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Biodiversity and Molecular Characterization of *Trichoderma* spp. and Exploring its Synergistic Action for the Management of Cucumber Damping Off Incited by *Pythium aphanidermatum*

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Bioversity of fungal antagonist, *Trichoderma* from different locations of Nilgiri district of Tamil Nadu, India, were characterized through molecular methods. Thirty four isolates were tentatively identified as *Trichoderma* and confirmed upto species level through molecular tools. PCR amplification of the 18s-28s rRNA gene region revealed that all thirty four isolates produced amplicon size of 600bp and were further confirmed through sequencing. The sequences of various *Trichoderma* spp. were compared with both NCBI and TrichOKEY database to validate their molecular identity. Among 34 isolates, 31 isolates were identified as *T. asperellum* (KT462693, KU361372, KX533978 to KX533999, KX523262 to KX523264, KX555650, KX147092 to KX147094, KX5334000), 2 isolates as *T. harzianum* (KX533989, KX533990) and 1 isolate as *T. virens* (KU666466) through NCBI data base. However, all the *T. asperellum* isolates identified through NCBI database were identified as *T. koningiopsis* using TricHOKEY data base. Identity of *T. harzianum* isolates (TRI 35 and TRI 36) and *T. virens* isolate (TRI 37) were same in both NCBI and TricHOKEY database. Antagonistic assay with diverse species of *Trichoderma* revealed that *T. virens* (TRI 37) was effective in inhibiting the radial growth of *Pythium aphanidermatum* (87.78%) followed by *T. harzianum* (TRI 35), (TRI 36) and *T. asperellum* (TRI 9) *in vitro*. The effective isolates *T. virens* (TRI 37), *T. harzianum* (TRI 35, TRI 36) and *T. asperellum* (TRI 9) were compatible with each other. Biopriming of cucumber seeds with talc based formulation of the consortia comprising of *T. virens* isolate (TRI 37), *T. harzianum* isolates (TRI 35 and TRI 36) and *T. asperellum* TRI 9 @ 10^8 cfu/g and soil application suppressed damping off to an extent of 76.82% over untreated control.

Keywords: Chlamydospore ,Conidiophore , ITS , Phylogenetic analysis, *Trichoderma*.

Trichoderma is a filamentous, soil-borne, mycoparasitic fungus found in plant root ecosystem[32] with antifungal activity against several soil-borne plant pathogens[15]. Antagonistic action is attributed through competition for space and nutrients[23], production of siderophores [3,29], synthesis of inhibitory compounds (pyrone antibiotics)[5] and the release of cell wall lytic enzymes including cellulytic, chitinolytic, pectinolytic, proteolytic and lipolytic enzymes[6]. Besides the antagonistic activity, *Trichoderma* is an opportunistic, avirulent plant symbiont. Rhizosphere colonization by *Trichoderma* stimulate plant defense, plant growth and reproductive capacity[4,12]. Meyer[21] demonstrated the diversity in conidial ornamentation, mitochondrial DNA and plasmids among strains having warted conidia and warranted for taxonomic revision. Recently Polymerase Chain Reaction (PCR) has been used for identification of fungal species. Genotypic techniques involving the amplification of a phylogenetically informative target, such as the small-subunit (18S) rRNA gene are increasingly gaining importance[35]. rRNA gene is essential for the survival of all cells and the genes encoding the rRNA are highly conserved in the fungal kingdom. The

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rRNA genes are universally conserved, while the ITS region and intergenic spacer (IGS) are highly variable[19]. The ITS and IGS region are the fastest evolving regions and varies among the species within a genus. Thus, the sequences of these regions were used for identification of closely related species[34]. The diversity of Trichoderma has been used for the management of soil-borne diseases. Among the soil-borne diseases, damping off caused by the genus *Pythium* is a common problem in fields and greenhouse grown crops which kills the seedlings. This disease complex usually involves other pathogens such as *Fusarium*, *Phytophthora* and *Rhizoctonia*. Pre - and post-emergence damping-off caused by *Pythium* spp. in vegetable crops are economically important worldwide[33]. Rapid germination of sporangia of *Pythium* in 1.5–2.5 h after exposure to exudates or volatiles from seeds or roots[22] followed by immediate infection makes management of the pathogen very difficult[33]. *Pythium* spp. tends to be generalistic and non-specific in their host range, which causes extensive and devastating root rot is often very difficult to prevent or control[17]. With this background the present study was undertaken for characterization of bio geographical diversity of *Trichoderma* by morphological and molecular means to explore the antagonistic potential against damping off pathogen in cucumber under protected cultivation.

MATERIALS AND METHODS

Sampling and Isolation of *Trichoderma*

Rhizospheric soil samples were collected from different crop fields of Nilgiri district, Tamil Nadu, India. *Trichoderma* were isolated from the rhizospheric soil samples on *Trichoderma* selective medium[10] using serial dilution technique[26]. The plates were incubated at 28±2°C for 4 to 7 days. Visible fungal colonies were transferred to Potato dextrose agar (PDA) plates and incubated at 28±2°C for 5 days and maintained on PDA medium for subsequent studies.

Molecular characterization of *Trichoderma* spp.

Genomic DNA extraction from *Trichoderma* isolates

Extraction of genomic DNA of all the isolates of *Trichoderma* spp. were extracted by harvesting the mycelium grown in potato dextrose broth for 3-4 days at 28±2°C. Mycelial mat was collected on filter paper, washed with distilled water for 2-3 times , frozen and used for DNA extraction. Genomic DNA was extracted as per the protocol described by Raeder and Broda[24]. DNA was suspended in 50µl of TE buffer and quantified with ethidium bromide fluorescence.

PCR amplification and sequencing

Primers ITS1 (5'-TCCGTAGGTGAACCT GCGG-3') and ITS4 (5'-TCCTCCGCTTAT TGATATGC-3') described by White *et al*[34] were used to amplify a fragment of rDNA including ITS1 and ITS2 and the 5.8S rDNA gene. The PCR amplification reactions were performed in a 50 ¼l mixture containing 50 mM KCl, 20 mM Tris HCl (pH 8.4), 2.0 mM MgCl2, 200 ¼M of each of the four deoxynucleotide triphosphates (dNTPs), 0.2 ¼M of each primer, 40 ·g/¼l of template and 2.5 U of Taq polymerase. The cycle parameters included an initial denaturation of 1 min at 95°C, followed by 35 cycles of 1 min at 95°C, 30 s at 60°C and 1.5 min at 72°C, with a final extension of 10 min at 72°C. The PCR products were resolved in 1% agarose gel, purified PCR product was sequenced in SciGenome Labs Pvt Ltd , Kerala.

Phylogenetic analysis

The rDNA homology searches were performed using ITS gene sequences by BLAST program (http://www.ncbi.nlm.nih.gov). Seqeces were compared with *Trichoderma* spp. isolates retrieved from the Genbank database. Newly obtained sequences were submitted in

Genbank database (NCBI). Sequences were analyzed in pairwise and multiple sequence alignment and the identity was scored with the Bio-Edit V 7.0.5[11]. Phylogenetic tree was constructed by the neighbor joining method and tree topologies were evaluated by performing bootstrap analysis of 1000 data sets performed with MEGA 6 (Molecular Evolutionary Genetic Analysis) software[31]. The rDNA homology searches were performed using ITS gene sequences by TrichOKEY program (http://www.isth.com). Sequences were compared with *Trichoderma* spp. isolates retrieved from the TrichOKEY database.

Isolation of *Pythium* from infected cucumber plants

The pathogen *Pythium* was isolated from damping off affected cucumber plants collected from major cucumber growing areas of Coimbatore, Erode and Madurai districts of Tamil Nadu. The infected plant tissue was washed with sterile water and cut into small pieces from the leading edges of lesions. Then surface sterilized with 0.1% mercuric chloride, washed with sterile distilled water thrice and shade dried on sterile filter paper. The dried pieces were plated on PDA and incubated at 28°C ± 2°C for 5 days.

Molecular Characterization of *Pythium* isolate

Isolation of genomic DNA of *Pythium* sp.

Genomic DNA was extracted from the suspension cultures of *Pythium* by the Cetyl Trimethyl Ammonium Bromide (CTAB) method as described by Lee and Taylor[18]. The isolate of *Pythium* was grown at room temperature (28 ± 2°C), and transferred into 250 ml conical flasks containing 150 ml potato dextrose broth (PDB). It was incubated at 28 ± 2°C for 5 days. After complete colonization of the medium, the mycelium was harvested by filtration through sterile filter paper and stored at -80°C until used for DNA extraction. DNA was extracted from the harvested

mycelia according to the procedure described by Mahuku[20] . Mycelia were ground to a fine powder in liquid nitrogen and suspended in CTAB buffer.

The mixture was incubated at 65°C for 30 min. DNA was precipitated using ice-cold isopropanol and the pellet was washed with 70% ethanol, dried and dissolved in TE buffer.

Identification of *Pythium* sp.

To identify the species of *Pythium* isolates of 16S rDNA intervening sequence specific Pa1-(5'TCCACGTGAACCGTTGAAATC3');ITS2-(5'GCTGCGTTCTTCATCGATGC-3') primers were used to get an amplicon of 210 bp size[13]. PCR amplification reactions were performed in a 50 ¼l mixture containing 50 mM KCl, 20 mM Tris HCl (pH 8.4), 2.0 mM MgCl2, 200 ¼M of each of the four deoxynucleotide triphosphates (dNTPs), 0.2 ¼M of each primer, 40 ·g/¼l of template and 2.5 U of Taq polymerase. Amplification was conducted with a total reaction volume of 50µl in Eppendorf Master Cycler, German. The PCR settings used were as follows: a hold of 2 min at 95°C, 30 cycles of 1min at 94°C, 30 sec at 54°C and 1min at 72°C and a final extension of 10min at 72°C. The PCR products were resolved on 1% agarose gel at 50 V, stained with ethidium bromide (0.5µg/ml) and analyzed using gel documentation system.

Screening of *Trichoderma* spp against *P. aphanidermatum*

The antifungal activity of *Trichoderma* spp. was tested by dual culture technique[7]. The pathogen and *Trichoderma* were grown on PDA for a week at room temperature (28 ± 2°C), about nine mm diameter mycelial disc of the pathogen *(Pythium aphanidermatum)* was cut from the periphery and transferred to the Petri plate with PDA and nine mm diameter mycelial disc of *Trichoderma* was placed simultaneously at opposite sides of same Petriplate aseptically and incubated at room temperature 28 ± 2°C with alternate light and darkness for 7 days and observed periodically. The experiment was replicated thrice and per cent growth inhibition was calculated by the formula of I = (C-T)/C x 100, where C is mycelial growth in control plate, T is mycelial growth of test organisms in inoculated plate and I is inhibition of mycelial growth. Hyperparasitism was calculated by measuring the overgrowth of *Trichoderma* isolates on the pathogen from the zone of interaction of *Trichoderma* with pathogen in centimeter.

Testing the efficacy of *Trichoderma* spp. against *P. aphanidermatum* in green house

The efficacy of *Trichoderma* spp. against damping off pathogen was evaluated with the four effective *Trichoderma* isolates *viz.,T. virens* (TRI 37), *T. harzianum* isolates (TRI 36, TRI 35) and *T. asperellum* isolate (TRI 9) in pot culture. Treatment details include T1- Biopriming (BP) with TRI 37 @ 10g/kg of seeds, T2- BP with TRI 36 @ 10g/kg of seeds, T3- BP with TRI 35 @ 10g/kg of seeds,T4- BP with TRI 9 @ 10g/kg of seeds,T5- BP with (TRI 37+TRI 36+TRI 35+TRI 9) @ 10g/kg of seeds, T6- Soil application (SA) with TRI 37 @ 2.5kg/ha at 15 and 30th days after seeding, T7 - SA with TRI 36 @ 2.5kg/ha at 15 and 30th days after seeding,T8- SA with TRI 35 @ 2.5kg/ha at 15 and 30th days after seeding, T9- SA with TRI 9 @ 2.5kg/ha at 15 and 30th days after seeding, T10- SA with (TRI 37+TRI 36+TRI 35+TRI 9) @ 2.5kg/ha at 15 and 30th days after seeding, T11- BP+SA with TRI 37 @ 10g/kg of seeds+@ 2.5kg/ha at 15 and 30th days after seeding,T12- BP+SA with TRI 36 @ 10g/kg of seeds+@ 2.5kg/ha at 15 and 30th days after seeding,T13- BP+SA with TRI 35 @ 10g/kg of seeds+@ 2.5kg/ha at 15 and 30th days after seeding, T14- BP+SA with TRI 9 @ 10g/kg of seeds+@ 2.5kg/ha at 15 and 30th days after seeding, T15- BP+SA with (TRI 37+TRI 36+TRI 35+TRI 9) @ 10g/kg of seeds+@ 2.5kg/ha at 15 and 30th days after seeding ,T16- BP+SA with Metalaxyl 2g/kg of seeds + 0.1% @ 15 and 30th days after seeding,T17- Un treated control. The treatments were replicated thrice and pathogen inoculated control was maintained. Five cucumber seeds were planted in each pot containing sterile potting medium (red soil: sand: FYM at 1:1:1 w /w/w). The pathogen was multiplied in sand maize medium and incorporated @ 10g per pot up to the depth of 10cm @10^5cfu/g. *Trichoderma* was delivered through bio priming of seed and soil application with different combinations. Seeds were bioprimed with talc based bio formulation @ 10 g/ kg followed by two soil applications on 30 and 45 days after sowing @ 2.5 kg/ha. Plants inoculated with the pathogen alone served as control. Healthy controls were also maintained. Disease incidence was recorded after 20 days of sowing and per cent disease incidence was calculated as follows.

$$\text{Disease incidence (\%)} = \frac{\text{Number of plants affected}}{\text{Total number of plant}} \times 100$$

The experimental design was completely randomized with three replicates (pots) for each treatment and repeated twice.

RESULTS AND DISCUSSION

Isolation of *Trichoderma* spp.

A total of 34 isolates of *Trichoderma* were isolated from different rhizosphere soil samples of different crop plants. Isolate code, species identification, location, NCBI accession numbers, TrichOKEY identification and isolation details of *Trichoderma* strains are furnished in Table 1.

Molecular characterization of *Trichoderma* spp.

PCR amplification with the conserved primer

(ITS 1 -5'TCTGTAGGTGAACCTGCG 3') and ITS 4-5'TCCTCCGCTTATTGATATGC 3') of ITS region yielded the genomic product of 600 bp (Fig 1) in the reactions performed with 34 isolates of *Trichoderma* species. Absence of size variation among the isolates collected suggest that, majority of the isolates belong to *Trichoderma.*

DNA sequencing

The size of the amplicon containing the ITS1, ITS2 and 5.8S r RNA was around 600 bp. In order to ascertain the *Trichoderma* orgin of sequence, the sequences were initially analyzed in BLAST. In the BLAST analysis isolates TRI 1 to TRI 16, TRI 19 to TRI 21, TRI 23- TRI 29, 38, 50, 60 and 70 had highest identity with *T. asperellum*. The query coverage was between 93-100% and identity was between 96-100%. In the case of other next three isolates TRI 35, 36 and TRI 37, isolate TRI 35 and 36 exhibited maximum identities with *T. harzianum* isolate TRI 37 with *T. virens*. On the basis of identity search in BLAST the isolates collected in the present study could be clearly categorized into three groups.1. Major group comprising isolates were identified as *T. asperellum* and another small group of two isolates belonging *T. harzianum* and one belonging to *T. virens*(table) To overcome this problem the International Commission of Taxonomy of Fungi has recommended the use of DNA barcode tools for correct identification of *Hypocrea* and *Trichoderma* species. Therefore the ITS neucleotide sequence of the 34 isolates of the present study were analysed in TrichOKEY programme (www.isth.info) contrasting to results obtained in BLAST search in TrichOKEY analysis. The 34 isolates could be differentiated into 4 groups, one major group comprise isolate TRI 2-13, 16, 19, 20, 24, 26, 27, 28, 38, 50, 60 and 70 belonging to *T. koningiopsis* pertaining to the *Rufa clade*. The second group consists of isolates TRI 35 and TRI 36 which were confirmed as *T. harzianum* under catopteron clade, third group comprised isolate TRI 37 which was identified as *T. virens* belonging to virens clade, fourth group comprised of isolate TRI 15 which was identified as *T. asperellum* belonging to pachybasium A clade. Comparision of the results of TrichOKEY with BLAST indicate that the isolates TRI 2-13, 16, 19, 20, 24, 26, 27, 28, 38, 50, 60 and 70 under BLAST search were identified as *T. asperellum*. The other two group of isolates were identified as *T. harzianum* and *T. virens* by BLAST and appeared to be similar to TrichOKEY analysis. The six isolates (TRI 1, 14, 21, 23, 25 and 29) identified as *T. asperellum* in BLAST were found to have only genus specific hall mark sequences in TrichOKEY. However further species identification was

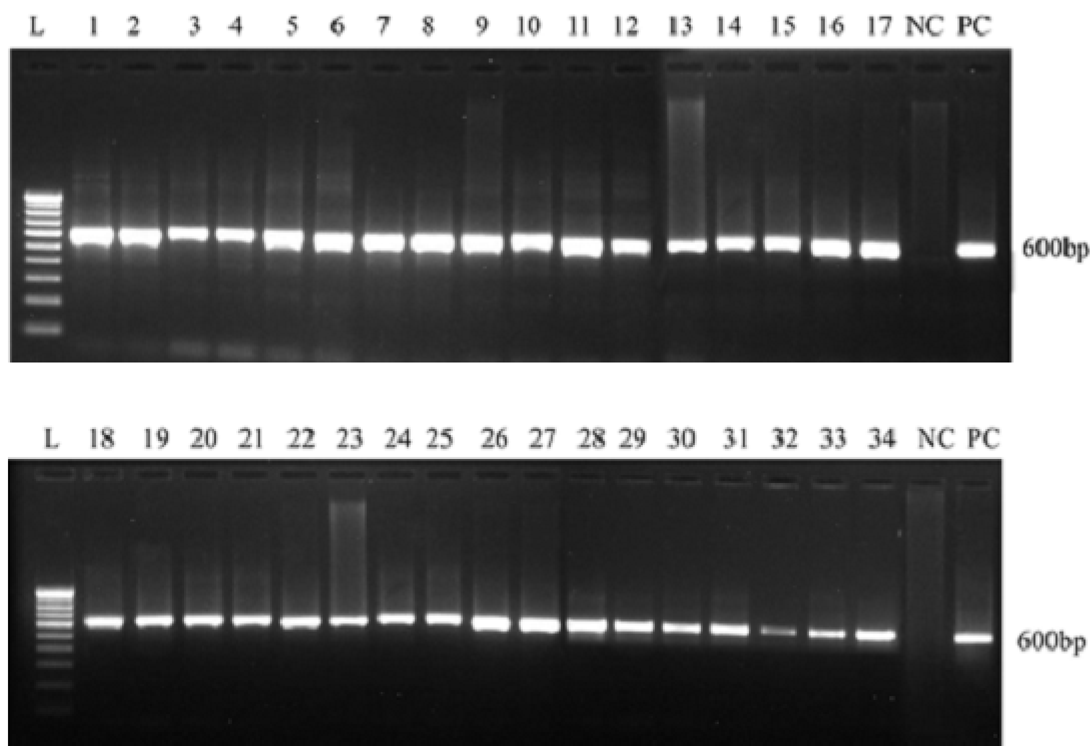

Fig. 1. Molecular confirmation of *Trichodema* isolates with ITS primers

not possible in TrichOKEY since species specific hall mark were not detectable, the isolates are therefore considered as unidentified species under the genus *Trichoderma*.

Diversity study of *Trichoderma* spp.

The result of the phylogenetic analysis based on the 18S-28S-rRNA gene sequences of different species of *Trichoderma* isolates were analyzed and results revealed that three different clusters were formed in phylogenetic tree (Fig 2). The evolutionary history was inferred using the Neighbor-Joining method Saitou and Nei[25]. The optimal tree with the sum of branch length = 1.69400390. The difficulty in identification of species using NCBI similarity search tool, BLAST (http://blast.ncbi.nlm.nih.gov). has been expressed by several workers[8]. The lacunae in identifying species on the basis of similarity search in BLAST are absence of quality control of species authentification, sequences deposited under the original names and not under the names after verification. Kredics *et al*[16] suggested that more than 40% of *Hypocreae* and *Trichoderma* sequences available in Genbank database are unidentified or misidentified at the species level . In the present study the isolate which had maximum hit with *T. asperellum* were identified as

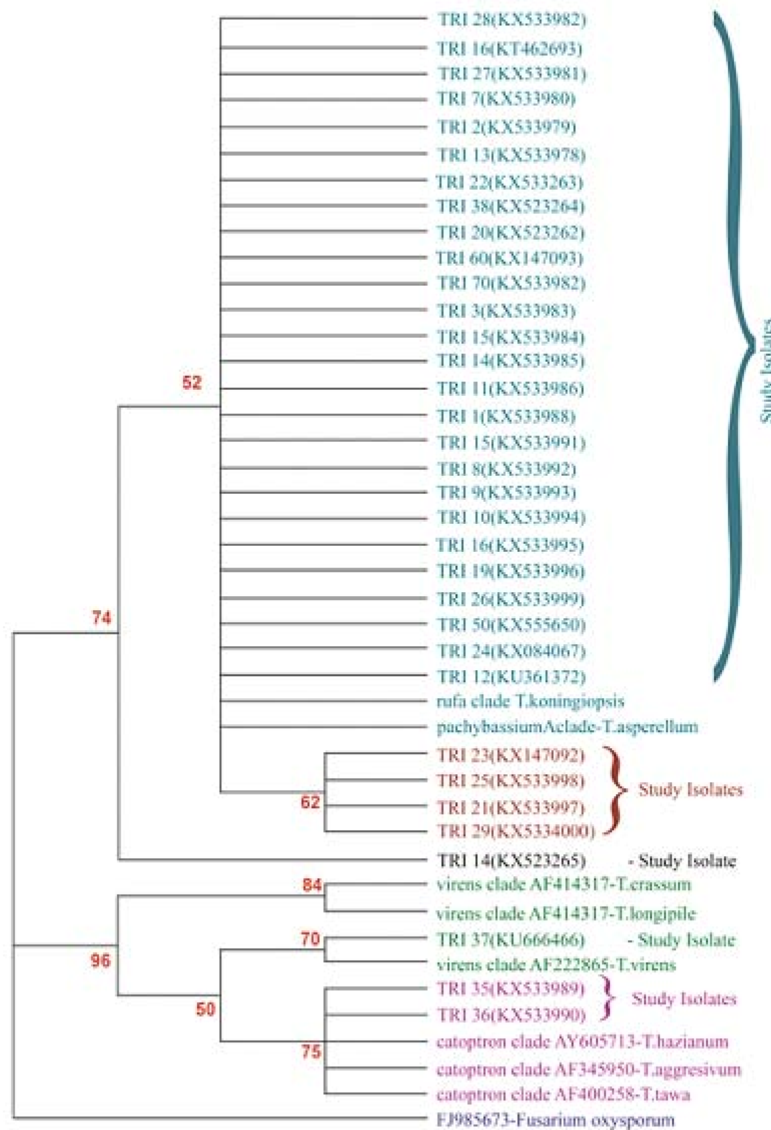

Fig. 2. Phylogenetic tree of the *Trichoderma* isolates. The numbers given over branches indicate bootstrap co-efficient

T. koningiopsis in TrichOKEY. However identification of the other isolates as *T. virens* , *T. harzianum* tally both in BLAST and TrichOKEY search.

The relationship between the *Trichoderma* isolates of the present study and the established species in the TrichOKEY programme were assessed further. The type sequences of the identified species, *T. koningiopsis*, *T. asperellum*, *T. virens* and *T. harzianum* belonging to clade Rufa, Pachybasium A,Virens and Catopteron (*harzianum* clade) were retrived from database and analyzed in multiple alignment in CLUSTAL-W programme. A phylogenetic tree was constructed based on alignment clearly revealed the species identity of the isolates under study. The TRI isolates 2-13, 15, 16, 19, 20, 22, 24, 26-28, 38, 50, 60 and 70 occupied the same position as the *T. koningiopsis* the member of rufa clade, it is intresting to note here that *T. asperellum* which belong to pachybasium clade also occupy the same branch as that of rufa clade which suggest that on the basis of nucleotide identity these two species are not easily distinguishable. The isolate TRI 37 was grouped with virens clade, TRI 35 and TRI 36 was aligned with catopteron clade. Very clearly these three rufa,virens and catopteron clade branch off distinctly. Interesting results are observed with TRI isolates TRI 23, 25, 21, 29 and 14 which could not be identified at species level in TrichOKEY. They have origin along with rufa and pacybacium clade but branch off distinctly. They may represent a new species group which need to be validated by taking more phylogenetic marker genes.

The shortcoming in identification of species by using only ITS marker has been reported by several workers[30,17]. Druchian and kubich[9] evaluated along 11 gene loci and formed that the 4th and 5th introns of translation elongation factor 1 alpha (tef1-EF-1±) and the coding region of endochitinase -42(ech 42) aid in resolving the species

Antagonistic activity

Dual culture assay revealed that all the isolates of *Trichoderma* spp inhibited the mycelial growth of *P.aphanidermatum* more than 50% over control (Table 2, Fig 3). However, the maximum inhibition of 87.78% of the mycelia growth of *P.aphanidermatum* was observed with the *T. virens* isolate TRI 37. It was followed by the *T. harzianum* isolates TRI 35 and TRI 36, which inhibited the mycelial growth to an extent of 85.5% over control. The next best isolates TRI 7, TRI 9, TRI 26 and 38 which inhibited the growth of pathogen to an extent of 81.5, 81.3, 80.0 and 80.0 per cent over the control were *T. asperellum* respectively. Similarly Anita *et al*[2] reported that, interaction between *Trichoderma* and isolated *Pythium* species in dual culture technique, range of inhibition was observed ranging from 56.92 -86.67%. The significant inhibition was observed in case of *T. viride* against *P. viniferum* 86.67% . Studies on hyperparasitism indicated that the *T.virens* isolate (TRI 37) overgrew on *P. aphanidermatum* up to 2.16

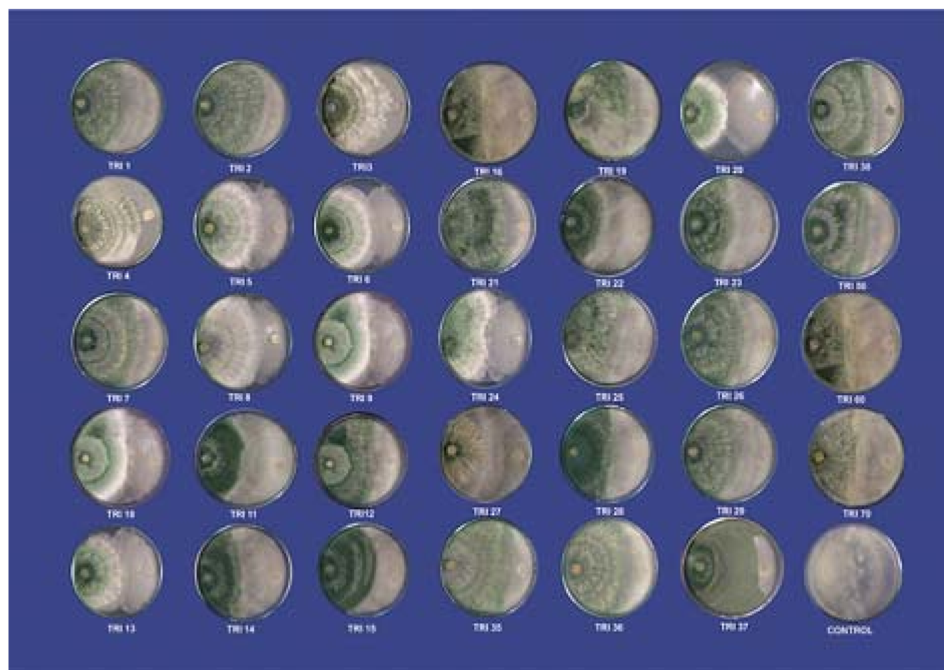

Fig. 3. Antagonistic efficacy of *Trichoderma* spp. against cucumber damping off pathogen under *in vitro* condition

Table 1. Identification,NCBI Genebank accession number and isolation details of different isolates of *Trichoderma*

S. No.	Isolate code	Area	GPS Location Longitude	Latitude	Source of culture	Genebank accession number	Identity of Species NCBI	TricHOKEY
1	TRI 1	Coonoor	11.3530 f N	76.7959 f E	Lillium	KX533988	*T.asperellum*	*T.koningiopsis*
2	TRI 2	Coonoor	11.3530 f N	76.7959 f E	Lillium	KX533979	*T.asperellum*	*T.koningiopsis*
3	TRI 3	Gudalur	11.5029 f N	76.4917 f E	Tea	KX533983	*T.asperellum*	*T.koningiopsis*
4	TRI 4	Gudalur	11.5029 f N	76.4917 f E	Tea	KX533985	*T.asperellum*	*T.koningiopsis*
5	TRI 5	Baraliayur	11.3429 f N	76.8500 f E	Tea	KX533991	*T.asperellum*	*T.koningiopsis*
6	TRI 6	Chinnaka-rumpalam	11.3347 f N	76.7705 f E	Silver oak	KT462693	*T.asperellum*	*T.koningiopsis*
7	TRI 7	Chinnaka-rumpalam	11.3347 f N	76.7705 f E	Tea	KX533980	*T.asperellum*	*T.koningiopsis*
8	TRI 8	Baraliayur	11.3429 f N	76.8500 f E	Chrysanthemum	KX533992	*T.asperellum*	*T.koningiopsis*
9	TRI 9	Kaikatti	11.3128 f N	76.6909 f E	Tea	KX533993	*T.asperellum*	*T.koningiopsis*
10	TRI 10	Edavanalli	12.5207 f N	77.9855 f E	Lillium	KX533994	*T.asperellum*	*T.koningiopsis*
11	TRI 11	Edavanalli	12.5207 f N	77.9855 f E	Lillium	KX533986	*T.asperellum*	*T.koningiopsis*
12	TRI 12	Kallar	11.3390 f N	76.8652 f E	Tea	KU361372	*T.asperellum*	*T.koningiopsis*
13	TRI 13	Kallar	11.3390 f N	76.8652 f E	carnation	KX533978	*T.asperellum*	*T.koningiopsis*
14	TRI 14	Kasolai	11.3151 f N	76.6992 f E	Carnation	KX533987	*T.asperellum*	Unidentified
15	TRI 15	Kasolai	11.3151 f N	76.6992 f E	Gerbera	KX533984	*T.asperellum*	*T.asperellum*
16	TRI 16	Kasolai	11.3151 f N	76.6992 f E	Tea	KX533995	*T.asperellum*	*T.koningiopsis*
17	TRI 19	Katteri	11.3341 f N	76.7879 f E	Chrysanthemum	KX533996	*T.asperellum*	*T.koningiopsis*
18	TRI 20	Kunnakombai	11.3149 f N	76.7111 f E	Tea	KX523262	*T.asperellum*	*T.koningiopsis*
19	TRI 21	Kunnakombai	11.3149 f N	76.7111 f E	Silver oak	KX533997	*T.asperellum*	Unidentified
20	TRI 22	Kothagiri	11.4086 f N	76.8720 f E	Rose	KX523263	*T.asperellum*	*T.koningiopsis*
21	TRI 23	Kothagiri	11.4086 f N	76.8720 f E	Lillium	KX147092	*T.asperellum*	*T.koningiopsis*
22	TRI 24	Kodanad	11.5015 f N	76.9063 f E	Lillium	KX084067	*T.asperellum*	*T.koningiopsis*
23	TRI 25	Kodanad	11.5015 f N	76.9063 f E	Chrysanthemum	KX533998	*T.asperellum*	Unidentified
24	TRI 26	Kodanad	11.5015 f N	76.9063 f E	Carnation	KX533999	*T.asperellum*	*T.koningiopsis*
25	TRI 27	Denad	11.44906916	76.9420 f E	Gerbera	KX533981	*T.asperellum*	*T.koningiopsis*
26	TRI 28	Masiangudi	11.5634 f N	76.6338 f E	Tea	KX533982	*T.asperellum*	*T.koningiopsis*
27	TRI 29	Masiangudi	11.5634 f N	76.6338 f E	Chrysanthemum	KX5334000	*T.asperellum*	Unidentified
28	TRI 35	Devasola	11.3204 f N	76.6868 f E	Lillium	KX533989	*T.harzianum*	*T.harzianum*
29	TRI 36	Devasola	11.3204 f N	76.6868 f E	Silver oak	KX533990	*T.harzianum*	*T.harzianum*
30	TRI 37	Devasola	11.3204 f N	76.6868 f E	Tea	KU666466	*T.virens*	*T.virens*
31	TRI 38	Chinnaka-rumpalayam	11.3347 f N	76.7705 f E	Tea	KX523264	*T.asperellum*	*T.koningiopsis*
32	TRI 50	Kallar	11.3390 f N	76.8652 f E	Chrysanthemum	KX555650	*T.asperellum*	*T.koningiopsis*
33	TRI 60	Maniyapuram	11.3157 f N	76.7080 f E	Carnation	KX147094	*T.asperellum*	*T.koningiopsis*
34	TRI 70	Maniyapuram	11.3157 f N	76.7080 f E	Gerbera	KX147093	*T.asperellum*	*T.koningiopsis*

cm from the zone of interaction, indicating the hyperparasite nature of the *T.virens* isolate TRI 37. Hyperparasitism by the *T.virens* indicate the capability of these isolate produce hydrolytic enzyme followed by lysis of pathogen. Similarly hyperparasitism nature reported by Yang *et al*[36], In co-culture *in vitro,* isolates of *Trichoderma* spp., including Tri01003, Tri01090 and Tri01091, displayed the ability to steadily colonize and aggressively attack the mycelia of *P. ultimum,* and finally produce conidia on the *Pythium* colony.

Bioefficacy of *Trichoderma* formulation on the management of cucumber damping- off

The effective isolate s of *T. virens*, *T. harzianum* isolates (TRI 35 and 36) and *T. asperellum* isolate (TRI9) were evaluated for the management of cucumber damping off under pot culture in green house through biopriming of seeds and soil application either as individual isolate or as consortia. Results of the investigation emphasized ingeneral that bio-priming, soil application and bio priming coupled with soil application

Table 2. *In vitro* efficacy of *Trichoderma* spp. *against Pythium aphanidermatum* by dual culture method

S. No.	Isolates No.	Mycelia growth(cm) *Pythium*	Mycelia growth(cm) *Trichoderma*	Hyperpar-asitism(cm)	Inhibition over control (%)
1	TRI 1 (*T.asperellum*-KX533988)	2.67	6.3	1.8	70.33^{n} (56.99)
2	TRI 2(*T.asperellum* -KX533979)	2.20	6.8	1.7	75.56^{h}(60.37)
3	TRI 3(*T.asperellum* -KX533983)	2.37	6.6	1.8	73.67^{i}(59.78)
4	TRI 4(*T.asperellum* -KX533985)	2.70	6.3	1.9	70.00^{o}(56.78)
5	TRI 5(*T.asperellum* -KX533991)	2.63	6.4	1.62	70.78^{m}(57.27)
6	TRI 6(*T.asperellum*-KT462693)	3.60	5.4	1.60	60.00^{s}(50.76)
7	TRI 7(*T.asperellum*-KX533980)	1.40	7.6	1.98	84.44^{e}(66.76)
8	TRI 8(*T.asperellum*-KX533992)	2.10	6.9	1.8	76.67^{f}(61.12)
9	TRI 9(*T.asperellum*-KX533993)	1.37	7.6	1.08	84.81^{d}(67.06)
10	TRI 10(*T.asperellum*-KX533994)	2.70	6.3	1.08	70.00^{o}(56.78)
11	TRI 11(*T.asperellum*-KX533986)	2.50	6.5	1.98	72.22^{l}(58.19)
12	TRI 12(*T.asperellum*- KU361372)	2.10	6.9	1.44	76.67^{f}(61.11)
13	TRI 13(*T.asperellum* -KX533978)	3.57	5.4	0.72	60.37^{r}(50.98)
14	TRI 14(*T.asperellum* -KX533987)	3.63	5.4	1.8	59.63^{t}(50.55)
15	TRI 15(*T.asperellum* -KX533984)	2.37	6.6	1.44	73.70^{i}(59.14)
16	TRI 16(*T.asperellum* -KX533995)	3.07	5.9	1.98	65.93^{p}(54.28)
17	TRI 19(*T.asperellum* -KX533996)	2.37	6.6	1.44	73.70^{i}(59.14)
18	TRI 20(*T.asperellum* -KX523262)	4.30	4.7	0.36	52.22^{w}(46.27)
19	TRI 21(*T.asperellum* -KX533997)	2.13	6.9	1.8	76.30^{g}(60.86)
20	TRI 22(*T.asperellum*- KX523263)	3.63	5.4	1.08	59.63^{t}(50.55)
21	TRI 23(*T.asperellum*- KX147092)	2.40	6.6	1.98	73.33^{j}(58.90)
22	TRI 24(*T.asperellum*- KX084067)	4.30	4.7	0.36	52.22^{w}(46.27)
23	TRI 25(*T.asperellum*- KX533998)	2.67	6.3	1.98	70.37^{n}(57.02)
24	TRI 26(*T.asperellum*- KX533999)	2.43	6.6	1.98	72.96^{k}(58.66)
25	TRI 27(*T.asperellum*- KX533981)	4.20	4.8	1.08	53.33^{v}(49.90)
26	TRI 28(*T.asperellum*- KX533982)	3.70	5.3	1.98	58.89^{u}(50.11)
27	TRI 29(*T.asperellum*- KX5334000)	2.63	6.4	1.8	70.74^{m}(57.25)
28	TRI 35(*T.harzianum*- KX533989)	1.33	7.7	1.98	85.19^{c}(67.36)
29	TRI 36(*T.harzianum*- KX533990)	1.30	7.7	1.98	85.56^{b}(67.66)
30	TRI37(*T.virens* -KU666466)	1.10	7.8	2.16	87.78^{a}(69.53)
31	TRI 38(*T.asperellum*- KX523264)	2.20	6.8	1.98	75.56^{h}(60.37)
32	TRI 50(*T.asperellum*- KX555650)	2.13	6.9	1.8	76.30^{g}(60.86)
33	TRI 60(*T.asperellum*- KX147094)	3.40	5.6	1.97	62.22^{q}(52.07)
34	TRI 70(*T.asperellum* -KX147093)	3.57	5.4	1.8	60.37^{r}(50.98)
35	Control	9.0	-	-	-

Means followed by a common letter are not significantly different at the 5% level by DMRT; Figures in parentheses are square root transformed values

with consortia of *Trichoderma* isolates comprising of *T. virens* (TRI 37), *T. harzianum* isolates (TRI 35 and 36) and *T. asperellum* (TRI 9) were effective in the suppression of damping off rather than the application of individual isolates of *Trichoderma* compared to untreated control. However, bio priming and soil application with the consortia comprising of *T.virens* (TRI37), *T.harzianum* isolates (TRI 35 and 36) and *T.asperellum* (TRI 9) suppressed damping off to an extent of 76.82% over untrated control and was followed by the soil application of consortia comprising of (TRI 37+TRI 36+TRI 35+TRI 9),which was applied on 15 and 30th days after seeding(74.08% reduction over control).

Comparison of *Trichoderma* consortia, delivered through biopriming and soil application with *T. virens* isolate (TRI 37) and *T. harzianum* isolates (TRI 35 and 36) was only next to seed treatment with metalaxyl coupled with soil application of metalaxyl 0.1% on 15 and 30th days after seeding, which reduced damping off upto 85.02% over control(Table 3). Similar results were also reported by Abd-El- Khair *et al*[1] and Singh *et al*[28] that confirms our findings. They reported that the incidence of damping-off was found maximum

Table 3. Effect of bioformulations of *Trichoderma spp.* on the incidence of cucumber damping off under glasshouse conditions

S. No	Treatments	Damping off incidence (%)	Per cent reduction over control
T1	BP with TRI 37 @10g/kg of seed	38.00^{k} (37.16)	53.44
T2	BP with TRI 36 @10g/kg of seed	39.10^{l} (38.70)	53.15
T3	BP with TRI 35 @10g/kg of seed	39.07^{l} (38.68)	53.19
T4	BP with TRI 9 @10g/kg of seed	39.20^{l} (38.76)	53.03
T5	BP with TRI37+TRI 36+TRI 35+TRI9 10g/kg of seed	37.17^{j} (37.56)	55.46
T6	SA with TRI 37 @ 2.5 kg/ha at 15 and 30th days after seeding	29.66^{g} (32.99)	64.46
T7	SA with TRI 36 @ 2.5 kg/ha at 15 and 30th days after seeding	32.71^{h} (34.88)	60.81
T8	SA with TRI 35 @ 2.5 kg/ha at 15 and 30th days after seeding	32.69^{h} (34.87)	60.83
T9	SA with TRI 9 @ 2.5 kg/ha at 15 and 30th days after seeding	35.27^{i} (36.43)	57.74
T10	SA with TRI37+TRI 36+TRI 35+TRI 9 @ 2.5 kg/ha at 15 and 30th days after seeding	21.13^{c} (27.36)	74.68
T11	BP +SA with TRI 37 @10g/kg of seed+2.5 kg/ha at 15 and 30th days after seeding	23.04^{d} (28.68)	72.39
T12	BP +SA with TRI 36 @10g/kg of seed+2.5 kg/ha at 15 and 30th days after seeding	23.00^{d} (28.65)	72.44
T13	BP +SA with TRI 35 @10g/kg of seed+ 2.5 kg/ha at 15 and 30th days after seeding	26.45^{e} (30.95)	68.31
T14	BP +SA with TRI 9 @10g/kg of seed+ 2.5 kg/ha at 15 and 30th days after seeding	27.24^{f} (31.46)	67.36
T15	BP +SA with TRI37+TRI 36+TRI 35+TRI9 @10g/kg of seed+ 2.5 kg/ha at 15 and 30th days after seeding	19.34^{b} (26.09)	76.82
T16	BP +SA with Metalaxyl 2g/kg of seed+ 0.1% at 15 and 30th days after seeding	12.50^{a} (20.70)	85.02
T17	Control	83.47^{m} (66.01)	-

in the pathogen inoculated control (54.67%) and lowest in the plants treated with the consortium of *Trichoderma* isolates BHU51+BHU105 (22.00%) rather than the individual application of *Trichoderma* isolate BHU51 and BHU105 on to seeds. Singh and Singh[27] also reported that the use of mixture of *Trichoderma*, increase the level of defence releted enzymes in the plant that protect the plant from the infection caused by *Macrophomina*.

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REFERENCE

1. Abd-El-Khair, H., Khalifa, R. Kh. M., Haggag, K. H. E. Effect of *Trichoderma* species on damping off diseases incidence, some plant enzymes activity and nutritional status of bean plants. *J. Amer. Sci*. 2010; **6**(9): 486-497.
2. Anita, P., Aarti, L., Ashwin, L., Hariprasad, P., Shubhada, M. *In vitro* antagonistic properties of selected *Trichoderma* spp. against tomato root rot causing *Pythium* species. *Int. J. Sci. Environ. Technol*., 2012; **1**(4): 302-315.
3. Anke, H., Kinn, J., Bergquist, K.E., Sterner, O. Production of siderophores by strains of the genus *Trichoderma*: Isolation and characterization of the new lipophilic coprogen derivative, palmitoylcoprogen. *Biol.Met.*, 1991; **4**(3): 176 180.
4. Bailey, B.A., Bae, H., Strem, M.D., Roberts, D.P., Thomas, S.E., Crozier, J., Samuels, G.J., Choi, I.Y., Holmes, K.A. Fungal and plant gene expression during the colonization of cacao seedlings by endophytic isolates of four *Trichoderma* species. *Planta.,* 2006; **224**: 1449–1464.
5. Benítez, T., Rincón, A. M., Limón, M. C., Codón, A. C. Mecanismos de biocontrol de cepas de *Trichoderma*. *Int. Microbiol.*, 2004; **7**(4): 249-260.
6. Bisset, J. A. revision of the genus *Trichoderma*. II. Infrageneric classification. *Can. J. Bot*., 1991; **69**: 2357-2372.
7. Chernin, L., Chet, I.: Microbial enzymes in the bio control of plant pathogens and pests. In: Enzyme in the environment (Dick, R.P. and Burns, R.G. eds). Marcel Dekker,New York, 2002; pp171-225
8. Dennis, C. Webster, J. Antagonistic properties of species groups of *Trichoderma* III. Hyphal interaction. *Transaction of British mycological Society*.1971; **57:** 363-369.
9. Druzhinina, I., Koptchinski, A., Komon, M., Bissett, J., Szakacs, G., Kubicek, C.P. An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. *Fungal Genet. Biol*., 2005; **42**: 813–828.
10. Druzhinina, I., Kubicek, C.P. Species concepts and biodiversity in *Trichoderma* and *Hypocrea*: from aggregate species to species clusters. J. *Zhejiang Univ. Sci.,* 2005; **6**: 100–112.
11. Elad, Y., Chet, L., Henis, Y. A selective medium for improving quantitative isolation of *Trichoderma* spp. from soil. *Phytoparasitica*, 1981; **9** (1): 59-67.
12. Hall, T.A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98NT. *Nucleic Acids Symposium Series* , 1999 ; **41**: 95-98.
13. Harman, G.E., Howell, C.R., Viterbo, A., Chet, I. Lorito, M. *Trichoderma* species opportunistic, avirulent plant symbionts. *Nat. Rev. Microbiol*., 2004; **2**: 43–56.
14. Jarvis, W. R.(ed): Managing diseases in green house crops. Saint Paul, Minnesota: APS Press. 1992; 122-7.
15. Jash,S., Pan, S. Evaluation of mutant isolates of *T.harzianum* against *R.solani* causing seedling blight of green gram. *Ind. J. Agric. Sci.*, 2004; **74**: 190-193
16. Kredics, L., Antal, Z., Doczi, I., Manczinger, L., Kevei, F., Nagy, E. Clinical importance of the genus *Trichoderma*. A review. *Acta. Microbiol. Immunol. Hung.,* 2003; **50**:105–117.
17. Kullnig-Gradinger, C.M., Szakacs, G., Kubicek, C.P. Phylogeny and evolution of the fungal genus *Trichoderma*: a multigene approach. *Mycol. Res*., 2002; **106**: 757–767.
18. Lee, S.B., Taylor, J.W. Isolation of DNA from fungal mycelia and single spores. In: PCR protocols: A guide to method and applications (Innis, M.A., Gelfand, D. H., Sninsky, J.J., White, T.J. eds). New York, USA, Academic press,1990; pp 282-287.
19. Lieckfeldt, E., Samuels, G.J., Helgard, H.I., Petrini, O. A morphological and molecular perspective of *Trichoderma viride*: is it one or two species. *Appl. Environ. Microbiol*., 2002; 65: 2418-2428.
20. Mahuku, G. A Simple Extraction Method Suitable for PCR-Based Analysis of Plant, Fungal and Bacterial DNA. *Plant Mol. Biol. Rep*., 2004; **22**: 71-81.
21. Meyer, R. J. Mitochondrial DNAs and plasmids as taxonomic characters in *Trichoderma viride.*

Appl. Environ. Microbiol., 1991; **57**: 2269-2276.

22. Osburn, R.M., Schroth, M.N., Hancock, J.G., Hendson, M. Dynamics of sugarbeet colonization by *Pythium ultimum* and *Pseudomonas* species: Effects on seed rot and damping-off. *Phytopathol.*,1989 ; **79**: 709–716.
23. OzbayNusret, S. E. N. Fusarium crown and root rot of tomato and control methods. J *Plant Pathol.,* 2004; **2**: 1-4.
24. Raeder, U., Broda, P. Rapid preparation of DNA from filamentous fungi. *Lett. Appl. Microbiol* .,1985; **1**: 17–20
25. Saitou, N., Nei, M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. and Evol.,* 1987; **4**:406-425.
26. Sinclair, J.B., Dhingra, O.D. (ed): Basic plant pathology methods, 2nd edn. CRC press, 1995; pp 448.
27. Singh, S. P. and Singh, H. B. 2014. Effect of mixture of *Trichoderma* isolates on biochemical parameters in leaf of *Macrophomina phaseolina* infeceted brinjal. *J. Environ. Biol.* 35: 871-876.
28. Singh, S.P., Singh, H. B., Singh, D. K. Biocontrol potential mixture of *Trichoderma* isolates on Damping off and collar rot of tomato. *The Bioscan*, 2014; 9(3): 1301-1304.
29. Srivastava, M., Tiwari, R., Sharma, N. Effect of different cultural variables on siderophores produced by *Trichoderma* spp., 2013; **1**(7): 1-6.
30. Taylor, J.W., Jacobson, D.J., Kroken, S., Kasuga, T., Geiser, D.M., Hibbett, D.S., Fisher, M.C. Phylogenetic species recognition and species concepts in fungi. *Fungal Genet. Biol*., 2000; **31**: 21–32.
31. Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol. Biol. and Evol.,* 2013; **30**: 2725-2729.
32. Vinale, F., Sivasithamparam, K., Ghisalberti, E.L., Marra, R., Woo, S.L., Lorito, M. *Trichoderma*–plant–pathogen interactions. *Soil Biol. Biochem.,* 2008; **40**: 1–10.
33. Whipps, J.M., Lumsden, D.R. Biological control of Pythium species. *Biocontrol Sci. and Techn.*, 1991; **1**: 75–90.
34. White, T.J., Bruns, T., Lee, S.,Taylor, J.W. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR protocols: a guide to methods and applications. (Innis, M.A., Gelfand, D.H., Shinsky, J.J., White, T.J., eds). Academic Press, San Diego, 1990; pp 315–322.
35. Woese, C.R., Fox, G.E. Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *Proc. Natl. Acad. Sci*., USA. 1977; **74**: 5088-5090.
36. Yang, Y., Chang, K. F., Hwang, S.F., Callan, N. W., Howard, R.J., Blade, S.F. Biological control of *Pythium* damping off in *Echinacea angustifolia* with *Trichoderma* species. *J.Plnt.Dis.Protn*.,2004; **111**(2): 126-136.