TROPICAL AGRICULTURIST, VOL. 164, 2016

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF TRICHODERMA ISOLATES USED AS BIO-CONTROL AGENTS IN SRI LANKA

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ABSTRACT

Trichoderma, commonly available in soil and root ecosystems has gained immense importance since last few decades due to its biological control ability against several plant pathogens. Strain identification in situ is an important factor in the monitoring of microorganisms used in the field. In this study, six Trichoderma isolates which were identified as bio-control agents were used for characterization and identification up to species level on the basis of phenotypic characters and genotypic variations. The morphological characteristics studied were colony appearance, growth rate on PDA, the shapes and sizes of conidia, phialide and conidiophores, branching patterns of conidiophores and production of chlamydospores. However, due to the uncertainty of morphological data, it is often difficult to identify the Trichoderma isolates precisely. Therefore, DNA of the isolates were subjected to PCR amplification with ITS-1 and ITS-4 primers and sequenced. According to the molecular analysis, five isolates were identified as T. Asperellum and one as T. Longibrachiatum.

KEY WORDS: Bio-control agents, characterization, Trichoderma spp.

INTRODUCTION

A key element of sustainable agriculture is the ecological approach to solve problems with plant pathogens by the application of Bio-Control Agents (BCA). These BCAs include strains belonging to fungal genera such as Trichoderma, Candida, Gliocladium and bacterial genera such as Bacillus and fluorescent Pseudomonas. Among the BCAs, Trichoderma spp. is the most intensively studied fungal species. Trichoderma spp. are beneficial plant symbionts that act as natural bio-control agents against several important phytopathogenic fungi and it is one of the best known mycoparasites that could be used as a bio-control agent against many soil borne plant pathogens such as Fusarium, Rhizoctonia, Phytophthora, Sclerotinia and Alternaria. Trichoderma spp. develop exactly on other fungi's hyphae, coils around them and degrades the cell walls. This action of parasitism restricts the development and activity of pathogenic fungi (Agrilinks, 2013).

CHARACTERIZATION OF TRICHODERMA

Studying the behavior of these fungi as antagonists is essential for their effective use since they can act against target organisms in several ways. Therefore, the commercial use of Trichoderma BCAs must be headed by precise identification. Characterization and identification of strains at the species level is the first step in utilizing the full potential of fungi in specific applications. Morphological characterizations were used in the past and in recent years, several molecular biological techniques based on Polymerase Chain Reactions (PCR) have been used to detect and discriminate among microorganisms. Sequence analysis of the internal transcribed spacer (ITS) region has been helpful in the neotypification, description and characterization of species in the genus Trichoderma (Shahid et al., 2013). Therefore, the objective of this study is to characterize the Trichoderma spp. Phenotypically, based on cultural and microscopic observations, and genotypically, at species level with sequence analysis of the ITS region, for future use of producing large scale BCAs for Sri Lankan agricultural fields.

MATERIALS AND METHODS

Sample collection

The six Trichoderma isolates identified as bio-control agents were used in this study namely MAR1, MAR3, JAP, KON, HAR and VIR. Those were obtained from the culture collection at Horticultural Crop Research and Development Institute, Gannoruwa.

Morphological characterization of isolates

Morphological observations were made from single spore cultures grown on Potato Dextrose Agar (PDA) at 28 oC under laboratory conditions. For observing colony characteristics and growth rate, a five mm mycelia disc taken from the actively growing cultures was placed at the center of petri dishes by using a cork-borer. The morphological characterization and identification were performed based on the online interactive key provided by Samuels et al. (2015) which included the phenotypic characters classified under colony appearance, sporulation pattern, growth rate, size and shape of conidia/ phialides/ conidiophores, branching patterns of conidiophores, and the appearance of chlamydospores. Treatments were six times replicated and arranged as CRD and incubated at 28 oC. Daily observations were made for 4 days. Growth rate was measured using the following equation.

$$\text{Growth rate of Trichoderma} = \frac{\text{Mean radius of Trichoderma colony (cm)}}{\text{Time taken for colony measurements (days)}}$$

Lengths and widths of conidiophores, phialide and conidia of the Trichoderma isolates were measured with a stereo microscope attached to a Nikon camera (Figure 1). The mean growth rates and the measurements of the Trichoderma structures were analyzed by MSTATC statistical software.

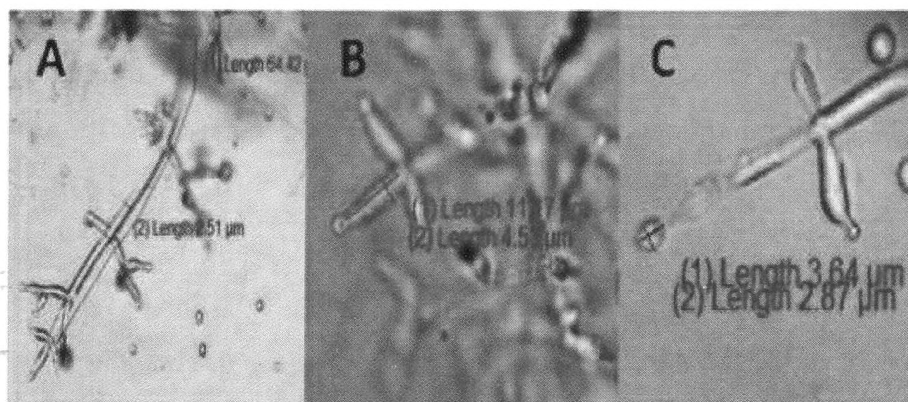

Figure 1. Measurements taken under stereo microscope attached to a Nikon camera. A- Conidiophores (X200), B- Phialide (X400), C- Conidia (X400). (1) - Length (μm), (2) - Width (μm).

Molecular characterization

DNA extraction and PCR amplification of ITS region

The total genomic DNA was extracted from isolates of Trichoderma based on the Cetrimide Tetradecyl Trimethyl Ammonium Bromide (CTAB) extraction method of Crowhurst et al. (1995). All the samples were subjected to PCR amplification with universal primers, ITS-1 and ITS-4. The set of primers: ITS1: 5'-TCCGTAGGTGAACCTGCGG-3', which anneals at the end of 18S rDNA and ITS4: 5'-TCCTCCGCTTATTGATATGC-3', which anneals at the beginning of 28s rDNA (White et al., 1990) amplify a fragment of 600 bp size. PCR amplifications were performed in a total volume of 10 μl by mixing 5μl of Taq PCR master mixture (Qiagen- 25 units Taq DNA polymerase, 200 μm of each dNTP and 1X PCR buffer and 1.5 mm MgCl2), 0.8 μl of each primer (10 mm), 0.5 μl of diluted (1:10) DNA template and 2.9 μl of sterilized distilled water. PCR program included an initial denaturation of 5 min at 94 oC, followed by 35 cycles of 1 min at 94 oC, 1 min at 55 oC and 1 min at 72 oC with a final extension at 72 oC for 10 min using a thermo cycler (Labnet Gradient). PCR products were electrophorized in 1.4 % agarose gel for 01 h at 80 V and visualized with a gel documentation system (ENDUROTM GDS). Promega– G571A 1 kb ladder was used as a marker.

Sequencing and phylogenetic analysis of amplification products

The PCR products of the samples were sequenced (by Macrogen, Korea) and FASTA form of the result sequences were edited and analyzed using Basic Local Alignment Search Tool (BLAST). Multiple sequence alignment was then performed using Clustal W program. Phylogenetic dendogram was constructed by the neighbour-joining method and tree topologies were evaluated by performing bootstrap analysis of 1000 data sets using MEGA 4.

RESULTS AND DISCUSSION

Several Trichoderma spp. may occupy one ecological niche. Therefore, single spore cultures for isolates of Trichoderma were prepared. Due to homoplasy of morphological characters, it is often difficult to discriminate species as it requires wide fungal taxonomical knowledge (Druzhinina and Kopchinskiy, 2008). The morphological descriptors used for the characterization of Trichoderma isolates are presented in Table 1.

The growth rate of Trichoderma spp. is normally rapid. Colony radius was measured at 24, 48, 72 and 96 h and radial growth rate was calculated. According to the results, Trichoderma isolates exhibited significantly different growth rates. The colony color changed from light green to dark green with the production of conidia after 48 h. The conidial shapes were observed to be globose to subglobose in all cultures while oblong to narrowly ellipsoidal in KON and the phialide shapes were observed to be straight and ampulliform in all cultures while cylindrical in KON. Chlamydospores were present in all five samples except for KON. However, the appearance of chlamydospores did not provide much information for the identification of Trichoderma isolates as all the chlamydospores observed were unicellular and uniform in appearance ranging from subglobose to globose.

Table 1. Key morphological descriptors used for the characterization of Trichoderma

Morphological Characters	Isolates					
	MAR1	MAR3	JAP	HAR	KON	VIR
Mean growth rate (cm/day)*	1.86b**	1.78a	2.05d	1.87b	2.04d	1.98c
Colony color	Light to dark green	Light to dark green	Light to dark green	Light to dark green	Light to dark green	Light to dark green
Phialide shape	Ampulliform	Ampulliform	Ampulliform	Ampulliform	Cylindrical	Ampulliform
Conidia shape	Globose to Subglobose	Globose to Subglobose	Globose to Subglobose	Globose to Subglobose	Oblong to narrowly ellipsoidal	Globose to Subglobose
Branching pattern of conidiophores	Regular	Regular	Regular	Regular	Regular	Regular
Appearance of clamydospores	Present	Present	Present	Present	Absent	Present

*CV-12.81%

**Means followed by the same letters are not significantly different at $\alpha = 0.05$.

CHARACTERIZATION OF TRICHODERMA

Trichoderma spp. used in this study had been identified as BCAs in the fields. According to the literature, the most common BCAs identified are T. harzianum, T. viride and T. koningii. According to Samuels et al. (2015), the cultural characteristic of T. harzianum is conidia forms densely over the center and in undulating concentric rings towards the edge and in many colonies conidia were first yellow in color and becomes yellow-green; often with yellow pigment diffusing in medium and conidia of T. viride is forming abundantly in conspicuous concentric rings with no diffusing pigment. The cultural characteristic of T. koningii is conidia typically forming moderately well in concentric rings with a diffusing yellow pigment. However, these characteristics were not observed in the respective Trichoderma samples used for this study.

Species of this genus produce a broad array of pigments from bright greenish-yellow to reddish in color, although some are colorless. Therefore, species identification within this genus through pigmentation is difficult because of the narrow range of variation of the simplified morphology in Trichoderma. Furthermore, the shapes of phialides and conidia were not applicable with the shapes of the known isolates. Nevertheless, the descriptions of the shapes of phialide and conidia were not categorically useful in identifying most of the isolates due to the confusion caused by the use of different terms in different literatures in describing the shapes of the phialide and conidia. Furthermore, noticeable differences in the sizes of phialides, conidia and conidiophores were observed in the cultures (Table 2).

There was a significant variation observed among the mean lengths and widths of the Trichoderma isolates. The measurements of conidiophores could not be served as an exact distinguishing characteristic for Trichoderma species identification because the conidiophores were observed to be highly branched and thus were difficult to define or measure. However, phialide and conidial sizes of these isolates were useful in narrowing down the possible number of Trichoderma species in the process of species identification (MAR1 = MAR3).

The morphological characteristics were generally found to be highly variable. Druzhinina and Kubicek (2005) stated that morphological analyses are highly prone to error. Therefore, based on these morphological results, it is often difficult to precisely identify the Trichoderma isolates. Hence, there was a necessity to use molecular techniques to compensate for the limitations of morphological characterization.

Table 2. Lengths and widths of phialides, conidia and conidiophores of Trichoderma isolates

Isolates of Trichoderma	Lengths and widths (µm)					
	Phialide		Conidia		Conidiophore	
	Length*	Width*	Length	Width*	Length*	Width*
MAR 1	14.950a	5.985a	4.308a	4.123b	62.263cd	3.517ab
MAR 3	9.962cd	3.223c	2.965cd	2.860c	78.240bc	2.532c
JAP	9.370cd	2.855c	2.795d	2.615c	69.415bc	3.070bc
KON	10.582c	2.595c	3.553bc	3.210c	39.736d	1.583d
HAR	11.585b	3.022c	4.253b	4.283a	46.125d	2.385cd
VIR	6.552d	4.063b	3.590bc	3.175c	5.865ab	3.488ab

*. Data based on the mean of 20 measurements of phialide, conidia and conidiophore. Within each column, the values followed by the same letters are not significant at $\alpha = 0.05$.

Molecular characterization

The PCR conducted using genus specific ITS1 and ITS4 primers successfully amplified an approximately 600bp fragment from all samples (Figure 2). The results were in accordance with Mukherjee et al. (2002) who studied the identification and genetic variability of the Trichoderma isolates.

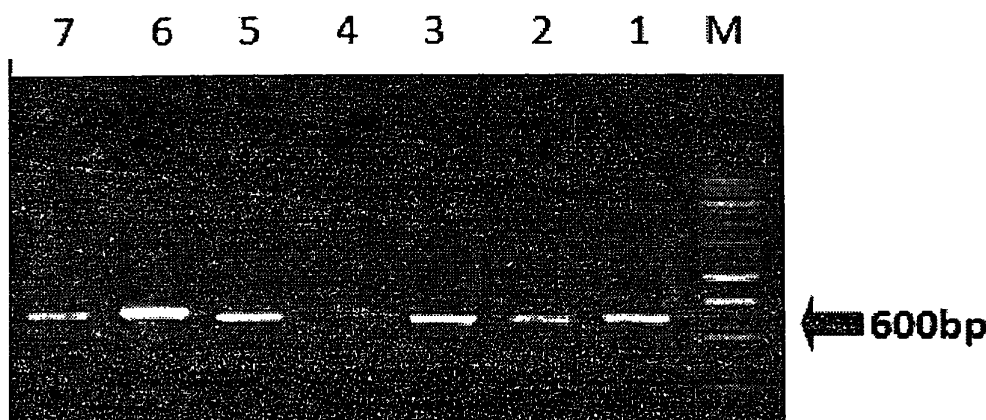

Figure 2. ITS region amplified using primer pair ITS1 and ITS4. Lane M- 1 kb marker, 1)-MAR 1, 2)-MAR 3, 3)-JAP, 4)-Water control, 5)-KON, 6)-HAR, 7)-VIR

Hermosa et al. (2000) stated that any taxonomical study with Trichoderma spp. should include a higher number of strains for phylogentic analysis. Therefore, the phylogenetic trees (Figure 3) for each isolate were obtained by sequence analysis of ITS region based on 10 highly similar sequences obtained from Genbank. Although phylogenetic trees were drawn for each isolate with higly similar sequences for all the isolates (MAR3, JAP, KON, HAR, VIR), they were clustered separately. According to the BLAST and phylogenentic results (Table 3), MAR3 showed 98 % nucleotide identity with T. asperellum from Vietnam, China, India and Brazil. JAP showed 100 % nucleotide identity with T. asperellum from Japan, China, Brazil and Italy. KON showed 98 % identitiy with T. longibratiachum from India, Vietnam, Australia and Malaysia. HAR showed 99 % nucleotide identity with as T. asperellum from China and Brazil while VIR showed 99 % nucleotide identity with T.asperellum from China, Indonesia, Brazil and India.

Table 3. Highly similar identities of the isolates with other closely related species.

S.No	Description	Abbreviation	Accession number	Max ident
MAR3				
1	Trichoderma asperellum strain T164	T.asperellum_Vietnam_1	GU176465.1	98%
2	Trichoderma asperellum strain Q1	T.asperellum_Shandong_China	HQ293149.1	98%
3	Trichoderma asperellum isolate NBAII Th-4	T.asperellum_India	JQ013144.1	98%
4	Trichoderma asperellum strain IBLF-897	T.asperellum_Brazil_1	KJ646883.1	98%
JAP				
1	Trichoderma asperellum strain T13	T. asperellum_Japan_1	LC002601.1	100%
2	Trichoderma asperellum strain T-1	T.asperellum_ Hangzhou_China_1	KM277355.1	100%
3	Trichoderma asperellum strain IBLF-897	T. asperellum_ Sao Paulo _Brazil	KJ646883.1	100%
4	Trichoderma asperellum isolate 2046	T. asperellum_Italy	KF454870.1	100%
KON				
1	Trichoderma longibrachiatum isolate TL10	T. longibrachiatum _Delhi_India_1	JN039083.1	98%
2	Trichoderma longibrachiatum strain T36	T. longibrachiatum_Vietnam	GU176487.1	98%
3	Trichoderma longibrachiatum strain IHEM 5747	T. longibrachiatum_Australia	KP132794.1	98%
4	Trichoderma longibrachiatum isolate A3S1-D3	T. longibrachiatum_Malaysia	KJ767089.1	98%
HAR				
1	Trichoderma asperellum strain PTN-10	T. asperellum_China_1	KF589303.1	99%
2	Trichoderma strigosellum isolate C42-19	T. strigosellum_ Brazil	KJ439091.1	99%
VIR				
1	Trichoderma asperellum strain YNKM1067	T. asperellum_Shangai_China	JQ040322.1	99%
2	Trichoderma asperellum isolate T4	T. asperellum_Indonesia	KC479809.1	99%
3	Trichodermaa asperellum strain CEN503	T. asperellum_Brasilia_Brazil	KC561066.1	99%
4	Trichoderma asperellum strain BPLMBT1	T. asperellum_India	KC243781.1	99%

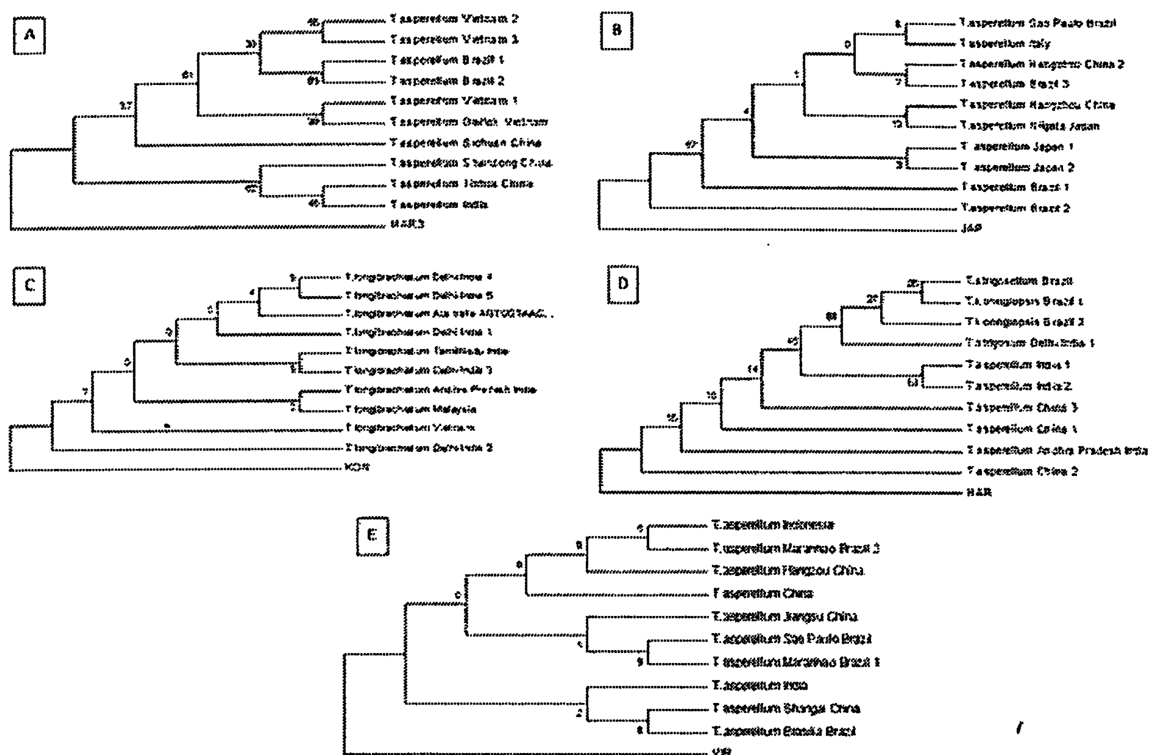

Figure 3. Phylogenetic trees constructed using MEGA 4.0 at 1000 bootstrap value showing the relationship of (A) MAR 3, (B) JAP, (C) KON, (D) HAR and (E) VIR with other closely related species.

CONCLUSIONS

Based on the molecular analysis through ITS regions, among the 6 isolates, five were identified as T. asperellum and one as T. longibrachiatum. Morphology based technique was not accurate.

ACKNOWLEDGEMENTS

Authors are grateful to all the staff members of the Plant Pathology Division, Horticultural Crops Research and Development Institute, Gannoruwa, Peradeniya for their support in conducting this study.

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