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RESEARCH ARTICLE

Morpho-molecular characterization, diversity analysis and antagonistic activity of *Trichoderma* isolates against predominant soil born pathogens

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Abstract

Trichoderma is an important antagonist fungi used in biological plant disease management. The present study was carried out to identify the antagonistic efficiency of native *Trichoderma* isolates against soil born pathogens and to characterize them based on morphological and molecular markers. Twenty-nine *Trichoderma* cultures were isolated from the agri-horticulture and forest tree rhizosphere. These isolates were identified as *T. asperellum, T. aureoviride* and *T. virens* based on the ITS sequences. The isolates produced dark green with or without border (n = 16), light green (n = 9) with white borders and alternate green and white (n = 4) with a flat or uneven appearance. The efficacy of these antagonists was investigated by employing the dual plate confrontation assay technique against fungus causing pigeon pea wilt (*Fusarium oxysporum* f. sp. *udum*) and stem rot of tomato (*Sclerotium rolfsii*). The inhibition zone varied from 46.74 to 100 per cent against *F. oxysporum* f. sp. *udum* and the highest inhibition (100%) was exhibited by five *T. asperellum* isolates. Whereas, the suppression of the *Sclerotium* was varied from 47.89 to 72.78 per cent. The phylogenetic analysis based on the ITS sequences revealed two distinct clusters, cluster-I (n = 26) and cluster-II (n = 3). To find the diversity, twelve SSR primers were employed and Jaccard's similarity coefficient was estimated to construct a genetic similarity matrix. The present study has identified five effective native isolates of *Trichoderma* which can be used against *Fusarium* and *Sclerotium* pathogens in the disease management programs.

Keywords *Trichoderma* · Biocontrol agents · Antagonism · Soil pathogens · ITS · SSR

Introduction

Plant pathogens reduce the quality and yield of agricultural produce (Savary et al. 2019). In recent years, managing plant pathogens has been shifted from chemical management to eco-friendly biological control (Singh et al. 2012). Biological control involves the suppression of pathogens employing living organisms (Heimpel and Mills 2017). Many biocontrol agents are used in managing plant diseases; the most important are *Trichoderma, Pseudomonas,* and *Bacillus* (O'Brien 2017). Among these agents, *Trichoderma* has gained immense importance as a biological control agent in the agricultural industry due to its varied activities ranging from being an essential antagonist against soil-borne pathogens to acting as a provider of nutrients to the plants as well (Vinale et al. 2008). *Trichoderma* species are free-living fungi commonly present in soil and root ecosystems and are opportunistic, virulent plant symbionts and parasites on other pathogenic fungi (Harman et al. 2004). Among the *Trichoderma* species, some are used commercially as effective biocontrol agents viz., *T. atroviride, T. harzianum, T. viride, T. hamatum* and *T. asperellum,* which affect the phytopathogens through several mechanisms (Howell 2003; Papavizas 1985; Verma et al. 2007).

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The *Trichoderma* strains compete with fungal pathogens for space and nutrients (Simon and Sivasithamparan 1989), inhibit pathogens by producing extracellular enzymes (Haran et al. 1996) and antifungal antibiotics (Ghisalberti and Rowland 1993), promote plant growth and induce host plant resistance (Inbar et al. 1994; De Meyer et al. 1998). The hydrolytic enzymes partially degrade the cell wall of fungi and lead to mycoparasitism limiting fungal growth and activities (Björkman et al. 1998). Several antagonistic *Trichoderma* isolates were reported from different places with varied soil types, climate, vegetation and cropping system etc., which influences their effectiveness in disease management (Rai et al. 2016). Therefore, the specific isolates from a particular rhizosphere were isolated and used against the locally distributed pathogens.

Soil-borne diseases are considered a major limitation to crop production (Panth et al. 2020). The pathogens namely, *Fusarium*, *Sclerotium*, *Verticillium*, *Phytophthora* and *Pythium* cause 50–75 per cent yield loss in different crops (Mihajlovic et al. 2017). Among these, *Fusarium* and *Sclerotium* are important and difficult to manage due to the production of soil born sclerotia and air-borne ascospores (Panth et al. 2020; Smolińska and Kowalska 2018). *Fusarium* wilt disease in pigeon pea is devastating and causes yield loss of up to 97,000 tonnes per year in India (Saxena et al. 2010). The pathogen is soil and seed-borne, therefore, difficult to manage through fungicide alone. Continuous use of fungicides results in a detrimental effect on the environment and development of resistant strains of the pathogen, health hazards to an applicator as well as to a consumer of the treated material. Their toxic forms persist in soil and contaminate the whole environment (Hemanth et al. 2016).

Sclerotium rolfsii is a soil-borne fungus that causes foot-rot or collar rot disease, which is a polyphagous and most destructive soil-borne fungus. It has become a major problem and challenging to both farmers and scientists. Owing to its soil-borne nature and wide host range, management through chemicals as well as bio-agents is important (Sahana et al. 2017). Many *Trichoderma* spp. colonizes the pathogen hyphae, disrupts mycelial growth and kills the organism. For the management of such soil inhabitant pathogenic organisms, biological control offers a novel approach when applied either alone or with other management practices, which cannot be controlled by chemicals alone (Harman 2000; Howell 2003; Manoranjitham et al. 2000).

Like other fungal genera, *Trichoderma* isolates were defined based on morpho-cultural characteristics (Park et al. 2005; Rifai 1969; Sharma and Singh 2014). However, *Trichoderma* species are difficult to distinguish morphologically due to variability in species across different geographical locations and hosts (Blaszczyk et al. 2011; Hoyos-Carvajal et al. 2009). The studies also reported overlapping morphological features among species in the genus *Trichoderma* (Chaverri and Samuels 2013). Therefore, morphological methods are not conclusive, and there is a need for molecular methods such as DNA sequencing and genealogical concordance phylogenetic species recognition using several unliked genes to give accurate identification (Druzhinina et al. 2006). Many molecular techniques are used in recent times, viz., random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR) and amplified fragment length polymorphisms (AFLP) (Muthu Kumar and Sharma 2011; Shahid et al. 2013; Shalini Devi et al. 2012) to study the genetic variability of the organisms. However, the dominant nature of such markers limits their use in comparative studies (Rai et al. 2016). The use of simple sequence repeat (SSR) markers has gained potential importance in comparative studies since the SSR markers are reproducible, multiallelic, co-dominant, relatively abundant, and have good coverage of the genome (Rai et al. 2016; Singh et al. 2014).

Trichoderma spp. isolated from the different crop rhizosphere need to be evaluated against different soil-borne pathogens. By considering these factors, in the present investigation, we have isolated *Trichoderma* spp. from different agricultural and horticultural crop rhizosphere soils, including forest trees and identified them based on their morphological and ITS sequencing and evaluated their antagonistic nature against *F. oxysporum* f.sp. *udum* and *S. rolfsi*. Further, the diversity of isolates was also analyzed by employing the SSR markers. The present study has contributed in identifying the effective antagonistic *Trichoderma* species and also in understanding the native species diversity in the different crop rhizosphere soils of Karnataka, India, which can be used effectively in integrated plant disease management.

Material and methods

Sampling and isolation of *Trichoderma* isolates

Trichoderma species were isolated from soil samples collected from rhizospheres of banana (n = 7), pigeon pea (n = 6), arecanut (n = 6), pongamia (n = 5), teak (n = 4) and tomato (n = 1) during an exploratory survey carried at Karnataka, India (Table 1). The antagonists were isolated by serial dilution technique on Potato Dextrose Agar (PDA) and were maintained initially on PDA at 25 ± 1 °C for seven days. After an incubation period, colonies were transferred and purified on *Trichoderma* specific media (TSM) (Elad and Chet 1983) which were further confirmed through *Trichoderma* morphological keys and molecular techniques.

Table 1 Description of *Trichoderma* isolates used in the study

Code	Biological origin	Geographical origin	Accession number	Culture	Growth Inhibition against *Fusarium*[a] (%)	Growth Inhibition against *Sclerotium*[a] (%)
Tr 1	Teak	Haranahalli	MH351209	*T. asperellum*	64.55 (53.49)	72.78 (58.58)
Tr 2	Teak	Mathodu	MH383521	*T. asperellum*	66.56 (54.70)	65.93 (54.32)
Tr 3	Teak	Belalakatte	MH383522	*T. asperellum*	64.96 (53.73)	52.07 (46.21)
Tr 4	Pongamia	Harakere	MH383523	*T. asperellum*	72.44 (58.37)	65.59 (54.11)
Tr 5	Arecanut	Yadavala	MH383524	*T. asperellum*	55.26 (48.04)	54.44 (47.57)
Tr 6	Arecanut	Belalakatte	MH383525	*T. asperellum*	93.89 (75.73)	63.33 (52.76)
Tr 7	Arecanut	Harakere	MH383526	*T. asperellum*	55.63 (48.26)	65.56 (54.09)
Tr 8	Arecanut	Hosahalli	MH383527	*T. asperellum*	46.74 (43.15)	53.27 (46.90)
Tr 9	Banana	Mathodu	MH383528	*T. asperellum*	61.30 (51.56)	65.45 (54.03)
Tr 10	Banana	Harakere	MH400805	*T. asperellum*	100.00 (90.05)	60.00 (50.80)
Tr 11	Banana	Honnavile	MH400806	*T. asperellum*	56.74 (48.90)	61.33 (51.58)
Tr 12	Banana	Belalakatte	MH400807	*T. asperellum*	66.85 (54.88)	54.63 (47.68)
Tr 13	Tomato	Navile	MH400808	*T. asperellum*	67.48 (55.26)	58.22 (49.76)
Tr 14	Pigeon pea	Ayanuru	MH351210	*T. virens*	89.44 (71.08)	63.52 (52.87)
Tr 15	Arecanut	Mathodu	MH400809	*T. asperellum*	79.44 (63.07)	69.96 (56.80)
Tr 16	Pigeon pea	Thyajavalli	MH400810	*T. asperellum*	77.19 (61.50)	50.56 (45.34)
Tr 17	Pongamia	Ayanuru	MH400811	*T. asperellum*	100.00 (90.05)	62.04 (52.00)
Tr 18	Pigeon pea	Haranahalli	MH400812	*T. asperellum*	91.11 (72.69)	64.26 (53.31)
Tr 19	Teak	Muduvala	MH400813	*T. asperellum*	100.00 (90.05)	51.30 (45.77)
Tr 20	Banana	Hosahalli	MH400814	*T. asperellum*	93.33 (75.08)	52.44 (46.42)
Tr 21	Banana	Gejjanahalli	MH400815	*T. asperellum*	100.00 (90.05)	51.11 (45.66)
Tr 22	Pigeon pea	Honnavile	MH351211	*T. aureoviride*	77.80 (61.91)	71.30 (57.64)
Tr 23	Banana	Yadavala	MH400816	*T. asperellum*	92.30 (73.93)	55.37 (48.11)
Tr 24	Pigeon pea	Navile	MH400817	*T. asperellum*	70.11 (56.78)	47.89 (43.81)
Tr 25	Pigeon pea	Melinahanasavadi	MH400818	*T. asperellum*	100.00 (90.05)	50.11 (45.09)
Tr 26	Arecanut	Haranahalli	MH400819	*T. asperellum*	63.30 (52.78)	54.55 (47.63)
Tr 27	Pongamia	Hubbanahalli	MH400820	*T. asperellum*	76.67 (61.15)	52.78 (46.62)
Tr 28	Pongamia	Muduvala	MH400821	*T. asperellum*	88.33 (70.07)	57.93 (49.59)
Tr 29	Pongamia	Yadavala	MH400822	*T. asperellum*	63.89 (53.09)	48.96 (44.43)
S.Em ±					0.23	0.25
CD at 1%					0.88	0.94

[a]In vitro antagonistic activity of *Trichoderma* isolates against *Fusarium oxysporum* f. sp. *udum* and *Sclerotium rolfsi* studied by dual culture assay (Solanki et al. 2012). Figures in parenthesis are arc sine transformed values. The experiment was replicated trice

Isolation of *Fusarium oxysporum* f. sp. *udum* and *Sclerotium rolfsii*

The pigeon pea crop showing typical symptoms of the wilt (caused by *Fusarium oxysporum* f. sp. *udum*) was collected and the symptomatic part was cut into small pieces. The pieces were surface sterilized in sodium hypochlorite (1%) solution for one minute and washed thrice using sterilized distilled water and aseptically transferred on PDA. The plates were incubated at room temperature (27 ± 1 °C) for seven days to facilitate the growth of the fungus. The pathogen was identified morphologically by mycelia and conidial character (Booth 1971).

The tomato plants with symptoms of stem rot (caused by *S. rolfsii*) were collected and isolated using a similar technique as described for *F. oxysporum*. The pathogen was morphologically identified based on mycelia and sclerotial characters (Farr et al. 1995). A pathogenicity test for both the isolated organisms was performed as described previously (Ravikumara et al. 2022; Xie et al. 2014).

Morphological characterization of *Trichoderma* isolates

Morphological identification was accomplished through morphological keys (Bissett et al. 2015; Rifai 1969; Samuels

et al. 2012), considering macroscopic characteristics like colour and texture of the colony surface and reverse, presence or absence of pigmentation, a pattern of growth and sporulation. A loopful mycelium of each isolate was dyed with cotton blue to visualize and analyse the microscopic characters according to morphology, size, disposition of the conidia and the phialides with a Lawrence and Mayo microscope with scope image 9.0 (X3). The size of the conidiophores and conidia were recorded while microscopic observation of each isolates and statistically analysed.

Multivariate analysis was done to understand the role of each morphological trait in diversity. The factors number was selected based on roots number larger than 1, and the number of primary variables used in the factor analysis was equal to 8.

Fungal confrontation assay

Antagonistic activity of *Trichoderma* isolates was evaluated in vitro against two soil-borne pathogenic fungi viz., *F. oxysporum* f. sp. *udum* and *S. rolfsii* by dual culture technique according to Morton and Strouble (1955). The inhibition of pathogen growth was calculated by using the formula given by Vincent (1947).

Molecular characterization of *Trichoderma* isolates

Extraction of genomic DNA

Total genomic DNA of *Trichoderma* species was extracted by CTAB (Cetyl-trimethyl ammonium bromide) method (Rogers and Bendich 1994). Single-spore isolates of *Trichoderma* were cultured in Borosil conical flasks (250 ml) containing 100 ml of potato dextrose broth at 25 ± 1 °C for seven days. The mycelia were harvested by filtration through Whatman filter paper and dried with sterilized blotting paper. The dried mycelia were used immediately for DNA extraction. Samples were frozen in liquid nitrogen and grounded in mortar and pestle to a fine powder and preserved at – 20 ℃ for further studies (Shahid et al. 2014). The quantity of DNA was estimated by Qubit™ 4.0 Fluorometer (ThermoFisher Scientific, USA).

PCR amplification

ITS-1F and ITS-4 (White et al. 1990; Gardes and Bruns 1993), and ten SSR primers (Mohammad et al. 2013) were selected for amplification of the *Trichoderma* isolates (Table 2). PCR amplification was performed by mixing 50 ng of the template DNA with 0.2 mM concentrations of each primer, 200 mM concentrations of each dNTP, and 2.5 U of Taq DNA polymerase in Taq Buffer (100 mM Tris–HCl, pH 8.3; 500 mM KCl) and volume was made to 25 µl using Millipore water. The PCR reaction was performed with initial denaturation for 5 min at 94 °C, followed by denaturation, annealing and extension for 60 s at 94 °C, 60 s at 55 °C, and 3 min at 72 °C, respectively of 30 cycles and with a final extension of 5 min at 72 °C in an ABS-VeritiTM 96-well thermocycler (Applied Biosystems, USA). In the case of SSR primers, the reaction condition was similar to ITS but performed for 35 cycles.

Analysis of PCR products

To verify the efficiency of the PCR reaction, 4 µl of the obtained products were analyzed by 1.2% gel electrophoresis in 0.5 × TAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA solution with pH 8) and stained with ethidium bromide. Electrophoresis was conducted at 75 V for 40 min. After the run, the gels were visualized under an ultraviolet transilluminator to confirm the amplification.

Sequencing and phylogenetic analysis

The PCR products of ITS were purified using HiPura® PCR Product Purification Kit (HiMediaTM Laboratories Pvt Ltd, Mumbai, India). The sequencing of ITS PCR amplified products of 29 isolates was carried out using a commercial facility (Agri Genome Labs, Kerala, India). The sequencing was done using ITS-1 and ITS-4 primers, and sequences obtained were checked and edited in BioEdit 2.0 software (Version 7.2.5) (Hall 1999). Subsequently, the concurrent sequences obtained were compared with the GenBank database using the basic local alignment search tool (BLAST) algorithm (http://www.ncbi.nlm.nih.gov). Sequences were deposited in GenBank, and accession numbers were obtained for all the isolates (Table 1). The isolates (n = 29) belonging to the *Trichoderma* species from the study and *T. viride* (strain ATCC 28038), retrieved from the GeneBank database (out grouping), were used to construct the phylogenetic tree. The phylogeny was inferred by comparing the region of ITS phylogenetic groupings by analysing Maximum Likelihood Estimates (MLE) on Mega X software (Kumar et al. 2018) and estimated with a bootstrap value of 1000 pseudoreplicates. Sequences were blasted on BLASTn, and those with a high score (1000) and e-value equal to zero were used for comparison.

Scoring of bands and data analysis

The image of the gel electrophoresis was documented through the Bio-system 1D gel documentation system. All polymorphic bands were scored and analysed. Polymorphic information content (PIC) values were calculated according

Table 2 Nucleotide sequences of ITS and SSR primers used in the study

Primer name	Sequence (5′ to 3′)	Oligonucleotide length	Tm	GC (%)
ITS primer pairs				
ITS 1F	TCTGTAGGTGAACCTGCGG	19	63.9	57
ITS 4R	TCCTCCGCTTATTGATATGC	20	61.5	45
SSR Primers				
SSR 3 F	CAAGCTGACGCCTATGAAGA	20	55.70	56
SSR 3 R	CTTTCACTCACTCAACTCTC	20	55.00	48
SSR 4 F	CATGGTGGAATAGTGATGGC	20	53.70	50
SSR 4 R	CTCCATACACCACTCATTCAC	21	54.65	47
SSR 6 F	CCATGCATACGTGACTGC	18	58.00	55
SSR 6 R	GTTGACTGTTGGTGTAAGTG	20	56.77	51
SSR 10 F	CCGTAAGAATAGGTGTC	17	60.17	56
SSR 10 R	GGAAAATAGGGTGGAAAG	18	63.33	49
SSR 11 F	GAACTCAGTTTCTCATTG	18	60.00	57
SSR 11 R	GAACATATCCAATTATCATC	20	58.88	44
SSR 13 F	CCACGTATGTGACTGTATG	19	65.55	50
SSR 13 R	GAAAGAGAGGCTGAAACTTG	20	63.77	48
SSR 15 F	GGAATTTATCACACTATCTC	20	58.88	53
SSR 15 R	GACTCCCAACTTGTATG	17	60.55	48
SSR 16 F	GTACATTGAACAGCATCATC	20	59.45	48
SSR 16 R	CAATAGGGCATGAAAGGAG	19	60.15	44
SSR 18 F	GTGTGTACCTAAAGCCTTG	19	58.25	55
SSR 18 R	GTAAGTTGATCAAACGCCC	19	59.99	47
SSR 20 F	CACGACTATCCCACTTG	17	63.33	57
SSR 20 R	CTTACTTTCTTAGTGCTATTAC	22	61.45	45

to Anderson et al. (1993) for each SSR locus. PIC values were determined using Power marker Version 3.25 (Liu and Muse 2005). The binary data (1 or 0) was generated for each marker based on the presence or absence of bands. Jaccard's similarity coefficient and a dendrogram were constructed based on unweighted pair group methods (UPGMA) and analyzed by numerical taxonomy system (NTSYS) statistical package version 2.2 (Rohlf 1998).

Results

Morphological characterization of *Trichoderma* isolates

Among 29 *Trichoderma* isolates, 16 isolates showed morphological colonies as dark green; nine isolates were exhibited light green with white colour borders and four isolates represented colony pattern as an alternate green and white colour with a flat appearance (Fig. 1A). In contrast, most isolates were light green on reverse visualization, but some isolates exhibited reverse colony as white to dark green colour (Table 3). Most isolates possessed uneven mycelial form and produced one to five concentric rings. Morphometry of all isolates indicated that the conidiophore was hyaline, narrow and branched and the conidial shape varied from oval to round and ellipsoid (Table 3). The spore colour varied from olive green to green, and the length of conidia varied from 5.7 to7.23 µm. Based on the morphological characters, the isolates were classified into three morpho-groups (MG) (Table 4). The MG-1 consist of 16 isolates followed by MG-2 with 9 and MG-3 with 4 isolates, respectively.

In the factorial analysis employing major factors analysis and based on specific numbers greater than 1, five factors were identified, and they all together justified 78.68 per cent of existing variations among the morphological traits (Table 5). The 5th factor (possessing 76.68% of the variance among traits) played an essential role in justifying morphological variation changes. A Bi-axis plot was created based on phenotypic correlation and dissimilarity between morphological traits. Bi-axis screen plot (Fig. 1B) analysis indicated the importance of each factor in distributing all the *Trichoderma* isolates among the axis. The isolates were distributed in all the directions of the plots evidencing higher variability and diversity among them. Cluster analysis of 29 isolates of *Trichoderma* on an average of eight morphological parameters was performed (Fig. 1C) and three main clusters were distinguished. The first cluster consists of two

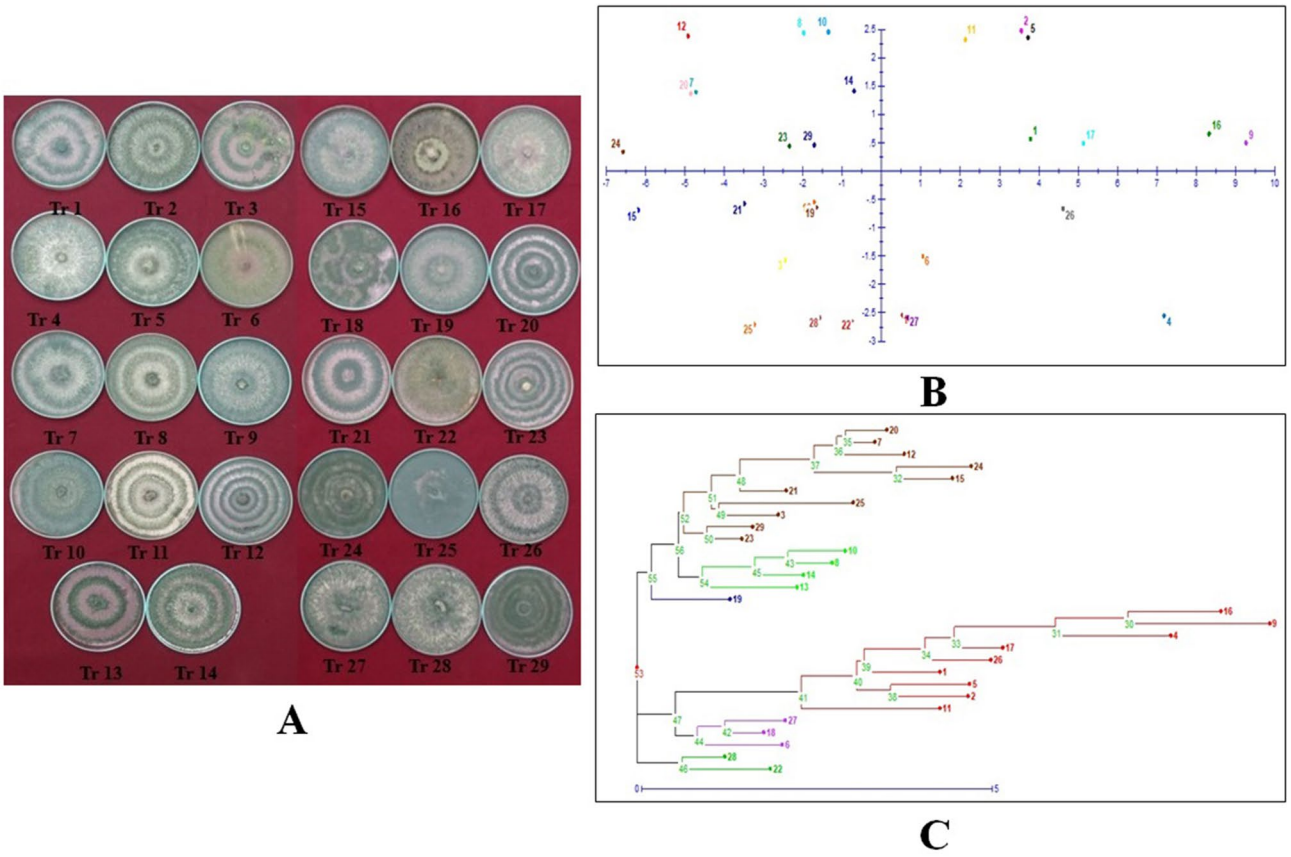

Fig. 1 Morphological characterization of *Trichoderma* isolates. **A** The morphological variability of 29 isolates as exhibited on PDA. **B** Bi-axis plot representing the distribution of isolates based on estimated morphological variables. The isolates were distributed in all the directions of the plots evidencing higher variability and diversity. **C** Dendrogram derived from the cluster analysis (UPGMA) shows the relationship among *Trichoderma* isolates based on morphological traits

isolates, followed by the second cluster with 12 isolates and the third cluster with 15 isolates.

In-vitro confrontation assay of *Trichoderma* isolates

The antagonistic potential of 29 *Trichoderma* isolates was evaluated against *F. oxysporum* f. sp. *udum* and *S. rolfsii* by dual culture technique (Table 1; Fig. 2A and B). The inhibition zone varied from 46.74 to 100 per cent against *F. oxysporum* f. sp. *udum* (Fig. 2C). The highest inhibition (100%) was observed in five *T. asperellum* isolates viz., Tr-10, Tr-21 (banana rhizosphere), Tr-17 (pongamia rhizosphere), Tr-19 (teak rhizosphere), and Tr-25 (pigeon pea rhizosphere) and least inhibition (46.74%) was recorded by *T. asperellum* Tr-8 isolate (arecanut rhizosphere). Whereas against *S. rolfsii*, the zone of inhibition varied from 47.89 to 72.78 per cent (Fig. 2C). The highest inhibition (72.78%) was observed in *T. asperellum* Tr-1 isolate (teak rhizosphere) and the least (47.89%) in *T. asperellum* Tr-24 isolate (pigeon pea rhizosphere).

Molecular characterization using ITS sequences

Genomic DNA subjected to PCR using ITS-1 and ITS-4 yielded ~ 600 bp products which were confirmed on 0.5 per cent agarose by gel electrophoresis. PCR products of amplified ITS regions were Sanger sequenced using ITS-1 and ITS-4 primers from both directions (Table 1). The BLAST program was used to determine the species identity of *Trichoderma* isolates and found that the Tr-14 isolate was identified as *T. virens*, the Tr-22 isolate was identified as *T. aureoviride,* whereas the remaining isolates were identified as *T. asperellum.* The sequences obtained were deposited in the NCBI GenBank database (Table 1).

The evolutionary analysis of the ITS sequence of 29 isolates of Karnataka revealed two distinct clusters, cluster-I and cluster-II (Fig. 3). The majority of the isolates (n = 26) were clustered together in cluster I, whereas only three *T. asperellum*, viz., Tr 13, Tr 23 and Tr 28, were diverged separately into cluster II. Within cluster-I, the 17 isolates were grouped under cluster-IA, whereas 9 isolates

Table 3 Colony and conidial characteristics of *Trichoderma* isolates

Isolate Code	Colony colour	Reverse colony colour	Mycelial form	Concentric rings	Conidial colour	Conidial size (µm)		
						Length (L)	Breadth (B)	L×B
Tr 1	Dark green	Light green	Uneven growth	3	Olive green	6.61	5.99	39.62
Tr 2	Dirty green	Whitish	Flat and even growth	5	Olive green	6.62	5.96	39.41
Tr 3	Dark green	Light green	Uneven growth	1	Green	6.33	5.25	33.32
Tr 4	Light green with white mycelium	White	Even growth	–	Olive green	7.14	6.00	42.83
Tr 5	Dark green with light green centre	Whitish	Flat and even growth	5	Green	6.64	5.91	39.32
Tr 6	Dirty green	Light green	Uneven growth	1	Green	6.49	5.67	36.85
Tr 7	Dark green	Light green	Uneven growth	4	Olive green	5.89	5.26	31.10
Tr 8	Dark green	White	Uneven growth	5	Olive green	6.03	5.59	33.90
Tr 9	Green with white mycelium	Light green	Uneven growth	3	Green	7.11	6.29	44.73
Tr 10	Dark green	Green	Uneven growth	5	Olive green	6.73	5.14	34.56
Tr 11	Alternate white and green mycelium	White	Uneven growth	5	Olive green	6.60	5.67	37.47
Tr 12	Dark green	Light green	Uneven growth	5	Olive green	6.06	5.10	30.95
Tr 13	Dark green	Dark green	Uneven growth	2	Olive green	6.58	5.16	34.21
Tr 14	Dark green with white mycelium	Light green	Uneven growth	4	Olive green	6.21	5.65	35.09
Tr 15	Dark green with white mycelium	Light green	Uneven growth	2	Olive green	5.70	5.17	29.57
Tr 16	Dirty green	White to light green	Uneven growth	3	Olive green	7.23	6.10	44.22
Tr 17	Light green	White	Uneven growth	3	Olive green	6.92	5.89	40.71
Tr 18	Dark green	Light green	Uneven growth	–	Olive green	6.28	5.78	36.24
Tr 19	Dark green with white mycelium	Light green	Uneven growth	2	Olive green	6.23	5.43	33.89
Tr 20	Dark green	Light green	Uneven growth	4	Green	5.83	5.29	30.96
Tr 21	Dark green	Light green	Uneven growth	2	Olive green	6.04	5.33	32.31
Tr 22	Light green	White	Uneven growth	–	Green	6.21	5.61	34.85
Tr 23	Dark green	Light green	Uneven growth	3	Olive green	6.20	5.40	33.51
Tr 24	Dark green	White	Uneven growth	3	Green	6.22	4.71	29.30
Tr 25	Dark green	White	Even growth	–	Olive green	6.19	5.25	32.50
Tr 26	Dark green with whitish mycelium	White	Uneven growth	2	Green	6.95	5.75	40.05
Tr 27	Light green with white mycelium	Light green	Uneven growth	–	Olive green	6.38	5.66	36.21
Tr 28	Light green with white mycelium	Light green	Uneven growth	–	Olive green	6.03	5.65	34.13
Tr 29	Dark green	Light green	Uneven growth	3	Olive green	6.25	5.46	34.10

were grouped under cluster IB. The cluster IA consists predominantly of *T. asperellum* (n = 15), followed by *T. aureoviridae* (n = 1), and *T. virens* (n = 1). The isolates from different rhizospheric soils were distributed across the tree, showing no correlation between the crop type and native *Trichoderma* species. The outgroup AY3800909.1 (*T. viride* strain ATCC 28038) was grouped in cluster II along with the *T. asperellum* isolates.

Diversity analysis using SSR primers

Ten simple sequence repeats (SSR) primers were used in which four were amplified (Fig. 4A). Polymorphism information content (PIC) values for four SSR primers were recorded and PIC values ranged from 0.50 to 0.39. The highest PIC value (0.50) was obtained for SSR 18, followed by SSR 13 (0.48) and SSR 6 (0.40). The lowest PIC value (0.39) was obtained for SSR 10. PIC value revealed that

Table 4 Morpho-groups of *Trichoderma* isolates used in this study based on macroscopic charcters

Morpho-group (MG)	Morphology of the colony	No.of isolates	*Trichoderma* isolates
MG-1	In the verse the colony reprented dark green with no or white colour borders and reverse with white or light green colony change	16	Tr1, Tr2, Tr3, Tr5, Tr6, Tr7, Tr8, Tr12, Tr13, Tr15, Tr16, Tr18, Tr22. Tr23, Tr24 and Tr26
MG-2	In the verse the colony presented light green with white colour in the borders, and reverse with white or light green color change	9	Tr4, Tr9, Tr11, Tr17, Tr19, Tr21, Tr25, Tr27 and Tr28
MG-3	In the verse the colony looks alternative green and white colour and reverse with no color change	4	Tr10, Tr14, Tr20 and Tr29

Table 5 Factor analysis by principle coordinates in morphological traits

Factor	Eighen values	Variance (%)
1	7.423	1.16
2	3.090	2.92
3	1.055	4.64
4	0.663	13.60
5	0.264	76.68

SSR 18 was a better marker to study isolates. Genotyping data generated by 29 *Trichoderma* isolates with four SSR primers were subjected to the estimation of Jaccard's similarity coefficient and the construction of a genetic similarity matrix. The UPGMA clustering system-generated ten genetic clusters with a similarity coefficient of 52.00 per cent (Fig. 4B). Cluster eight was the most significant with eight *Trichoderma* isolates, followed by clusters four and one with six, clusters six and seven with three isolates, cluster three with two isolates and clusters two, five, nine and ten with one isolate, respectively. The highest genetic distance was observed between the farthest clusters.

Discussion

The genus *Trichoderma* has paved immense significance due to its antagonistic nature against several plant pathogens (Panth et al. 2020). To develop and utilize an efficient fungal antagonist, the identification and characterization of *Trichoderma* is a prerequisite.

The morphological characteristics of *Trichoderma* concerning mycelia and spore production were studied on PDA since the mycelial development and pigmentation can be observed best on PDA than in other media (Samuels et al. 2002). Many workers reported green or yellow-green colonies (Kumar et al. 2014; Samuels et al. 2002). Initially, colonies appeared as a cream which later turned to dark green colour. Srivastava et al. (2014) reported that the colony colour changes from a light green shade to dull green with conidia production. The formation of conidia results in the blue-green to yellow-green pigmentation of the colony (Siddiquee 2017). Concentric rings can be observed occasionally for some species of *Trichoderma* (Samuels et al. 2002). The length and breadth of the conidia varied from 5.70 to 7.23 µm and 4.71 to 6.29 µm respectively. The total size of conidia varied from 29.30 to 44.73 µm. The results obtained were more or less similar to observations made by Soesanto et al. (2011), where the average size of conidia was 5 µm in length and 2 µm in width of *Trichoderma* isolate 3, and 7.20 × 7.20 µm in *Trichoderma* isolate 5. These results were also supported by Kumar et al. (2010) who reported 2.50–3.50 × 4.00–5.50 µm as the length and breadth of conidia.

To identify the efficient indigenous isolate of *Trichoderma* with prominent antagonist activity, isolates were subjected to a dual culture study. All the *Trichoderma* isolates inhibited the colony growth of *F. oxysporum* f. sp. *udum* and *S. rolfsii*. The earlier studies also reported the significant inhibition of the mycelial growth in the pathogenic strains of *F. oxysporum* (Athul and Jisha 2014; Altinok and Erdogan 2015; Kamala and Indira Devi 2012; Ramezani 2010; Sundaramoorthy and Balabaskar 2013) and *S. rolfsii* (Athul and Jisha 2014; Rekha et al. 2012).

The morphological study alone is insufficient to identify the *Trichoderma* spp. precisely due to the availability of limited variation within isolates that may overlap and leads to misidentification of the species (Anees et al. 2010). The expression of the morphological characters was also influenced by the kind of media used and the prevailing temperature (Hassan et al. 2014). Based on ITS sequencing of 29 isolates, 27 were identified as *T. asperellum*, one as *T. virens* and *T. aureo viride*. Identification of *Trichoderma* by using universal primers (ITS-1 and ITS-4) was also made by Mohammad et al. (2013) and Mohammad et al. (2014). Four SSR loci were employed to find the genetic diversity of 29 isolates of *Trichoderma* spp. PIC value is a

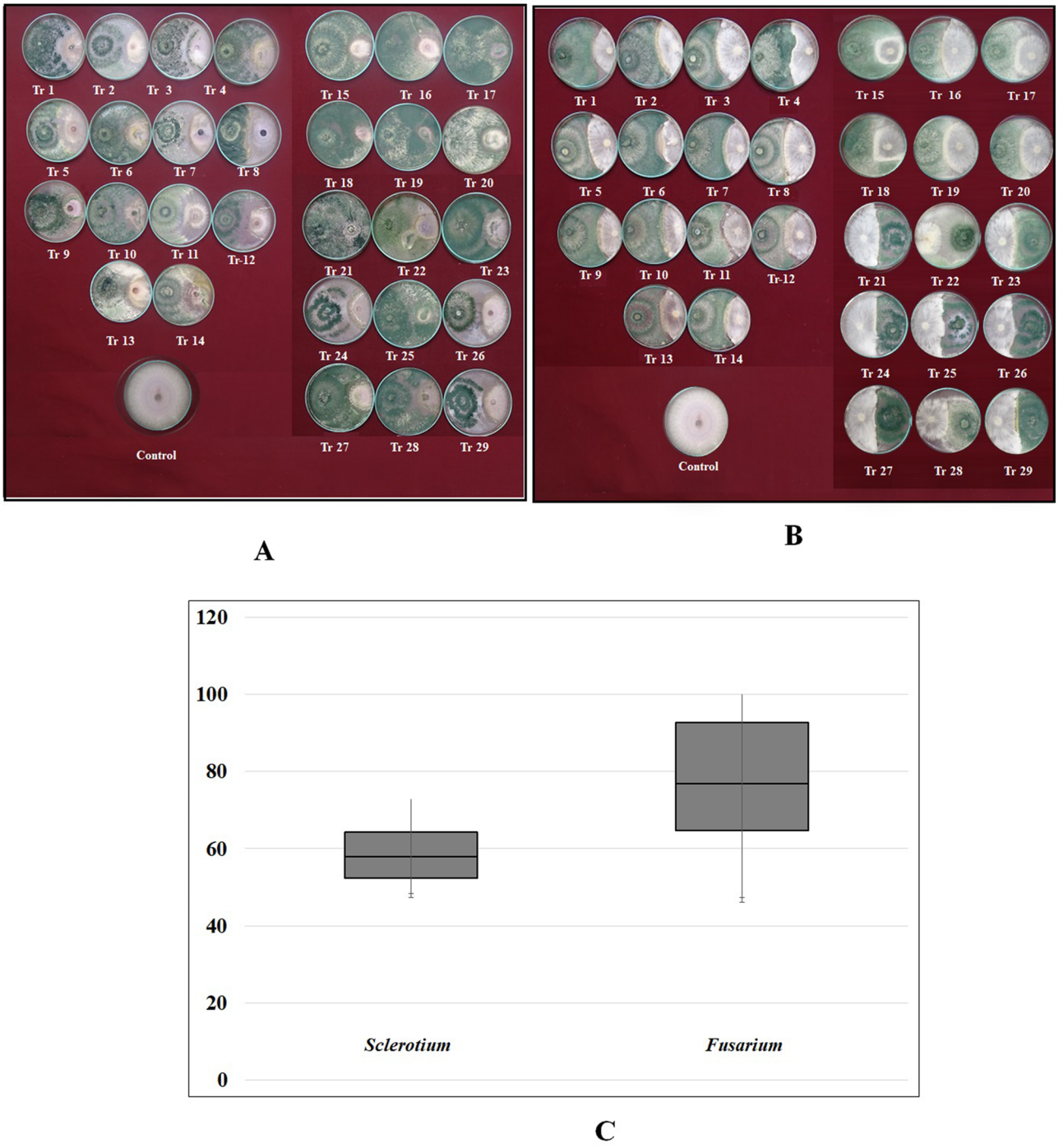

Fig. 2 Dual confrontation assay to evaluate the antagonistic effect of *Trichoderma* isolates against soil born pathogens. Trichoderma isolates were grown on PDA and placed against the pathogens namely **A** *Fusarium oxysporum* f.sp. *udum* and, **B** *Sclerotium rolfsii.* **C** The Box Plot analysis was used to express the efficacy of the isolates against pathogens

reflection of frequency and allele diversity among isolates. PIC values ranged from 0.50 to 0.39 which was similar to Rai et al. (2016), who reported PIC values > 0.40. Shahid et al (2013) evaluated the variability among *Trichoderma* isolates by using SSR and RAPD markers and obtained the highest polymorphism (> 77%) with SSR followed by RAPD (~ 50%).

In conclusion, the present study has identified the effective *Trichoderma* isolates from different crop rhizosphere for the management of soil-born plant diseases, especially

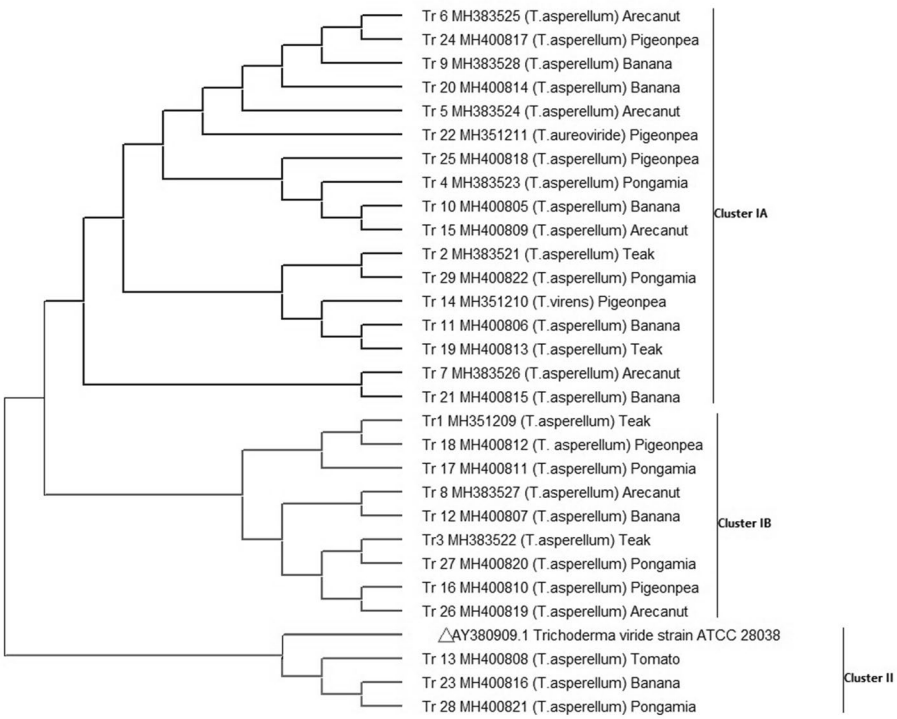

Fig. 3 Phylogenetic tree based on ITS sequences. The evolutionary history was inferred using the Maximum Composite Likelihood method. The optimal tree with the sum of branch length = 41.35094847 is shown. This analysis involved 30 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 647 positions in the final dataset

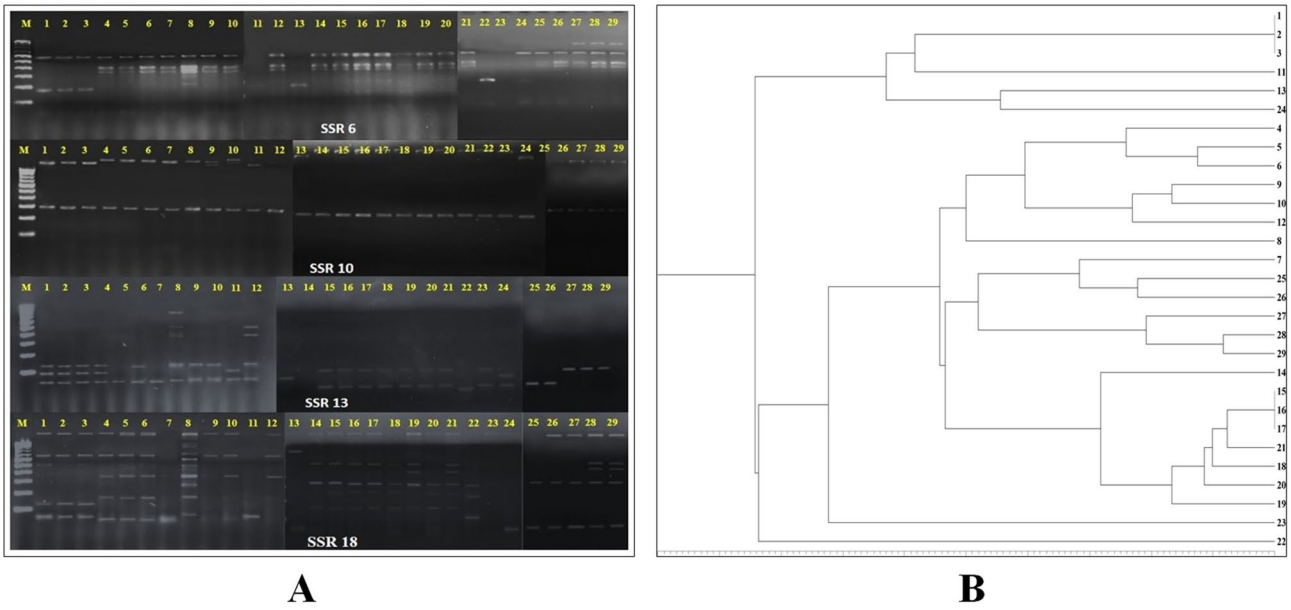

Fig. 4 Diversity analysis of *Trichoderma* isolates. **A** Polymorphism among accessions using SSR markers 6, 10, 13 and 18, M ladder (100 bp), **B** Dendrogram showing the relationship among 29 isolates of *Trichoderma* based on SSR analysis

against *Fusarium oxysporum* f. sp. *udum* and *Sclerotium rolfsii*. The rhizosphere of one crop contained the *Trichoderma* that was also effective against the pathogen from another rhizosphere and thereby providing an opportunity to use *Trichoderma* from any rhizosphere to contain the plant pathogen of any crop. The study identified the presence of *T. virens*, *T. aureoviride,* and *T. asperellum* in different crop rhizosphere and *T. asperellum* as predominant *Trichoderma* species in the soils of the present study.

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Availability of data and material The data relating to the ITS sequences are available at NCBI GenBank.

Code availability Not Applicable.

Declarations

Conflicts of interest Not Applicable.

Ethics approval Not Applicable.

Consent to participate Not Applicable.

Consent for publication Not Applicable.

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