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The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; 12(4): 1365-1370
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www.thepharmajournal.com
Received: 12-01-2023
Accepted: 16-02-2023

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Isolation characterization and genetic diversity analysis of *Trichoderma* isolates isolated from Gandheli, Aurangabad region

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Abstract
Trichoderma has gained immense importance since last few decades due to its biological control activity against several plant pathogens. In the present investigation five isolates of *Trichoderma* were collected from different geographical locations of Gandheli, Aurangabad region and characterized primarily on the basis of morphological characters. Molecular characterization of isolates of *Trichoderma* revealed homogeneity with RAPD primer. The genetic relatedness among five isolates of *Trichoderma* was analyzed by using six RAPD markers. In total 22 alleles were detected in five *Trichoderma* isolates. The number of alleles per loci ranged from 3 to 2 with an average of 2.6 alleles per locus (Table.6). All the RAPD loci show polymorphism. Analysis of genetic diversity among five isolates revealed that the polymorphic Information Content (PIC) value represents the relative informativeness and in this study, the average PIC value was found to be 0.44. A PIC value was found to be ranged from 0.26 to 0.56. Expected heterozygosity or Gene diversity computed according to Nei (1973) and varied from 0.32 to 0.64 with an average of 0.52.

Keywords: Characterization, genetic, *Trichoderma*, polymorphic information content

Introduction
The genus *Trichoderma* belongs to class *Ascomycota* and order *Hypocreales* is a filamentous fungus widely distributed in the soil, plant material, decaying vegetations and wood. *Trichoderma* spp. enhances plant growth and productivity in several agricultural crops is important for the control of other fungal diseases such as soil and seed borne (Vazquez-Angulo *et al.,* 2012) [14]. The use of *Trichoderma* spp. in the crop field can help to reduce the application of chemical pesticides and conserve the soil and its ecosystem. Thus, *Trichoderma* cultures were isolated from soil of Gandheli, Aurangabad, Marathwada region of Maharashtra to be used as local and adapted species for different crop plants. Identification of diverse species of *Trichoderma* isolated from soil is an important issue. Previously microorganisms were identified at species level by means of the application of the concept of Morphological species recognition i.e. MSR in combination with its other phenotypic traits.
One of the major problems in agricultural production in the world is soil borne diseases that cause significant economic losses in yield and quality of many important crops such as wheat, cotton, vegetables and temperate fruits. The symptoms include root rot, root blackening, wilt, yellowing, stunting or seedling damping- off, bark cracking and twig or branch dieback. They are difficult to predict and control because they form resistant structures that can survive for many years. Currently, many chemical fungicides have been used extensively to control these diseases. These chemicals are expensive, and result in resistant pathogens, environment pollution and bad effect on human health and living organisms. Therefore, biological control is the best alternative method because it is inexpensive, clean and simple. *Trichoderma* spp. has been used as biocontrol agents against pathogens such as *Alternaria alternata, Rhizoctonia solani, Fusarium oxysporum, Rhizoctonia solani and Scerotium* spp. in many crops. However, precise identification of *Trichoderma* fungi is essential in order to utilize its full application in biocontrol pathogens (Harman, 2006) [4].
Trichoderma spp. show different mechanisms such as antibiotic production, mycoparasitism, production of cell wall degrading enzymes. *T. harzianum* may be used as alternative to the chemicals to suppress the wilt pathogen and raise the yield of tomato.

The role of microorganisms in plant growth promotion, nutrient management and disease control is well known. These beneficial microorganisms colonize the rhizosphere/ endorhizosphere of plants and promote growth of the plants through various direct and indirect mechanisms (Saxena *et al*. 2005) [12]. However, of late, the role of microbes in management of biotic and abiotic stresses is gaining importance. The subject of PGPR elicited tolerance to abiotic stresses has been reviewed recently (Venkateswarlu *et al*. 2008; Yang *et al*. 2009) [15, 16]. Also some species of *Trichoderma* have the ability to promote plant growth, as a result of different mechanisms, such as solubilization of phosphates, micronutrient and minerals, such as Fe, Mn and Mg that have important role in plant growth as well as indirectly with the control of the major and minor root infesting pathogens in rhizosphere, and improve nutrient uptake (Hoyos-Carvajal *et al.,* 2009; Mushtag and Upadhyay, 2011) [5, 8], as well as improve plant defense level against biotic and/or abiotic stress (Mastouri Species of the genus *Trichoderma*, among them *T. viride*, are well known for their production of several lytic enzymes and/or antibiotics. Strains of those species are widely used in biocontrol of soilborne plant-pathogenic fungi.
The visual identifications become highly error prone due to lack of well defined morphological characteristics in cultures. So to limit the above drawbacks DNA based characterization of isolates may reflects the clear picture of relationships than do morphological characters. Purified and high concentration of genomic DNA is a perquisite for taxonomic studies based on molecular characterization. Various authors described different methods for DNA isolation from *Trichoderma* (Gadambe *et al.,* 2008; Vazquez-Angulo *et al*., 2012; Cassago *et al.,* 2002) [3, 14, 2]. The major challenges for isolation of fungal DNA of good quality lies Chloroform: phenol (1:1) was added to each in breaking of rigid cell wall, high tube and vortex briefly. The taxonomic confirmation of species of the genus *Trichoderma*, based only on morphological markers, can be considered limited and of low accuracy, due to the plasticity of its characteristics. Therefore, molecular techniques must be combined with adopting a variety of parameters in order to identify species correctly. There are several molecular methods to characterize *Trichoderma* species such as internal transcribed spacer rDNA (ITS) and translation elongation factor1-alpha (TEF1-α). This paper has following two objectives. Isolation and purification of *Trichoderma* isolated from Aurangabad region (Gandheli). Morphological and molecular characterization of isolated *Trichoderma* isolates.

Material and Method

The present investigation was carried out at Department of Plant Biotechnology, Mahatma Gandhi Mission (MGM), College of Agricultural Biotechnology, Aurangabad, India. To fulfill the set objective, soil samples were collected from Gandheli region and were used for *Trichoderma* isolation. Further the isolated pure cultures of *Trichoderma* were used for genetic diversity analysis. The particulars of the materials used, methods and protocols followed and statistical tools used for analysis, in different experiments were presented under the respective experiment separately.

Isolation and purification of *Trichoderma* isolated from Aurangabad region (Gandheli)

The *Trichoderma* isolates were isolated from the collected soil samples, purified on PDA. The methodology was used as describe below.

Collection of Soil Sample

Soil samples were collected from the fourteen different ecological habitats of crops and horticultural plant of gandheli, Aurangabad for isolation of *Trichoderma* spp (Table 1). They were collected randomly from the rhizosphere of soils at depths 10 to 15 cm, they were placed in sterile plastic bags and label the location and crop name and transfer in laboratory. The soil samples were air dry in shadow.

Table 1: List of soil sample collection sites of Gandheli, Aurangabad region

Sr. No.	Location	No. of samples collected
1	MGM, KVK, Fig plant (A-Block)	2
2	MGM, KVK, bhendi field (A-Block)	2
3	MGM, KVK, sorghum field (A-Block)	4
4	Dairy farm, Guava field (A-Block)	2
5	Dairy farm, sapota field (A-Block)	2
6	Dairy farm, pomegranate field (A-Block)	2
7	Dairy farm, Drumstick field (A-Block)	2
8	Dairy farm citrus field (A-Block)	4
9	Vidya-arnyam Chana field (B-Block)	4
10	Vidya-arnyam bamboo field (B-Block)	2
11	Vidya-arnyam wheat field (B-Block)	4
12	Vidya-arnyam mango field (B-Block)	2
13	CABT sorghum field (A-Block)	4
14	NKCA Crop cafeteria (A-Block)	10
Total sample collected=50		

Isolation of *Trichoderma* from soil samples

Isolation of *Trichoderma* spp. from rhizosphere soil was made using serial dilution technique in sterile distilled water. The 1 gm soil is suspended in 9ml sterile distilled water. The suspensions were made homogenous by agitation using a vertex mixture and further serial dilutions of 10-2, 10-3 and 10-4 were made. 0.5 ml aliquot from each dilution was poured in selective media *Trichoderma* selective media (TSM) in aseptic condition (Table 3). The plates were incubated for 7 days at 28 ± 2 °C. The culture plates were daily observed some different fungal colony are observed and individual green conidia forming *Trichoderma* fungal colony were observed. These fungal colonies were isolated and subculture on fresh PDA media.

Table 2: Composition of potatoes dextrose agar (PDA) media

Sr. No	Composition	g/lit
1	Potato	200
2	Dextrose	20
3	Agar	15
4	Distilled Water	1000ml
5	Ph	5.6+-0.2

Table 3: Composition of TSM media

Sr. No.	Chemicals	Amount(gm/lit)
1	KH_2PO_4	1
2	$MgSO_4$	0.5
3	Peptane	0.005
4	Glucose	10
5	Rose Bengal	0.017
6	Streptomycin Sulphate	30 mg/lit
7	Agar	20

Isolation of *Trichoderma* pure culture

Putative *Trichoderma* colonies were purified by sub culturing on PDA medium (Table 2). Also, hyphal tipping was performed using a dissecting microscope to view the species of interest at high magnification. In addition, if the spores were small and were difficult to manipulate, another way was used to isolate the pure culture of the particular species by selecting individual spores from the species of interest. Finally, pure culture were transferred and stored at -4 °C for further study.

Morphological characterization of *Trichoderma* spp.

Cultural characters of all the *Trichoderma* isolates under investigation were studied on 20 ml sterilized PDA in petri plates. Mycelial disc of approximately 5 mm diameter or spores were transferred aseptically from culture slants using inoculation needle at the center of each petri dish and incubated at 28±2 °C for 4 days. The cultured petri dishes were observed for growth pattern (presence or absence of aerial mycelium or subdued growth etc), pigmentation of varying shades of green of the vegetative growth and pigmentation of the secreted metabolite in the substrate medium against white background under sunlight. The cultural characteristics were photographed using digital camera.

Microscopic Characterization of *Trichoderma* spp.

Conidiophores and conidial morphology, branching pattern, critical for identification to the species level are best observed before the conidia are completely mature. Mounts from the actively growing (fungal growth from the growing colony margin) isolates of *Trichoderma* spp. were prepared in lactophenol cotton blue on glass microscopic slide (preferably young tufts where the conidia just begins to develop pigment in actively growing cultures). Mounts were prepared using a cello tape. A strip of cellotape was held in thumb and forefinger and the gum coated surface was impressed against the sporulating growth of the isolate. The cellotape thus lifted the intact sporulating growth (including conidiophores, philides, and sporulation) without forming any clump of mycelium and injuring the substrate. Microscopic morphological features of all the selected isolates were observed using a Leica binocular microscope and were micro photographed digitally.

Molecular characterization of isolated *Trichoderma* isolates

The DNA was extracted from five isolated *Trichoderma* isolates and used for PCR amplification to study the genetic diversity.

DNA Extraction and Quantification

The fresh cultures of five *Trichoderma* isolates were used for DNA extraction. The DNA was extracted according to the protocol describe by Kadu *et al*., 2019 and Samarrari and Schmidt, 2002 [6, 10]. The procedure of DNA extraction is described below.

Isolates grown on potato dextrose broth (PDA) at 25 °C for 48 h will be used to harvest Mycelium Fungal tissue and DNA isolate using CTAB method modified with EBA and EBB. After incubation at 65 °C, an RNase treatment was performed. DNA was precipitated with isopropanol, washed with ethanol (70%), and air-dried for 15 min. Store in TE for overnight an RNase treatment was performed on next day. DNA was rehydrated with 50 µl of DNA hydration solution which will further be quantified using Nanodrop Spectrophotometer (ND 1000). The acceptable absorbance ratio (A260/A280) for pure DNA is 1.8. Quantified DNA samples of the selected isolates will be subjected to PCR amplification using gene specific primers. The acceptable absorbance ratio (A260/A280) for pure DNA is 1.8.

PCR amplification using RAPD markers

The DNA extracted from the five isolates of *Trichoderma* was used for PCR amplification. The following mentioned primers were subjected to PCR amplification (Table 4) using Applied Biosystem thermal cycler. A series of optimization experiments was carried out in which concentrations of template DNA, primers, dNTPs, *Taq* polymerase and annealing temperature were varied to determine the optimum reaction condition. The 34 cycles were used for amplification in Applied Biosystems thermal cycler. The PCR was performed in a volume of 20 µl reaction containing 2 µl (50 ng) genomic DNA, 10X PCR buffer with 25 mM $MgCl2$ (2.0 µl), 2 mM dNTPs (2.0 µl), 10 µM primer (2.0 µl), 1 µl Taq DNA Polymerase (1U/µl) and Nanopore water. Amplification was performed in an APPLIED BIOSYSTEM thermal cycler with an initial denaturation at 94 °C for 5 mins followed by 34 cycles at 94 °C for 30 sec, 25-40 °C for 30 sec (depending on primer Tm) and 72 °C for 1min, followed by a final extensions at 72 °C for 7mins.

Table 4: List of RAPD Marker and their sequence used for PCR amplification

Sr. No.	Name of marker	Primer sequence (5-3)	No. of bp
1	GLA-06	GGTCCCTGAC	10
2	GLC-01	TTCGAGCCAG	10
3	GLC-08	TGGACCGGTG	10
4	GLJ-12	GTCCCGTGGT	10
5	GLK-10	GTGCAACGTG	10
6	GLK-12	TGGCCCTCAC	10

Agarose gel electrophoresis and Visualization of bands

After the PCR reaction was completed, 4 µl of 6 X loading dye was added to 20 µl PCR amplicons and from this 20 µl (PCR product with dye) sample was loaded in each separate well. Agarose gel of 2.5% was used for better resolution of PCR product. 20 µl of the sample was loaded into the wells in gel with 1X TAE buffer with 100 bp DNA ladder (Genei) and the electrophoresis unit was operated at 100 volts. The gel was visualized under UV transilluminator and Gel documentation unit and the fragment sizes for each marker were determined. The size of the fragments was determined using the 100 bp DNA ladder and the scoring of markers was performed for each fragment.

Data scoring

Based on the expected product size, the size of most intensely amplified bands around the expected product size for each marker was identified using standard molecular weight size marker (50/100 bp DNA ladder; NEB (ILS) Company).The band appeared in primers were scored in the form of matrix in term of base pairs, which indicate the different allelic forms of the same marker.

Statistical analysis
For the estimation of genetic diversity for each primer, parameters such as number of alleles per locus, heterozygosity, major allele frequency, gene diversity and polymorphic information content (PIC) for each marker were estimated using POWERMARKER version 3.25 (Liu *et al.,* 2005) [7]. Nei's distance (Nei *et al*., 1973) [17] was calculated and used for the construction of phylogenetic tree and unrooted phylogeny using neighbour-joining (NJ) method, bootstrapping of the data (10,000 permutations) as implemented in POWERMARKER, and MEGA 6.0 was used to visualize the Tree (Tamura *et al*., 2007) [13].

Result and Discussion
Isolation of *Trichoderma* from soil samples
In the present investigation to isolate the *Trichoderma* the soil samples were collected from different locations of Gandheli Aurangabad. The total number of locations surveyed was 14 and a total 50 samples were collected. The serial dilution technique was used to isolate *Trichoderma* isolates from soil samples. The *Trichoderma* selective medium (TSM) shows the green to white color colonies which were subculture on PDA media. Total five different isolates were obtained in the present investigation named as T1, T2, T3, T4, and T5 from five different locations namely KVK-fig plant, Dairy-Sapota field, Vidyaarnyam-Wheat field, CABT-Sorghum field, and NKCA-crop cafeteria respectively. The pure culture of each *Trichoderma* isolates was maintained on PDA for further morphological, microscopic, and genetic diversity study.

Morphological characterization of the isolates of *Trichoderma*
Through light microscopy we observed the typical characteristics features of the phialides and conidia. This helped us to classify / allocate the *Trichoderma* isolates collected from different geographical locations to different sections as per the characteristics key feature of the sections. Most species of the genus grow rapidly in artificial culture and produce large numbers of small green or white conidia from conidiogenous cells situated at the ends of widely branched conidiophores. This characteristic allows a relatively easy identification of *Trichoderma* as a genus, but the species concepts are difficult to interpret and there is considerable confusion over the application of specific names. Rifai divided *Trichoderma* into nine species aggregates on the basis of morphological features. Bisset revised the genus and also included some *Hypocrea* anamorphs in the genus, resulting in the establishment of five new sections. Species concepts within *Trichoderma* are very wide, and this has resulted in the establishment of many specific and subspecific taxa.
In the present investigation five isolates of *Trichoderma* were characterized primarily on the basis of morphological characters. All the isolates were grown on PDA medium and were observed for growth rate, colony color, diffusible pigments etc. All the isolates show different colony characteristics as describe in Table 5 and Fig. 1. It was observed that conidiophores differed in branching patterns. The key characteristics features of the isolates for conidiophore branching and the phialides corroborates with the keys to sections of *Trichoderma* as reported by Bisset, 1991a and Samuels *et al*., 1996 [1, 11], which justifies the *Trichoderma.* Conidiophores and branches narrow and flexous (wavy), phialides mostly in verticils (Whorls) of 2 or 3, lageniform (flask shaped) to subulate was observered.

Table 5: Colony and mycelia characters of different *Trichoderma* isolates on PDA after 7 days

Isolates	Colony Color	Growth Pattern	Appearence	Different Pigment	Odor
T1	Green Whitish	Areal Subduded	Uniform velvety cottony	Absent	Coconut
T2	White to Green	Areal Subduded	Uniform Cottony Uniform Slightly	Absent	Absent
T3	Dark green	Areal Subduded	Cottony	Absent	Absent
T4	White Green	Areal Subduded	Cottony	Absent	Absent
T5	Green	Areal Subduded	Uniform Cottony	Absent	Absent

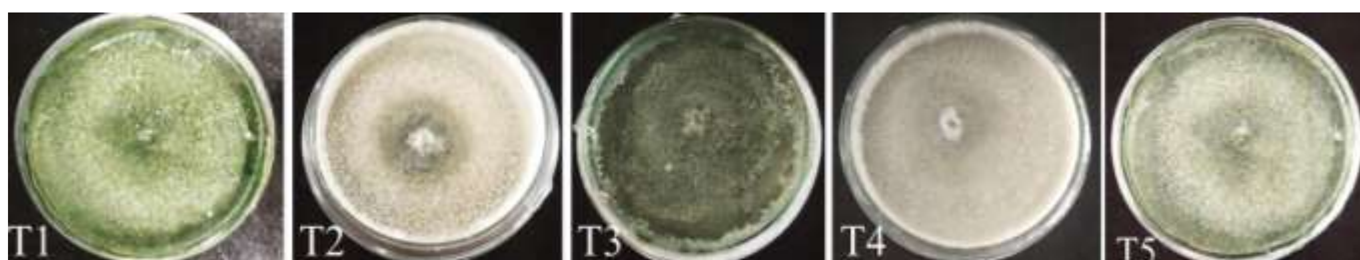

Fig 1: Morphological characterization of *Trichoderma* isolates on PDA media

Molecular characterization of isolated *Trichoderma* isolates
The isolated genomic DNA was quantified by using spectrophotometric measurement at 260 and 280 nm. The yield and purity of isolated DNA for five *Trichoderma* isolates in triplicate were noted in and was ranged from 500 to 1500 ng/ul. The samples were diluted to 50 ng/ul and used for PCR amplification using RAPD primers. The amplicons were resolved on 2.5% agarose gel. The genotypic data in term of base pairs (bps) were scored for each marker and used for diversity study.

Genetic diversity analysis
Analysis of genetic structure of the *Trichoderma* isolates is an important for their identification of species. In this study attempt has been made to analyses the genetic structure of isolated *Trichoderma* isolates using RAPD markers. The amplification shows polymorphism with different allele size. All the markers produce amplicons were used for analysis of genetic diversity among the five *Trichoderma* isolates. Thus,

all these markers should represent the genetic diversity. In total 22 alleles were detected in five *Trichoderma* isolates. The number of alleles per loci ranged from 3 to 2 with an average of 2.6 alleles per locus (Table.6). All the RAPD loci show polymorphism.

Genetic variation within sorghum accessions and cluster analysis

Analysis of genetic diversity among five isolates revealed that the polymorphic Information Content (PIC) value represents the relative informativeness and in this study, the average PIC value was found to be 0.44 was comparable to past and earlier reports. A PIC value was found to be ranged from 0.26 to 0.56. Expected heterozygosity or Gene diversity computed according to Nei (1973) [17] and varied from 0.32 to 0.64 with an average of 0.52. The major allele frequency across five isolates was ranged from 0.40 to 0.60 with the mean major allele frequency was 0.51 (Table 6). Thus, the present result revealed that five isolates used in this study have wide genetic diversity and are good candidates for further study.

Table 6: Mean Diversity of analysis of five *Trichoderma* isolates estimated using Powermarker software

S. No.	Diversity Traits	Number	Range	
1	Total No. of alleles	22	Maximum	Minimum
2	Mean No. of alleles/locus	2.6	3	3
3	Mean Measure allele frequency	0.5	0.6	0.4
4	Mean Diversity	0.52	0.64	0.32
5	Mean PIC	0.44	0.56	0.26

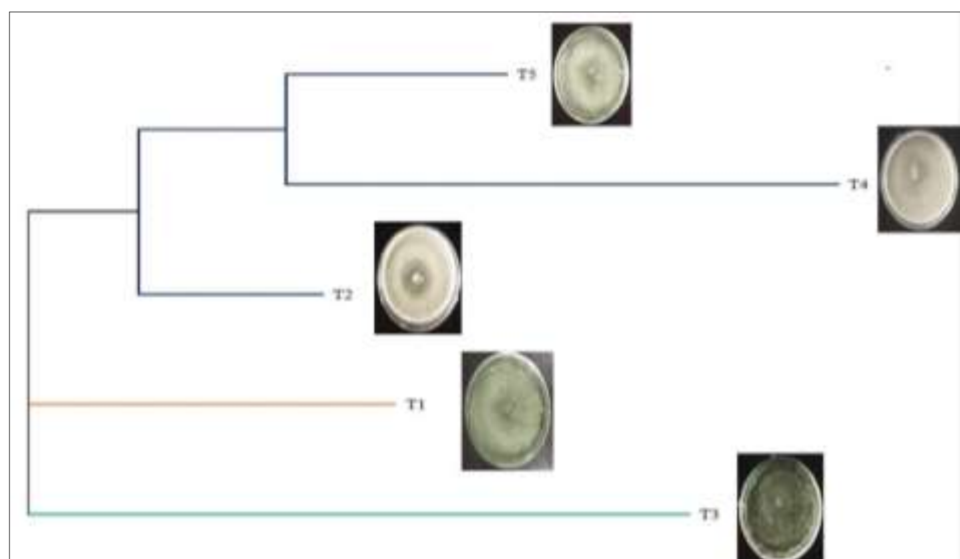

Fig 2: Neighbor-joining (NJ) tree of Five *Trichoderma* isolates was constructed using POWERMARKER software and the tree was viewed using MEGA software

Employing RAPD markers to analyse the genetic diversity is useful. Estimation of major allele frequency, allele number, gene diversity and PIC value with the use of molecular markers is more reliable and accurate than the use of other system. PIC provides an estimate of the discriminatory power of a locus or loci by the number of alleles expressed and the relative frequencies of those alleles.

Distance based approach which is based on calculating pair wise distance matrix was computed by calculating a dissimilarity matrix using a Nei distance (1973) [17] and used for the construction of phylogenetic tree by Neighbour-Joining (NJ) method, implemented in POWERMARKER version 3.25 and the tree was viewed using MEGA V 6.0. A neighbour-joining (NJ) tree was constructed based on genetic distance (Fig. 2). Total three groups were found. Amongst the five isolates tested, the isolate T2, T4 and T5 were grouped in one where as T1 and T3 were differentiate from each other and rest of the isolates. In the present study all isoalates were distinctly placed in this dendogram obtained by the molecular diversity analysis were more consistent with the some important characteristics. No much significant results were reported in the previous study based on the morphological data used to classify *Trichoderma* isolates. Limitations on use of morphological traits, their experimental costs, effect of environment, evaluation time and genotype × environment interactions are widely discussed. In this context, DNA-based markers have become powerful application tools for illustrating and quantifying genetic differences within and among the various *Trichoderma* species.

Conclusion

The *Trichoderma* spp. are used extensively as a biocontrol agent that colonize the rhizosphere of plants interacting with root system of plants and promote growth of the plants. All five isolates were grouped into three different clades. The RAPD marker analysis revealed genetic variation among the sorghum genotypes. Amongst the five isolates tested, the isolate T2, T4 and T5 were grouped in one where as T1 and T3 were differentiate from each other and rest of the isolates. While on molecular basis, maximum polymorphism was observed in the selected isolates. All the bands observed were polymorphic in nature indicating the genetic diversity among *Trichoderma* spp. The result of statistical analysis reflects the broad genetic variability was existed across the *Trichoderma* spp. that provided the scope to identify the variation across the *Trichoderma* isolates.

Acknowledgement

We gratefully acknowledge the MGM College of Agricultural Biotechnology, Aurangabad, India, for providing technical support, laboratory facility and space for growing plants.

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