction between *Rizoctonia solani*, *Fusarim oxysporum* and *Sclerotium rolfsii* and *Trichoderma* spp. as biocontrol fungi were studied following dual culture method. Interactions in the inhibition in percentage were recorded. *Trichoderma harzianum* tested was more virulent against *Sclerotium rolfsii* where inhibition percentage was greater in comparison to *T. asperellum*. and *T. erinacium*. In studying *Trichoderma* isolates and the pathogen in dual culture, all of the *Trichoderma* isolates had a marked significant inhibitory effect on the growth of the pathogens compared with their control. By 48 h after interaction between mycelia of *Trichoderma* isolates and the pathogens mycelia, a clear zone of interaction was formed in all *Trichoderma*-pathogen combination. Maximum inhibition was observed in *T. harzianum* (F-01955) pathogen combination (Table 4).

Disease suppression

In order to assess the biocontrol potential of the different species and isolates of *Trichoderma*, *T. harzianum*, *T. asperellum* and *T. erinaceum* were selected on the basis of their sporulation behaviour as *in vitro* antagonistic tests and tested against sclerotial blight of *Vigna radiata* caused by *Sclerotium rolfsii.* Results (Fig. 6) revealed that among the isolates tested, sclerotial blight disease development was markedly reduced with prior applications of one isolate each of *T. harzianum* (F-01955) and *T.asperellum* (F-01967). However all other isolates reduced the disease to some extent.

Defense enzymes in leaves of Vigna radiata in T. harzianum amended soil

The isolates of biocontrol agents (BCA) *T. harzianum* (F-01955), *T. asperellum* (F-01967) and *T. erinaceum* (F-01960) which showed significant disease reduction, were further tested *in vivo* and accumulation of defense enzymes in host plants (*Vigna radiata*) against *S. rolfsii* following application of *T. harzianum* were determined. Accumulation of three defense enzymes (peroxidase, ß-1, 3- glucanase and chitinase) were compared with untreated healthy control. Enzyme activities were higher in soil pretreated with *T. harzianum* as well as treated and inoculated plants in comparison to untreated healthy control (Table 5).

DISCUSSION

The ribosomal RNA genes (rDNA) possess characteristics that are suitable for the identification of fungal isolates at the species level. rDNA are highly stable and exhibit a mosaic of conserved and diverse regions within the genome. They also occur in multiple copies with up to 200 copies per haploid genome arranged in tandem repeats with each repeat consisting of the 18S small subunit (SSU), the 5.8S, and the 28S large subunit (LSU) genes. The most prominent fungal marker genes are the Internal Transcribed Spacers (ITS) and the 28S and 18S rRNA gene sequences. While the ITS is the best candidate for species barcoding, it lacks the phylogenetic power, that is why classification of environmental sequences with ITS is often limited to kingdom or phylum level. In contrast, the 18S rRNA gene sequence rarely resolves fungal taxa to species or genus level but is an important phylogenetic marker for a reliable classification of undescribed fungal taxa (Yarza *et al*. 2017).

We have demonstrated that the analysis of aligned rDNA sequences is a reliable clustering strategy for identification purposes in a variety of taxonomic groups and systemic levels. While this approach was previously applied in analyzing complete genome data, the present study shows that it is also applicable in analyzing much shorter DNA sequences from a single gene, which is going to be the fundamental block in the massive rDNA database. This analysis could have other applications in DNA barcoding besides in cluster analysis. The determination of frequencies of DNA strings would enable easy identification of taxon-specific strings that can be used as taxon specific probes in DNA chip for species identification. *Trichoderma harzianum, Trichoderma asperellum* and *Trichoderma erinaceum* under study could be identified very easily on the basis of available similar “r DNA-based sequence” from the available database.

Such comparative studies will provide intriguing new insights into the physiology of *Trichoderma* species with different lifestyles, e.g. mycoparasites and efficient plant cell wall degraders. Application of high-throughput genome, transcriptome, and metabolome analysis of native and mutant strains will result in the identification of novel genes with specialized functions that are relevant to biomass degradation, biocontrol, and human pathogenicity (Mukherjee *et al.* 2013).The activity of defense related enzymes were also greatly enhanced in this present study following treatment of the biocontrol agent. *Trichoderma* spp. have evolved multiple mechanisms that result in improvements in plant resistance to disease and plant growth and productivity (Chakraborty and Chakraborty, 2017).

The direct effects of *Trichoderma* spp. on plants are remarkable and at least as significant as their direct effects on other fungi and have only recently been described. First, the fungi are highly efficient inducers of systemic and localized resistance in plants. Another mechanism proposed to explain biocontrol activity by *Trichoderma* species is that of induction of resistance in the host plant by treatment with the biocontrol agent. The plant response was marked by an increase in peroxidase activity. Increased enzyme activities

were observed in both roots and leaves. Later, inoculation of cucumber roots with *T. harzianum* (T-203) also induced an array of pathogenesis-related proteins, including a number of hydrolytic enzymes. Several authors have reported the ability of *Trichoderma* to induce resistance in plants (Tucci *et al*. 2011; Yoshioka *et al*. 2012; Wu *et al*. 2017).

In our present investigation the peroxidase activity was significantly enhanced in treated seedlings than control. In a similar study, it has been demonstrated that seed treatment of cotton with biocontrol preparations of *T. virens* (G-6, G-11, G6-5) or application of *T. virens* culture filtrate to cotton seedling radicles induced synthesis of much higher concentrations of the terpenoids desoxyhemigossypol (dHG), hemigossypol (HG), and gossypol (G) in developing roots than those found in untreated controls. In addition to terpenoid synthesis, treatment of cotton roots with *T. virens* also induced significantly higher levels of peroxidase activity than that found in control roots. *Trichoderma* spp. have been known as biocontrol agents for the control of plant diseases for decades (Harman *et al.* 2004). Biocontrol, in many cases, is not only related to their abilities to produce antibiotics, establish parasitic interactions, or otherwise directly affect pathogens (Harman *et al.* 2004; Howell 2003). Instead, it is now clear that, in many cases, the best strains also will fully colonize roots as they expand and grow (Harman 2011). They clearly are endophytic plant symbionts that also have the ability to proliferate and grow in soil. The establishment of root colonization and chemical communication by *Trichoderma* strains strongly affect plant physiology by changing plant gene expression, as documented by several groups (Harman 2011). The beneficial fungi may induce systemic resistance that is mediated by alterations in plant gene expression. In our present study the selected isolate of *Trichoderma harzianum* could both enhance growth and defense enzymes in *Vigna radiata.*

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