

A study on corroboration between DNA markers (RAPD, ISSR, ITS) and bio-control efficacy of *Trichoderma* species

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Abstract

Twelve isolates of *Trichoderma harzianum* and *Trichoderma viride* were assessed for their mycoparasitic effect on phytopathogens *Pythium aphanidermatum* and *Sclerotinia sclerotiorum*. Though *T. harzianum* isolates were more aggressive than *T. viride* isolates, the percent inhibitory effect among *T. harzianum* isolates did not vary much (80% to 86%). The inhibitory effect of *T. viride* isolates ranged from 50% to 80%; however, TvChen, Tv4, and TvNir were distinguishable from other *T. viride* isolates in exhibiting higher degree of antagonism. The dataset generated through molecular markers (RAPD and ISSR) showed a comparable output grouping the isolates Tv4, TvChen and TvNir in one cluster and all *T. harzianum* isolates in another cluster. Multiple nucleotide alignment of ITS 1 and ITS 2 region produced 100% homology among *T. harzianum* isolates whilst the nucleotide substitution at 62nd and 150th position of ITS 1 region and 27th and 40th position of ITS 2 region differentiated Tv4, TvChen and TvNir from other *T. viride* isolates. Genetic assessment could not establish substantial disparity among *T. harzianum* isolates which was comparable with its antagonism. The genetic distinctness of Tv4, TvChen and TvNir isolates authenticated their higher degree of antagonism. It is obvious from the present study that genetic diversity analysis had a positive correlation with the antagonistic ability of *Trichoderma* isolates.

Keywords: *Trichoderma*; Antagonism; Molecular characters; RAPD; ISSR; ITS.

Introduction

Plant disease epidemics have created an ecologically unbalanced system in modern agriculture. Deterrence of such epidemics for the most part achieved through use of chemical fungicides which have greater repercussion on environment and human health. Also progressive confrontation in a midst of pathogen resistance to accessible chemical plant protectants has engrossed the need of alternative methods of disease control.

Fungi of the genus *Trichoderma* are important biocontrol agents of several soil borne phytopathogens. The *Trichoderma* species serves as a potential alternative to chemical control measure and growing pathogen resistance crop cultivars. This may be attributable to their diverse metabolic capability and aggressively competitive nature [1,2]. *Trichoderma* use different mechanism for control of phytopathogens which includes mycoparasitism, out compete pathogenic fungi for nutrients, secretion for antibiotics and fungal cell wall degrading enzymes [3,4]. *Trichoderma* sp. includes a plethora of strains which differ in their innocuousness and effectiveness as biocontrol agents. Furthermore it is difficult to predict the degree of synergism and the behavior of a biocontrol agent in a natural pathosystem. The molecular technique like Random Amplified Polymorphic DNA (RAPD) developed by Williams et al. [5] has been used for genetic and taxonomic studies for several fungi including *Trichoderma* sp. [6,7]. Inter Simple Sequence Repeats (ISSR) have been used as another effective method to characterize genetic variability. Since the evolutionary rate within ISSR is considerably higher than other types of DNA, the likelihood of finding polymorphism is greater compared to RAPD [8]. The Internal Transcribed Spacer (ITS) region is perhaps the most widely sequenced DNA region in fungi. It has typically been most useful for molecular systematic study at species level, and even within species [9-12]. Kindermann et al. [13] attempted a first phylogenetic analysis of the whole genus of *Trichoderma* using sequence analysis of the ITS1 region

of rDNA. Thus the present study was to characterize the cryptic species of *Trichoderma* associated with biological control and to establish the relation between bioefficacy and molecular characters existed if any.

Materials and Methods

Isolation and identification of *Trichoderma*

Soil samples were collected from various experimental fields of Indian Agricultural Research Institute (IARI), Pusa, New Delhi. A total no. of twelve isolates of *Trichoderma* species was isolated and identified in specific selective medium. The identification of *Trichoderma* isolates were confirmed and deposited in Indian Type Culture Collection (ITCC), IARI, Pusa, New Delhi.

Confrontation assays *in vitro*

In vitro confrontations were studied by performing dual culture technique described by Dennis and Webster [14] was used to test the antagonistic ability of *Trichoderma* isolates against the phytopathogenic fungi *Pythium aphanidermatum* and *Sclerotinia sclerotiorum*. The host fungus and *Trichoderma* were grown on potato dextrose agar (PDA) for a week at room temperature (28±2°C). Growth parameters in all dual cultures were read after 7 days. The plates containing only the target pathogenic organisms without *Trichoderma* were taken as control to evaluate the percent growth inhibition.

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Molecular characterization

The total genomic DNA was extracted from each isolate of *Trichoderma* based on Cetrinide Tetradecyl Trimethyl Ammonium Bromide (CTAB) mini extraction method of Crowhurst et al. [15] with modification.

Random Amplified Polymorphic DNA (RAPD) analysis

RAPD-PCR conditions for *Trichoderma* isolates in the present investigations were standardized. Forty random primers of 10bp long each (10mer) of OPA and OPX (Operon Technologies, USA) series were used to amplify genomic DNA of the isolates. Out of these, sixteen primers which presented strong band resolution were chosen for the study viz., OPA 1, OPA 2, OPA 4, OPA 5, OPA 9, OPA 11, OPA 12, OPA 16, OPX 2, OPX 5, OPX 8, OPX 12, OPX 13, OPX 17, OPX 18 and OPX 20. Following the experiments for optimization of component concentrations, PCR amplification was carried out with 20ng/μl of genomic DNA, 2mM MgCl₂, 1U *Taq* DNA polymerase, 1X PCR buffer with 15mM MgCl₂, 0.6μM decamer primers and 0.2mM of dNTP mix. The volume was made up to 25μl. PCR reactions were carried out in a Perkin Elmer Thermocycler under the conditions involving denaturation at 94°C for 2 minutes; 40 cycles of denaturation at 94°C for 1 minute, primer annealing at 35°C for 1 minute and primer extension at 72°C for 2 minutes; final extension step at 72°C for 4 minutes.

Inter Simple Sequence Repeats (ISSR) analysis

The procedure described by Bornet et al. [16] with minor modifications was used for carrying out the PCR reactions for ISSR analysis. Ten primers were tested for amplification at different annealing temperatures of genomic DNA of the isolates. Out of these, eight primers viz., 5'(AG)₈AT3', 5'(AG)₈AC3', 5'(GA)₈AC3', 5'(GA)₉T3', 5'(GA)₉AC3', 5'BDB(GA)₈T3', 5'YT(CA)₇T3' and 5'YC(TG)₇T3' consisting of anchored ISSR gave satisfactory amplification and band resolution. The PCR amplification was carried out with 25ng of genomic DNA, 2.5mM MgCl₂, 1U *Taq* DNA polymerase, 1X PCR buffer without MgCl₂, 1μM ISSR primer and 0.2mM dNTP mix. The volume was made up to 25μl. PCR reactions were carried out in a Perkin Elmer GeneAmp 9600 thermocycler under the conditions involving denaturation at 94°C for 7 minutes; 30 cycles of denaturation at 94°C for 30 seconds, primer annealing at temperature specific to each primer for 45 seconds and primer extension at 72°C for 2 minutes; final extension step at 72°C for 7 minutes.

The PCR products of both RAPD and ISSR reactions were resolved on 1.4% agarose gel in 1X TBE buffer pre-stained with ethidium bromide (1μg/1ml) and electrophoresis was carried out at 90 volts for 1.5hrs followed by 70volts for 2hrs and visualized under UV light on a UV- Transilluminator. The gel was photographed using a Gel documentation system.

Data analysis

The amplification products were scored for the presence "1" and absence "0" and missing data as "9". The genetic associations between isolates were evaluated by calculating the Jaccard's similarity coefficient for pair wise comparisons based on the proportion of shared bands produced by the primers. The similarity matrix was subjected to cluster analysis by unweighted pair group method for arithmetic mean (UPGMA) and a dendrogram was generated. The computations were performed using the program NTSYS – PC. version. 2.02h [17].

The Jaccard's similarity matrix was subjected to principal component analysis. This coordination method makes use of multidimensional solution of the observed relationships. PCA resolves complex relationships into a function of fewer and simpler factors. In this technique, the data matrix is derived from the distances (or similarities) between the operational taxonomic units. To simplify the description of these 'clouds' of points, the (principal) axes through the hyper-ellipsoid are calculated. The successive principal axes, representing the first major axis, the second axis etc., account for the greatest, the second greatest, etc. amount of variation.

Internal transcribed spacer region

The internal transcribed spacer (ITS) regions of the rDNA repeat from the 3' end of the 18s and the 5' end of the 28s gene were amplified using the two primers, ITS A and D which were synthesized on the basis of conserved regions of the eukaryotic rRNA gene (White et al. 1990). The PCR-amplification reactions were performed in a 50μl mixture containing 50mM KCl, 20mM Tris HCl (pH 8.4), 2.0mM MgCl₂, 200μM of each of the four deoxynucleotide triphosphates (dNTPs), 0.2μM of each primer, 40ng/μl of template and 2.5U of *Taq* polymerase. The cycle parameters included an initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 2 min and primer extension at 72°C for 3 min and a final extension for 10 min at 72°C. Amplified products were separated on 1.2% agarose gel in TAE buffer, pre-stained with ethidium bromide (1μg/ml) and electrophoresis was carried out at 60 volts for 3

Isolate no:	Species	<i>Pythium aphanidermatum</i>		<i>Sclerotinia sclerotiorum</i>	
		Percent inhibition of mycelial growth (%) of pathogen	Mean mycelial growth (cm ²) of <i>Trichoderma</i>	Percent inhibition of mycelial growth (%) of pathogen	Mean mycelial growth (cm ²) of <i>Trichoderma</i>
Th3	<i>T.harzianum</i>	86.4	23.04	90.2	25.00
Th10	<i>T.harzianum</i>	83.0	21.16	88.1	24.01
Th30	<i>T.harzianum</i>	78.3	18.49	85.0	23.04
Th31	<i>T.harzianum</i>	80.0	20.25	74.5	16.81
ThAg	<i>T.harzianum</i>	74.5	16.81	72.7	14.40
Tv2	<i>T.viride</i>	68.0	14.44	83.3	23.24
Tv4	<i>T.viride</i>	75.0	16.81	80.5	18.49
Tv12	<i>T.viride</i>	70.0	15.21	74.7	16.81
Tv15	<i>T.viride</i>	56.5	12.96	60.9	12.96
Tv32	<i>T.viride</i>	65.8	14.44	50.5	12.25
TvChen	<i>T.viride</i>	77.0	17.64	82.0	20.25

Table 1: Bioefficacy of *Trichoderma* isolates on *Pythium aphanidermatum* and *Sclerotinia sclerotiorum*.

hours in TAE buffer. One Kb ladder (MBI, Fermentas) was used as a marker. The gel was observed in a trans-illuminator over ultra violet light. The desired bands were cut from the gel with minimum quantity of gel portion using QIAGEN gel extraction kit.

Nucleotide sequencing and *In silico* analysis

The sequencing of the PCR product was carried out in automated Sequencer at Genome – Bio, Pune, India. Related sequences were searched using BLAST programme from the Gen-Bank database (<http://www.ncbi.nlm.nih.gov/blast/>) [18]. The multiple sequence alignment and pairwise alignment were made using BioEdit version 5.09 [19]. The neighborhood-joining bootstrap tree was created using CLUSTAL W 1.6 matrix by the CLUSTAL X programme ver. 1.81 [20].

Result

Confrontation assay *in vitro*

The isolates of *Trichoderma* spp. viz., Th3, Th10, Th30, Th31 and ThAg belonging to *T.harzianum*; Tv2, Tv4, Tv12, Tv15, Tv32, TvChen and TvNir of *T.viride* were evaluated for their antagonistic ability against the phytopathogenic fungi *Pythium aphanidermatum* and *Sclerotinia sclerotiorum* based on percent inhibition mycelial growth and mean mycelial growth (in cm²) (Table 1). The isolate Th3

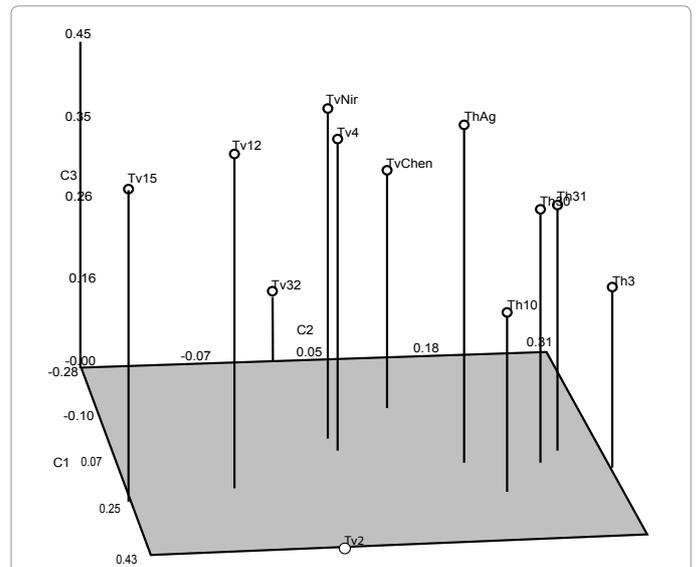


Figure 2: 3-D plot depicting the principal components of *Trichoderma* isolates based on RAPD primers.

of *T.harzianum* found to have the maximum inhibiting effect on the growth of *P.aphanidermatum* (86.45%) and *S.sclerotiorum* (90%) which directly corresponded to their mycelial growth rate of 23.04 cm² and 25 cm² against the target fungus. Among the *T.viride* isolates TvChen was more aggressive with 80% inhibiting effect on *P.aphanidermatum* and TvNir had 85% impact on *S.sclerotiorum*, closely followed by Tv4 in either of the cases.

Molecular characterization

Random amplified polymorphic DNA: The isolates of *Trichoderma* were subjected to RAPD analysis where 16 random primers resulted in robust and reproducible RAPD fragment patterns. The selected primers generated 112 RAPD bands and the size of the amplification products ranged from 250 bp to 900 bp. The percent polymorphism ranged from 60 to 100 with 4 primers OPA4, OPA5, OPX5 and OPX13 displayed 100% polymorphism (Plate 1). The genetic similarity between the isolates of *Trichoderma* was determined on the basis of Jaccard's similarity coefficient. The mean value of the Jaccard's similarity coefficient of the RAPD marker was 0.80. The highest genetic similarity was observed between the isolates Th31 and ThAg (80%) and the least was between the isolates Tv2 and Tv32 (30%). The dendrogram was generated using Unweighted Pair Group Method Arithmetic (UPGMA) based on NTSYS pc version 2.02i software resulted in three major clusters with isolates Tv2 and Tv32 as outliers (Figure 1). It was evident from the cluster analysis that the isolates representing to *T.harzianum* clustered in one group which had a similarity coefficient of 0.81 while isolates of *T.viride* segregated into two groups. Tv4, TvChen and TvNir formed second group with 78% similarity and Tv12 and Tv15 formed the third group which shared 82% similarity. The 3D depiction of principle components evidenced the close relatedness of *T.harzianum* isolates which formed into one subgroup and Tv4, TvChen and TvNir formed the other subgroup (Figure 2).

Inter simple sequence repeats

Fifteen ISSR primers were screened and eight of them giving satisfactory amplification and band resolution were taken for the

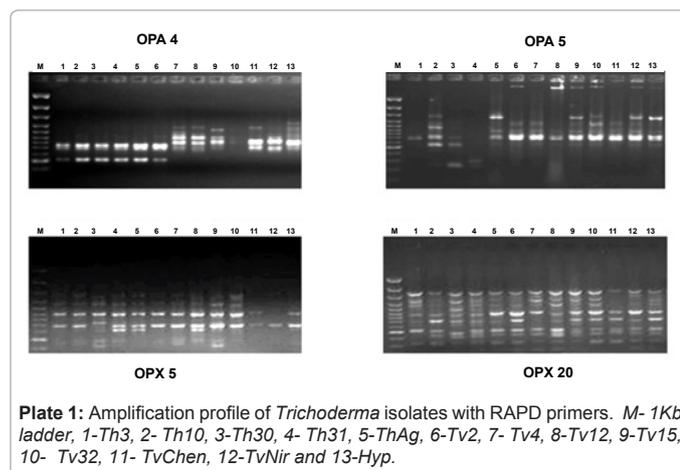


Plate 1: Amplification profile of *Trichoderma* isolates with RAPD primers. M- 1Kb ladder, 1-Th3, 2-Th10, 3-Th30, 4-Th31, 5-ThAg, 6-Tv2, 7-Tv4, 8-Tv12, 9-Tv15, 10-Tv32, 11-TvChen, 12-TvNir and 13-Hyp.

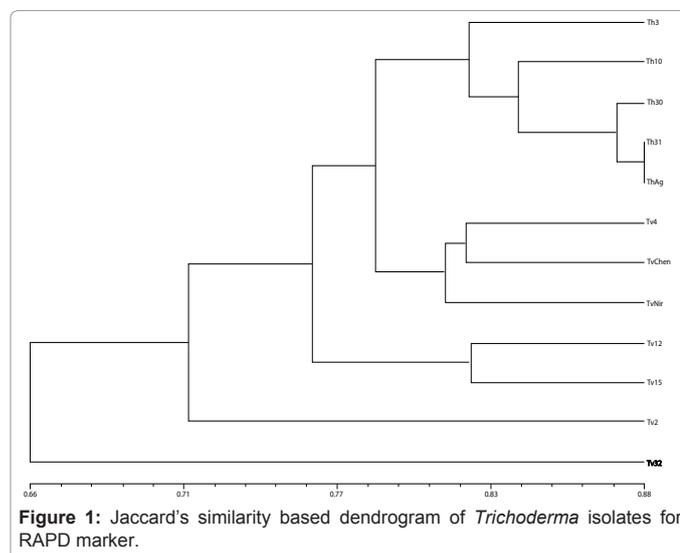


Figure 1: Jaccard's similarity based dendrogram of *Trichoderma* isolates for RAPD marker.

study. The selected primers generated 70 ISSR bands and the size of the amplification products ranged from 100 bp to 950 bp. The primer 5' (GA)₈AC 3' produced the maximum number of polymorphic loci (6) out of the total loci (7) followed by 5' (GA)₉AC 3' (6) (Plate 2). The percent polymorphism ranged from 40 to 86. The mean value of the Jaccard's similarity coefficient of the ISSR marker was 0.76. The dendrogram based on the above data separated the isolates into two major clusters with similarities ranging from 76% to 94% (Figure 3). All the five *T.harzianum* isolates clustered into a single group sharing similarity around 80%. The second major cluster consisted isolates of *T.viride*, however these isolates formed two subgroups within the cluster. The isolates Tv2, T15 and Tv32 formed the first subgroup while Tv4, TvChen and TvNir formed the other. However Tv12 did not cluster with any of these two subgroups but stood separately with only 65% similarity with main branch. The 3D depiction of principle components reconfirmed the results obtained through dendrogram analysis (Figure 4).

Internal transcribed spacer

The universal primers ITS A and ITS D were used to amplify the internal transcribed spacer regions of rDNA yielding products of approximately 600bp as estimated by agarose gel electrophoresis

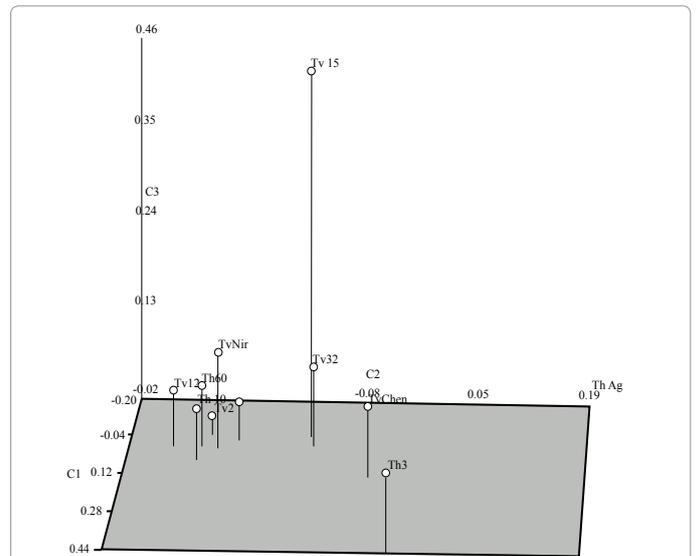


Figure 4: 3-D plot depicting the principal components of the *Trichoderma* isolates based on the ISSR primers.

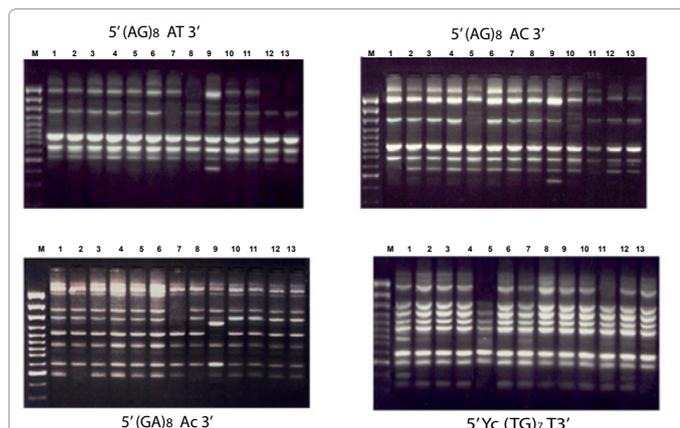


Plate 2: Amplification profile of *Trichoderma* isolates for ISSR primers. M- 1Kb ladder, 1-Th3, 2- Th10, 3-Th30, 4-Th31, 5-ThAg, 6-Tv2, 7- Tv4, 8-Tv12, 9-Tv15, 10- Tv32, 11- TvChen, 12-TvNir and 13-Hyp.

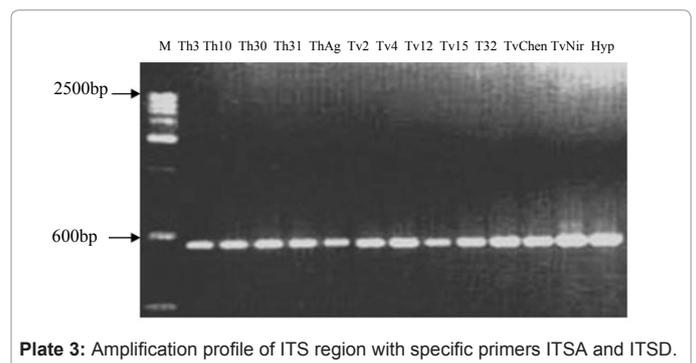


Plate 3: Amplification profile of ITS region with specific primers ITSA and ITSD.

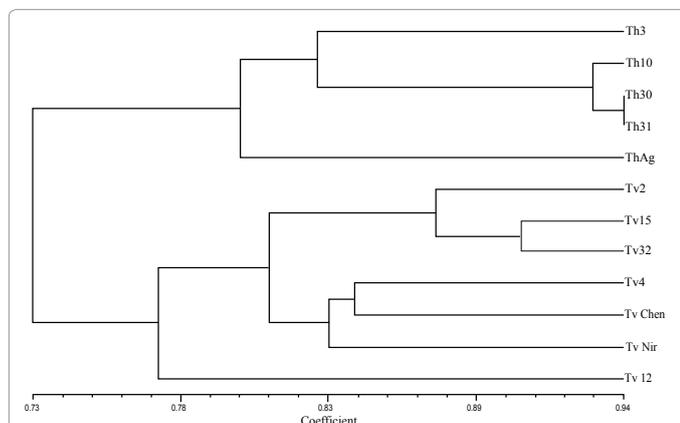


Figure 3: Jaccard's similarity based dendrogram of *Trichoderma* isolates for ISSR markers.

(Plate 3). The amplified PCR products was eluted and sequenced by automated sequencer which encompass ITS 1, ITS 2 and 5.8s rDNA gene and also 18s rDNA gene having base pairs ranging 35-49 bp at the 3' end and 20-25 bp of the 5' end of 28s rDNA. The sequences so obtained were subjected to BLAST search for its identity and confirmation, and subsequently submitted to National Centre for Biotechnology Information (NCBI) GenBank. The accession number for sequences of ITS 1 and ITS 2 region for the twelve isolates of *Trichoderma*. The multiple nucleotide alignment of ITS regions was analyzed using BioEdit programme. There was substantial disparity in length of ITS sequences between *T.harzianum* (199 bp) and *T.viride* (181 bp) isolates. The isolates of *T.harzianum* showed 100% homology in nucleotide sequence. Conversely *T.viride* isolates showed nucleotide divergence of 2.2% in ITS 1 and 2.26% in ITS 2 region. The isolates Tv4, TvChen and TvNir displayed 100% homology in nucleotide sequence, however they differed from other isolates at 62nd (A instead of C) and 150th (A instead of T) nucleotide position in ITS 1 region and 27th (T instead of G) and 40th (C instead of T) in ITS 2 region. To elucidate the genetic closeness of the *Trichoderma* isolates a phylogenetic tree was constructed based on sequence analysis of ITS 1 and ITS 2 regions using the neighbour-joining method in treecon for windows version 1.3b on sequences aligned using ClustalW 1.7 version (Figure 5 & 6). A random sequence was used as an out-group to demonstrate the situation of the root. Bootstrap analysis of ITS 1 region with 1000 bootstrap replication

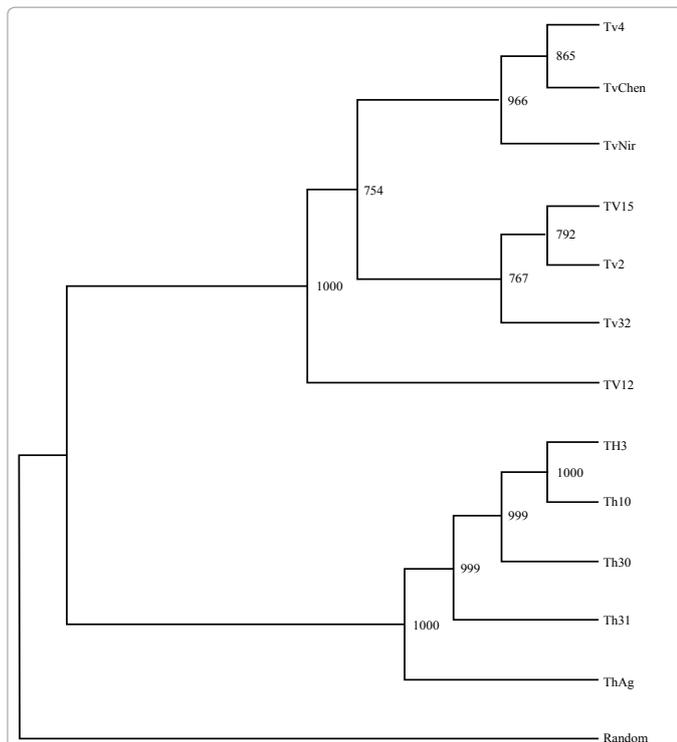


Figure 5: Genetic relationships of *Trichoderma* isolates based on analysis of ITS 1 sequences.

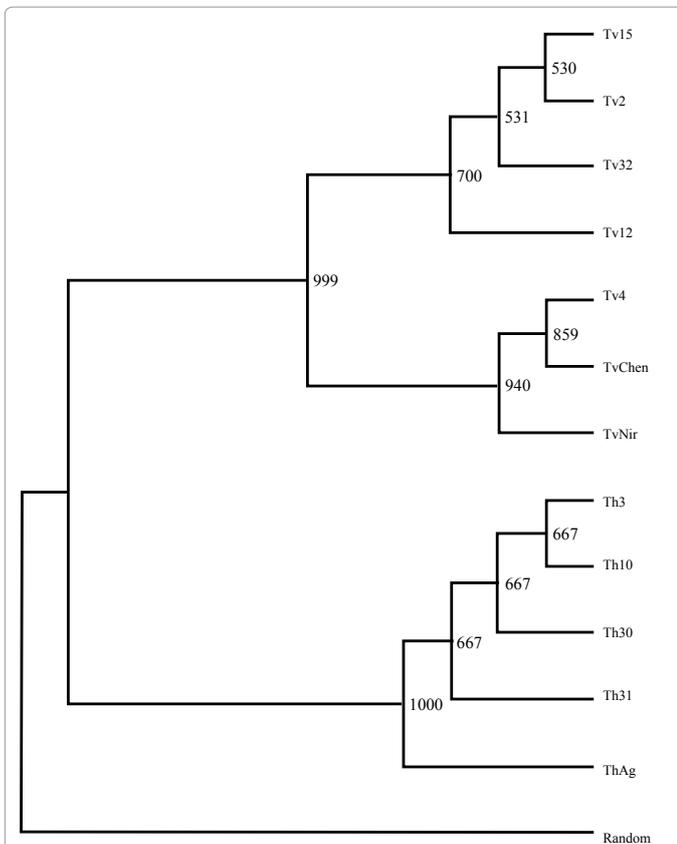


Figure 6: Phylogenetic relationships of *Trichoderma* isolates based on analysis of ITS 2 sequences.

demonstrated two main branches. All the isolates of *T.harzianum* formed one group which supported with a bootstrap value of 99.9%. The other cluster consisted of *T.viride* isolates which separated into two subgroups supported by bootstrap value higher than 75%. The isolates Tv4, TvChen and TvNir formed one subgroup, while Tv15, Tv2 and Tv32 formed the other subgroup. Tv12 did not group with any of the two subgroups but stood separately in this cluster. The bootstrap analysis of ITS 2 region also showed similar results, however Tv12 which stood separately in ITS 1 clustered into one of the subgroups consisting of Tv15, Tv2 and Tv32. This grouping was supported by more than 70 percent bootstrap values.

Discussion

In pursuit for a probability of corroborating the mycoparasitic antagonism ability of *Trichoderma* with respect to their molecular characters the current study was undertaken. Such corroboration will certainly be a needful aspect in elucidating the molecular mechanism involved in mycoparasitic antagonism. Dual petri-plate marriage of *Trichoderma* with pathogen *Pythium aphanidermatum*, and similarly with *Sclerotinia sclerotiorum* endorsed the mycoparasitic antagonism of *T.harzianum* and *T.viride*. *T.harzianum* isolates were more aggressive than *T.viride* in their antagonistic effect which ranged between 80% to 86% against *P. aphanidermatum* and 83% to 90% against *S. sclerotiorum*, on the other hand *T.viride* isolates ranged between 56% to 81% against *P. aphanidermatum* and 50% to 80% against *S. sclerotiorum*. However the percent inhibition of mycelial growth of Tv4, TvChen and TvNir was around 80% which was comparable with antagonistic ability of *T.harzianum*.

The molecular markers could not establish any variation within *T.harzianum* isolates instead grouped all the isolates into one cluster. The sequence identity matrix of ITS 1 and ITS 2 region showed 100% homology among these isolates of *T.harzianum*. None of the markers were able to identify distinct variation among *T.harzianum* isolates and also it is prominent that these isolates exhibited less variation in their antagonism. Therefore it can be interpreted that these isolates taken for study had no much dissimilarity among them genetically.

The RAPD markers characters divided the isolates of *T.viride* into two subgroups, but here Tv4, TvChen and TvNir formed a single group. Though RAPD marker formed 80% polymorphism it failed to differentiate Tv2 and Tv32. Singh et al. [21] reported that RAPD primers exhibited both inter and intra specific variation in the isolates and were able to distinguish the pathogenic isolates *T.harzianum* and *T.virens* causing green mould diseases in cultivated mushroom which was in conformity with our results. Though RAPD analysis provided good discrimination within and between species it is generally not considered a robust enough technique because of its poor reproducibility.

The ISSR technique which is more reliable reproduced similar dendrogram pattern as that of RAPD marker grouping TV4, TvChen and TvNir separately. The isolates Tv2 and Tv32 which stood as outliers in RAPD clubbed to form a subgroup, but Tv12 stood separately within the main branch. The combined ISSR and RAPD dendrogram also authenticated the above results.

The nucleotide sequence alignment of ITS 1 and ITS 2 region revealed the presence of hotspots with nucleotide substitution at four positions supported the distribution of Tv4, TvChen and TvNir into one subgroup. The dendrogram pattern obtained through ISSR was

almost indistinguishable from the phylogenetic tree obtained from ITS 1 and ITS 2 sequences. This authenticates the soundness of our perception that the bioefficacy of these three isolates were manifested at the molecular level which made them to distinguish from other *T. viride* isolates. Conversely Goes et al. (2002) reported that there was no relationship between the polymorphism showed by the *Trichoderma* isolates and their hardness against *R. solani* based on RAPD marker. Similar kind of result was reported by Shalini et al. [22] and Shanmugam et al. [23]. However the results of molecular markers employed in this present study was complementary and confirmatory in nature which substantiates our corroboration between bioefficacy of *Trichoderma* isolates and molecular characters.

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References

- Samuels GJ (1996) *Trichoderma*: A review of biology and systematics of the genus. Mycological Research 100: 923–935.
- Klein D, Eveleigh DE (1998) Ecology of *Trichoderma*. In: Kubicek CP, Harman GE (eds), *Trichoderma and Gliocladium* Vol. 1. Basic Biology, Taxonomy and Genetics. Taylor and Francis Ltd, London, 57-74.
- Harman GE, Howell CR, Viterbo A, Chet I, Lorito M (2004) *Trichoderma* spp. opportunistic avirulent plant symbionts. Nature Microbiology Reviews 2: 43-56
- Reino JL, Guerrero RF, Hernandez-Galan R, Collado IG (2008) Secondary metabolites from species of the biocontrol agent *Trichoderma*. Phytochemistry Reviews 7: 89-123.
- Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18: 6531–6535.
- Muthumeenakshi S, Mills PR (1995) Detection and differentiation of fungal pathogens of *Agaricus bisporus*. Mushroom Science 14: 603–610.
- Dodd SL, Hill RA, Stewart A (2004) A duplex-PCR bioassay to detect a *Trichoderma virens* biocontrol isolate in non-sterile soil. Soil Biology and Biochemistry 36: 1955-1965.
- Charlesworth B, Sniegowski P, Stephan W (1994) The evolutionary dynamics of repetitive DNA in eukaryotes. Nature 371: 215-220.
- Ospina-Giraldo MD, Royse DJ, Thon MR, Chen X, Romaine CP (1998) Phylogenetic relationships of *Trichoderma harzianum* causing mushroom green mold in Europe and North America to other species of *Trichoderma* from world-wide sources. Mycologia 90: 76-81.
- Kubicek CP, Mach RL, Peterbauer CK, Lorito M (2000) *Trichoderma*. From genes to biocontrol. Journal of Plant Pathology 83: 11-23
- Kullnig-Gradinger CM, Szakacs G, Kubicek CP (2002) Phylogeny and evolution of the genus *Trichoderma*: a multigene approach. Mycological Research 106: 757–767.
- Lee C, Hseu T (2002) Genetic relatedness of *Trichoderma* sect. *Pachybasium* species based on molecular approaches. Can J Microbiol 48: 831-840.
- Kindermann J, Ayouti YE, Samuels GJ, Kubicek CP (1998) Phylogeny of the genus *Trichoderma* based on sequence analysis of the internal transcribed spacer region 1 of the rDNA cluster. Fungal Genet Biol 24: 298-309.
- Dennis C, Webster J (1971) Antagonistic properties of species group of *Trichoderma*. I. Production of non-volatile antibiotics. Transactions British Mycological Society 57: 25-39.
- Crowhurst RN, King FY, Hawthorne BT, Sanderson FR, Choi-Pheng Y (1995) RAPD characterization of *Fusarium oxysporum* associated with wilt of angasana (*Pterocarpus indicus*) in Singapore. Mycological Research 99: 14-18.
- Bornet B, Goragner F, Joly G, Branchard M (2002) Genetic diversity in European and Argentinian cultivated potatoes (*Solanum tuberosum* subsp. tuberosum) detected by inter-simple sequence repeats (ISSRs). Genome 45: 481-484.
- Rohlf FJ (1997) NTSYS-PC. Numerical taxonomy and multivariate analysis system ver. 2.02h. Applied Biostatistics, Setuaket, NewYork, USA
- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, et al. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25: 3389–3402.
- Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98NT. Nucleic Acids Symposium Series 41: 95-98.
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24: 4876-4882.
- Singh SK, Sharma VP, Sharma SR, Kumar S, Tiwari M (2006) Molecular characterization of *Trichoderma* taxa causing green mould disease in edible mushrooms. Current Science 90: 427-431.
- Shalini N, Lata KP, Kotasthane AS (2006) Genetic relatedness among *Trichoderma* isolates inhibiting a pathogenic fungi *Rhizoctonia solani*. African Journal of Biotechnology 5: 580-584.
- Shanmugam V, Vivek Sharma, Ananthapadmanaban (2008) Genetic relatedness of *Trichoderma* isolates antagonistic against *Fusarium oxysporum* f. sp. *Dianthi* inflicting carnation wilt. Folia Microbiology 53: 130-138.