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Internal Transcribed Spacers (ITS) Based Identification of *Trichoderma* Isolates and Biocontrol Activity against *Fusarium oxysporum* and Chitinase Activity

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Twelve *Trichoderma* spp. isolated from different locations in Uttar Pradesh, India. Internal transcribed spacer (ITS) amplification and sequencing were used to identify isolates of Trichoderma that showed 99–100% identification with *Trichoderma harzianum*, *Trichoderma asperellum*, and *Trichoderma longibrachiatum*, the three species of Trichoderma. Tests were conducted *in Vitro* to evaluate the biocontrol potential of Trichoderma isolates against *Fusarium* species. After seven days

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of incubation, the isolate TBT6 (*T. harzianum*) exhibited the greatest antagonism against *Fusarium oxysporum*, with inhibition rates of 87.1%) by TBT7 (82.2%), and the least amount of inhibition (59.7%) by *T. longibrachiatum* isolate TBT10. Under field conditions, the antagonistic *T. harzianum* isolate TBT6 can be employed as a bio-control agent against *Fusarium oxysporum*. and for the development of formulations based on Trichoderma. Using the dual plate approach, twelve highly antagonistic Trichoderma isolates were selected for chitinolytic activity. Observing the breakdown of chitin substrates, Trichoderma isolates TBT6 was shown to have strong chitinolytic enzyme activity, making it a good candidate for endochitinase gene isolation. Using two particular primers, the genomic DNA of the Trichoderma isolate TBT6 was isolated and amplified.

Keywords: Trichoderma; internal transcribed spacers (ITS); antagonism; Fusarium oxysporum; chitinase activity; biocontrol.

1. INTRODUCTION

Farmers use chemical pesticides as their first option to manage plant diseases and maximize crop yields [1]. It was estimated that 12% of crop loss is due to plant pathogens [2]. Long-term pesticide use can lead to serious health and environmental issues in addition to being very expensive for developing nations. Famous saprophytic fungus Trichoderma may be isolated from any soil and, because of its high rate of colonization and reproduction, is an effective biocontrol agent [3]. Numerous fungal phytopathogens, including Phythium, Phytophthora, Macrophomina, Aspergillus, Rhizoctonia, and Fusariu, are combated by Trichoderma. employing a variety of biocontrol techniques, such as mycoparasitism, antibiotic synthesis, and pathogen competition for resources (food and space) [4-6]. Recently, global attention has been paid to *Trichoderma* as safe alternative of pesticides and this led to an increase in the number of *Trichoderma* biocontrol products [7]. “In agriculture 37% of crop loss is due to pests, out of which 12% is due to pathogens. Fungi are responsible for more than 70% of all major crop diseases. Significant loss has been observed in several crop species like rice, wheat, barley, cotton and groundnut due to fungi. In agriculture, annual crop losses due to pre and post-harvest fungal diseases exceed 200 billion euros, in United State alone, over $600 million are annually spent on fungicides” [2].

“Fusarium oxysporum species are ubiquitous soil-borne pathogens of a wide range of horticultural and food crops which cause destructive vascular wilts, rots, and damping-off diseases and several strains of pathogen to human or animals” [8].

“Chemical pesticides used by the farmers for the management of fungal and pest control. The chemical pesticides which used have been affecting on both human and environment. Chitinase is one of the enzymes that has been effectively used for management of fungal diseases. It targets chitin of the cell wall for management of plant pathogens. Plants have known to implicate chitinase in defense against plant pathogens. Chitinase provide an alternate solution over harmful chemicals to plant defense against fungal pathogens. *Fusarium oxysporum* and *Fusarium graminearum* are the representative species known as plant-pathogenic *Fusarium”* [9]. “The majority of *Fusarium* species are soil-inhabiting fungi,*Fusarium* conidia can be dispersed by water in rain splash and via irrigation systems but become airborne when dried, which makes them well-suited for atmospheric dispersal over long distances and which contributes to their worldwide distribution” [10]. “Although *Fusarium* utilizes multiple infection strategies, these fungi are considered to be hemibiotrophs capable of transitioning to necrotrophs depending on specific environmental and metabolic” [11]. “As plant pathogens, they cause root and stem rot, vascular wilt, or fruit rot in several economic crop species resulting in major yield losses (MT ha^{-1}) and in economic losses that value over $1 Billion” [12]. “*Fusarium* toxins are the most abundant natural contaminants of diets containing cereals and other grains” [13]. “Additionally, in clinical, several species are considered to be opportunistic pathogens in immune-compromised humans” [14].

Plant disease management is a significant cost component in crop production. Traditionally, the approaches to dealing with disease in agricultural ecosystems include breeding resistant varieties of the crop species, hygiene to prevent the spread of contaminated soil or seed, and fungicides to kill potentially infecting fungi.

Table 1. Isolation and identification of *Trichoderma* isolates based on ITS data

Isolate code	GenBank Acc. No. (ITS sequence)	BLAST results identity (%)	Molecular identification
TBT1	U.PMW776752	99	*T. asperellum*
TBT2	U.PMW776753	100	*T. asperellum*
TBT3	U.P MW776754	99	*T. asperellum*
TBT4	U.PMW776755	99	*T. asperellum*
TBT5	U.PMW776756	100	*T. longibrachiatum*
TBT6	U.PMW776756	99	*T. harzianum*
TBT7	U.PMW776758	99	*T. harzianum*
TBT8	U.PMW776759	99	*T. harzianum*
TBT9	U.PMW776760	99	*T. longibrachiatum*
TBT10	U.PMW776761	99	*T. longibrachiatum*
TBT11	U.P-	-	*Trichoderma spp.*
TBT12	U.P-	-	*Trichoderma spp.*

However, increasing concerns about the effects of fungicides in the environment and residues in food have resulted in the deregistration of a number of fungicides. Moreover, resistance of pathogens to fungicides has rendered certain fungicides ineffective [15-19]. There is a need to strengthen the practices and components of IPM in order to reduce the dependence on synthetic agrochemicals. Biological control is an essential part of these strategies as a substitute of agrochemicals. Biological control is the suppression of disease by the application of a Biocontrol Agent (BCA) usually a fungus, bacterium, or virus, or a mixture of these to the plant or the soil. The biological control agent acts to prevent infection of the pathogen by the plant. The main advantage of using a biological control agent they are highly specific for a pathogen and hence are considered harmless to non-target species [20-23].

“The Biocontrol agents exercise several antagonistic mechanisms such as nutrient competition, antibiotic production, mycoparasitism and induction of systemic resistance has been proposed as the major antagonistic mechanism by *Trichoderma* produces enzymes like chitinase and β-1, 3-glucanase which degrades the cell wall materials and also releases a number of toxic substances that can inhibit the growth of the pathogens funga”l [24]. **“**These chitinase genes have functions in the biocontrol mechanism such as cell wall degradation, hyphal growth, and parasitic activity” [25]. Chitinase helps in breakdown of the glycoside bonds. Glucose oxidase catalyses D- glucose to D-glucono-1, 5-lactone and hydrogen peroxide are known to have antifungal effect. Xylanase helps in breaking hemicelluloses a major component of plant cell walls. Chitinase gene (*ech42*) was produced by the biocontrol agent *Trichoderma harzianum* which was responsible for mycoparasitism [26-31]**.** These genes have their unique functions in the biocontrol mechanism such as cell wall degradation, hyphal growth, stress tolerance, and parasitic activity. The role of genes plays a major the biocontrol process by regulating some signals and leading to the secretion of some enzymes that help in the degradation of the pathogens and hence they are known as biocontrol genes. Increased expression of the genes helps in enhanced biocontrol activity which helps in promoting plant growth and prevents the plant from pathogen attack. The gene *ech42* from *T. harzianum*codes for endochitinase with significantly higher inhibitory activity against a broad range of phytopathogenic fungi than other chitinolytic enzymes [32-36]. The *ech42* gene involved in the biocontrol activity of *Fusarium* disease incidence has not been reported. Thus the genetic characterization of *ech42* is still remains an open field for the researchers.

“It is also known to have the ability to interact with plants, inducing resistance to biotic and abiotic stresses and promoting plant growth. These characteristics of fungi are useful in research of *Trichoderma* strains as biocontrol agent. *Trichoderma harzianum*being the most cited species as an active agent of commercial biopesticides and biofertilizers. Chitinases are found in a wide range of organisms including bacteria, fungi, higher plants, insects, and some vertebrates**.** Chitinases have been isolated from the *Trichoderma* species” [37]. So the *Trichoderma* chitinase encoding gene/s will help in crop improvement and disease management. They will also minimize the use of chemical pesticides.

It was reported that the identification of *Trichoderma* based on morphological characters can give misleading results (Fahmi *et al.,* 2016). Recently, molecular identification based on internal transcribed spacers (ITS) amplification and sequencing is common and highly trusted (Savitha and Sriam, 2015; Fahmi *et al*., 2016; Oskiera *et al*., 2015; Jiang *et al*., 2016). In this study, 10 isolates of *Trichoderma* spp. were isolated from soil rhizosphere of different locations in Egypt, characterized on molecular level and screened for their antagonistic *Fusarium oxysporum*.

2. MATERIALS AND METHODS

2.1 Isolation of *Trichoderma* Isolates

twelve isolated of *Trichoderma spp*. were recovered from the soil rhizosphere from different locations in Uttar Pradesh, India. A number of soil samples were taken at a depth of 15 cm, sealed in sterile bags, and brought into the lab for the isolation procedure. Fahmi *et al.* (2016) 1g air dried soil sample was added into 9 ml sterile water in a test tube to make 1:10 dilution (10^{-1}). The mixture was vigorously shaken on a vortex mixture for 5-10 minutes to obtain uniform suspension. One ml of soil suspension was transferred into a fresh sterile test tube containing 9 ml sterile water under aseptic conditions to make 10^{-2} dilution. Further, 10^{-3} dilution was made by pipetting 1 ml suspension into another fresh test tube containing 9 ml sterile water. One ml of soil suspension was taken and uniformly spread on PDA and TSM containing Petri plates and incubated at 25±2°C for 5-7 days in the dark. After incubation as the mycelial growth were appeared, the hyphal tips from the advancing mycelium were cut and transferred into the fresh PDA medium for further purification. The purified isolates were cultured on PDA slants and maintained at 4°C for further use.

2.2 Mycelial Growth Kinetics of Different *Trichoderma* Isolates

Growth kinetics of different *Trichoderma* isolates performed on PDA medium. Recorded data of growth kinetics observed by studying their regular time interwal for 12 h. By studying growth *Trichoderma* isolate viz., TBT1, TBT2, TBT3, TBT4,(*Trichoderma asperellum*), TBT5, TBT9, TBT10 (*T. longibrachiatum*), TBT6, TBT7, TBT8,(*T. harzianum*) TBT11 (T. *koningii*) and TBT12, (*T. koningiopsis*). Among different *Trichoderma* TBT6, TBT7 and TBT9 group fast growing were shown in (Table. 2).

2.3 Soil Borne Pathogens

The University of Svpuat Meerut's Faculty of Agri-Biotech kindly provided by isolated of Fusaiumspp.

2.4 DNA Extraction from *Trichoderma* Isolates

Trichoderma DNA was isolated according to Al-Samarrai and Schmid's [38] instructions. The sharp, separate bands served as a quality indicator for the DNA.

2.5 Isolation of Genomic DNA

The genomic DNA from twelve *Trichoderma* isolates of was extracted by CTAB methods with some modifications. The Genomic DNA of *Trichoderma* was isolated from cell which were in the vegetative phase. The cell suspension culture was obtained by incubation of Potato Dextrose Broth (PDB) *Trichoderma* mycelium and incubated for 36 hrs genomic DNA successfully isolated.

2.6 Purification of Crude Genomic DNA

Isolated DNA samples were treated with 1µl of RNAse (10mg/ml) mixed gently and incubated in boiling water bath at 37^0C for 1 h. This was done to single-stranded RNA. The purified DNA was precipitated and dissolved in 30µl TE buffer (pH 8.0). The quality of purified DNA of *Trichoderma* was checked on 0.8% agarose gel electrophoresis. The purified DNA did not show shearing or the presence of RNA contamination. This DNA was purified.

2.7 Molecular Identification of *Trichoderma* Isolates

Polymerase chain reaction (PCR) was utilized to amplify the internal transcribed spacer regions of *Trichoderma* using ITS1 (5' – TCC GTA GGT GAA CCT GCG G - 3') and ITS4 (5' - TCC TCC GCT TAT TGA TAT GC - 3') primers. PCR conditions were performed as described by Loc *et al*. (2011). PCR products were first purified using QIAquick PCR Purification Kit (QIAGEN Cat. No. 28104). Sequencing was performed using Big Dye Terminator v3.1 Cycle Sequencing Kit in a total volume of 20 µL using 3500 GeneticAnalyzer, Applied Biosystems (Daejeon, Korea).

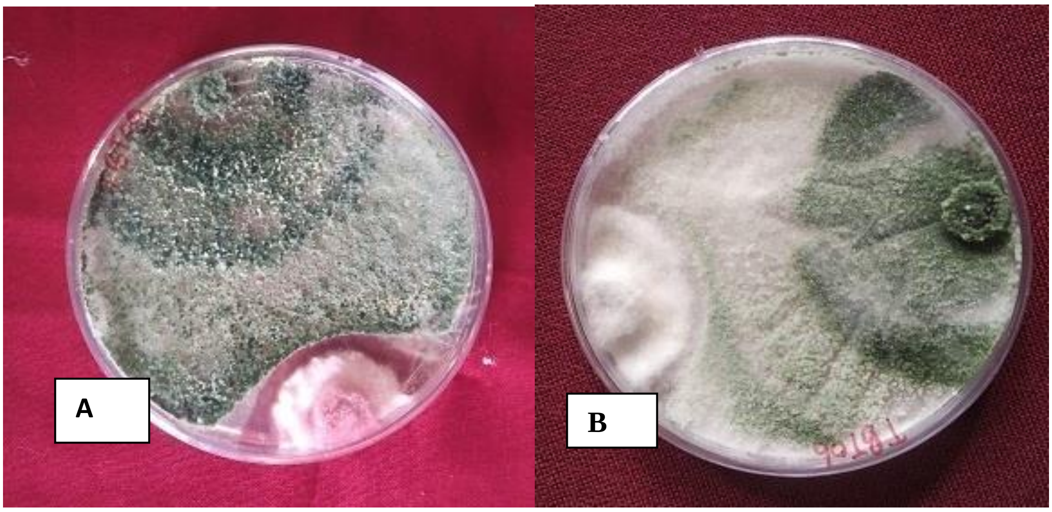

Fig. 1. Antagonism of *T.harzianum*against *Fusarium spp*

Table 2. Growth kinetics data mean value of different *Trichoderma* isolates (cm)

Isolates	12hr	24hr	36hr	48hr	60hr	72hr	84hr
TBT1	1.63	2.43	4.46	5.40	7.20	7.43	8.63
TBT2	1.26	2.30	4.30	5.20	7.15	7.27	7.83
TBT3	1.36	2.36	4.43	5.37	7.50	7.47	8.60
TBT4	1.46	2.40	4.40	5.27	7.30	7.50	8.50
TBT5	1.30	2.63	4.30	5.30	7.30	7.60	8.80
TBT6	1.90	2.80	4.80	5.90	7.80	8.20	9.00
TBT7	1.86	2.73	4.43	5.60	7.60	7.63	8.93
TBT8	1.36	2.60	4.80	5.20	7.50	7.50	8.87
TBT9	1.50	2.50	4.26	5.70	7.70	7.80	8.90
TBT10	1.10	2.10	4.20	5.10	7.10	7.30	8.30
TBT11	1.43	2.50	4.50	5.20	7.20	7.60	8.73
TBT12	1.33	2.40	4.30	5.23	7.13	7.50	8.87
CD@5%	0.138	0.150	0.238	0.324	0.210	0.205	0.242
C V	5.536	3.539	3.165	3.564	1.684	1.602	1.641

Values are the mean of three replications

2.8 PCR Amplification of Chitinase Gene

According to Loc *et al*. (2011), the chitinase gene was amplified using two particular primers: CHI-F (5-ATG TTG GGC TTC CTC GGA-3) and CHI-R (5-TTC GGG ATG GTT GTC ATA CTG-3).

2.9 Antagonism of *Trichoderma* against *Fusarium oxysporum*

The pathogen and antagonist were inoculated in the same Petri plate opposite with each other and observation was recorded at every 24 hours after inoculation of both organisms up to 96 hours. The interaction was observed between *Trichoderma* isolates and *Fusarium oxysporum*at 4th day. The growth of *Fusarium oxysporum*was increases at and 5th day thereafter this was checked by the antagonist at 6th and 7th day.

All *T. harzianum* isolates exhibited higher growth inhibition activity of *Fusariumoxysporum* compared to the other species isolates. Among the *T. harzianum* isolates highest growth inhibition percentage was recorded in TBT6 (87.1%) followed by TBT7 (82.2%), whereas the minimum growth inhibition TBT10 (59.7%) was recorded in *T. longbrachiatum* isolates TBT10 after 7 DAI (Table 3.). *T. harzianum* isolate TBT6 showed initial faster growth, overgrew on *Fusariumoxysporum* after 5 days of inoculation.

2.10 Data and Cluster Analysis

The NCBI website (http://www.ncbi.nlm.nih.gov/) was used to compare the findings of Trichoderma sequencing with known sequences using BLASTn. MEGA version 6 was used for the alignment and phylogenetic analysis [39]. The costat 6.3 version programme was used to analyse the antagonistic data that was obtained. Use of the Duncan's multiple range test [40] guided the analysis of variance and means comparison at the 5% level of significance.

2.11 Quantitative Estimation of Chitinase Activity of *Trichoderma* Isolates

All *Trichoderma* isolates were used for biochemical assay for production of cell wall degrading enzymes. Chitinase activity was assayed by measuring the release of reducing saccharides from colloidal chitin. Standard curve generated with N-acetyl-β-D-glucosamine (NAGA) was used to determine reducing saccharides concentration. The activity ranged from 5.77 unit/ml to 21.15 unit/ml commercial chitin derived colloidal chitins of the TBT5 and TBT6 isolates respectively; while TBT7 isolate presented 20.12 unit/ml chitinase enzyme activities.

3. RESULTS AND DISCUSSION

3.1 Isolation and Molecular Identification of *Trichoderma* Isolates

From rhizosphere soil samples taken from several locations in Uttar Pradesh, 12 strains of Trichoderma were identified (Table 1). The ITS region was amplified by PCR using ITS primers, yielding a single band of roughly 600 bp. After the PCR products were sequenced, the sequencing data was uploaded to the NCBI website for BLAST searches and comparison with previously released ITS data. Four isolates were found to belong to *Trichoderma asperellum* (TBT1, TBT2, TBT3, TBT4), three isolates were classified as *Trichoderma harzianum* (TBT6, TBT7, TBT8), and two isolate was identified as *Trichoderma longibrachiatum*. The amplified ITS regions of the Trichoderma isolates showed 99 to 99.99% identity with three species of Trichoderma (Table 1). The ITS identity was validated by the phylogenetic analysis.

Table 3. Growth inhibition of *Fusarium oxysporum*in dual culture by different *Trichoderma*isolates at 28 ±2^0 C

***Trichoderma* isolates**	**Growth inhibition efficiency of *Trichoderma* spp. in dual culture**					
	5th days after inoculation		**6th days after Inoculation**		**7th days after inoculation**	
	Radial Growth (cm)*	**Growth Inhibition (%)$^{\#}$**	**Radial Growth (cm)***	**Growth Inhibition (%)$^{\#}$**	**Radial Growth (cm)***	**Growth Inhibitio n (%)$^{\#}$**
TBT1	2.33	43.6	2.00	68.8	1.76	80.4
TBT2	3.66	11.4	3.40	48.2	2.83	68.6
TBT3	2.93	29.1	3.03	53.6	3.03	66.3
TBT4	3.03	26.6	2.86	56.1	2.63	70.8
TBT5	3.00	27.4	2.46	62.0	2.10	76.7
TBT6	2.63	36.3	2.33	64.0	1.16	87.1
TBT7	2.66	35.6	2.43	62.5	1.60	82.2
TBT8	2.63	36.3	2.53	61.0	2.16	76.0
TBT9	3.83	36.3	3.66	44.3	3.36	62.7
TBT10	4.03	2.4	3.86	41.4	3.63	59.7
TBT11	3.26	21.1	2.63	59.5	2.36	73.8
TBT12	3.66	11.4	3.5	46.7	3.13	65.2
Control	4.13	-	6.66	-	9.00	-
C.D.	0.20	-	0.19	-	0.176	-
C.V.	3.13	-	3.41	-	3.55	-

**Values are the mean of three replications, $^{\#}$Values are the mean percentage inhibition*

Table 4. Quantitative estimation of chitinase activity (U/ml) from different speciesof *Trichoderma*

S. N.	Isolates	Chitinase activity (Unit/ml)
TBT1	*Trichoderma asperellum*	12.30
TBT2	*Trichoderma asperellum*	18.23
TBT3	*Trichoderma asperellum*	14.26
TBT4	*Trichoderma asperellum*	9.17
TBT5	*Trichoderma longibrachiatum*	6.18
TBT6	*Trichoderma harzianum*	21.15
TBT7	*Trichoderma harzianum*	20.12
TBT8	*Trichoderma harzianum*	18.34
TBT9	*Trichoderma longibrachiatum*	18.63
TBT10	*Trichoderma longibrachiatum*	5.77
TBT11	*Trichoderma koningii*	17.28
TBT12	*Trichoderma koningiopsis*	7.29

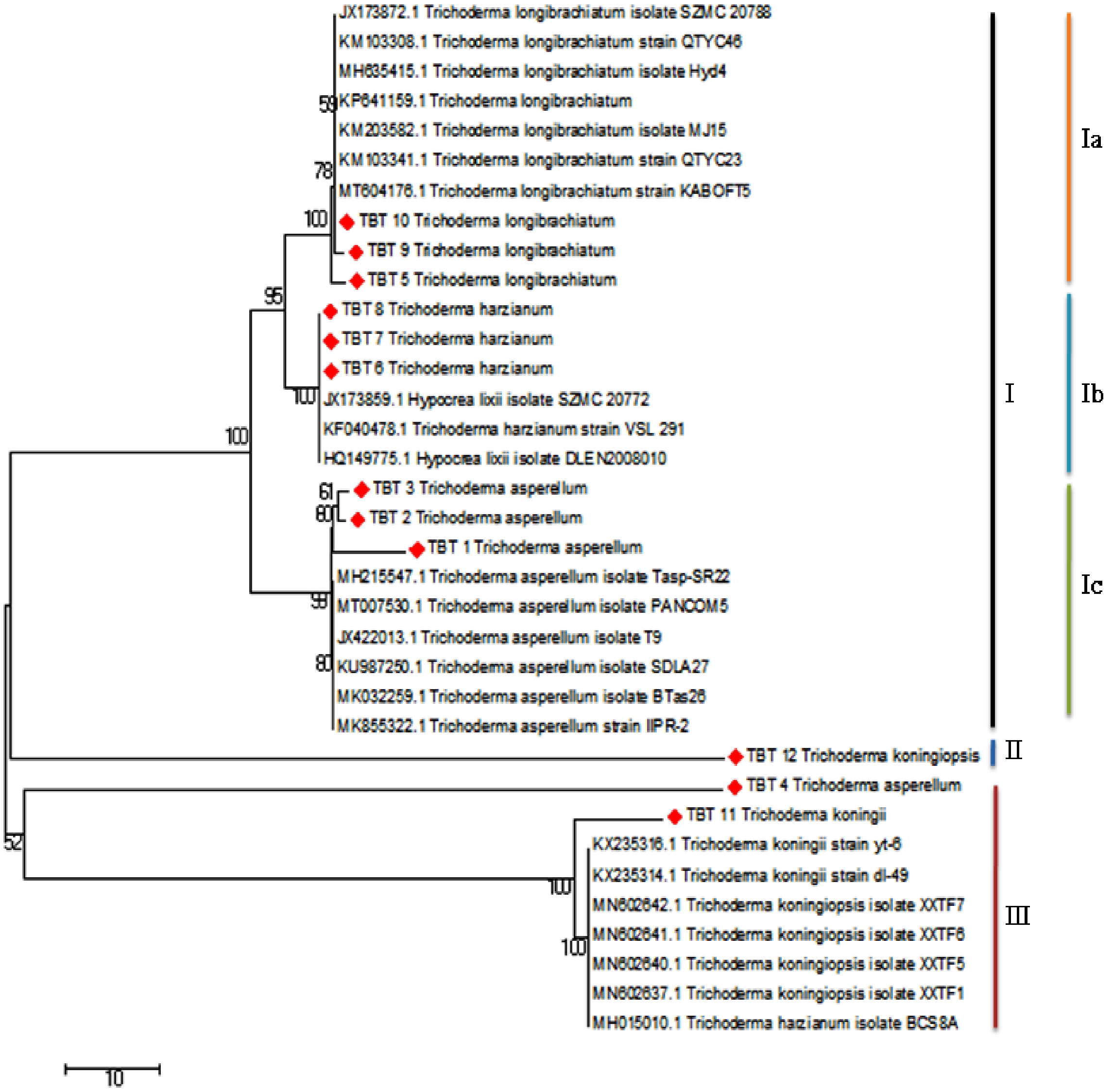

Fig. 2. Phylogeny analysis of *Trichoderma* isolates based on ITS data using MEGA

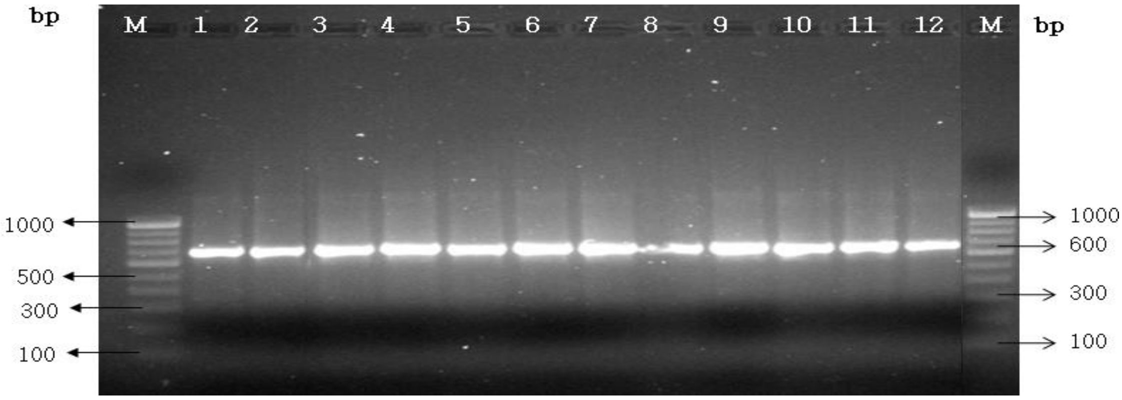

Fig. 3. Amplified PCR products of ITS region of 12 native *Trichoderma* spp. isolates from soil using ITS universal primers. Lane M: 100 bp ladder (Promega, USA), Lane 1-12: TBT1-TBT12

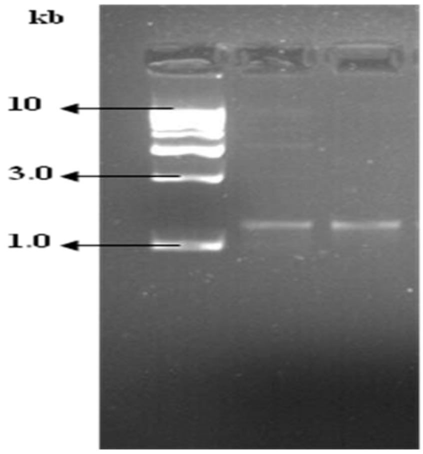

Fig. 4. Amplification of chitinase gene in *Trichoderma* isolates based CHI-F and CHI-R primers, M; 1 kb DNA ladder

3.2 Phylogenetic Analysis of *Trichoderma*

Phylogenetic relationship among the *Trichoderma* species was determined using the ITS sequence information of 12 *Trichoderma* spp. and 23 other *Trichoderma* sequences, including *T. asperellum, T. harzianum, T. longibrachiatum, T. koningii* and *T. koningiopsis* available in the NCBI database. A dendrogram was constructed by using the ClustalW and Mega7.0 software with the Neighbor-Joining (NJ) method.

3.3 Amplification of Chitinase Gene from *Trichodermaharzianum*

The genomic DNA isolated from *T. harzianum* was PCR amplified with specific primers for chitinase gene. The concentration (10 pM/µl) of respective primers was used in the PCR amplification. Through amplification, a good unique band was observed without any primer dimers. The large-scale amplification of putative full-length of chitinase gene approx (1.3 kb).

3.4 Gel Elution of the PCR Amplicon

The PCR products were separated in a low melting agarose gel of 1.2% along with 1kb DNA molecular marker. Very sharp bands of expected sizes were obtained. The 1.3 kb amplicon corresponding to chitinase gene were eluted out using QIAEX II Gel Extraction Kit.

3.5 Chitinase Gene of *Trichoderma*

"Chitin is an essential part of these pathogen cell walls, Trichoderma secretes extremely potent chitinases that may degrade and feed on them" [41]. "Chitinase 42 is one Trichoderma endochitinase that can hydrolyze the β-1, 4-glycosidic bonds that hold the N-acetyl glucosamine residues in chitin together". (Hassan *et al.,* 2015). Using PCR-based specialist primers (CHI-F and CHI-R), the chitinase 42 gene in Trichoderma isolates was amplified and discovered in this experiment, as shown in Fig. 4. The presence of this gene was confirmed in all investigated isolates by the PCR, which a single band around 1300 bp similar by Loc *et al*. (2011). Since the primers are unique to Trichoderma chitinases, the amplification of the chitinase gene verifies that these isolates are Trichoderma.

4. CONCLUSION

Identification of *Trichoderma* based on ITS markers exhibited high efficiency in discrimination among different *Trichoderma* spp. isolates. *Trichoderma* isolates showed variability in their aggressiveness against Fusarium oxysporum. In general, the isolate TBT6 of *T. harzianum*showed the best antagonism against Fusarium oxysporum and while the isolate. *Trichoderma* isolates improved. These results confirm the efficacy of *Trichoderma* as excellent biocontrol agent and also as plant growth promoting. *Trichoderma* under *in-vitro* conditions, against *Fusarium oxysporum*in dual culture bioassay.Among the *T. harzianum* isolates highest growth inhibition percentage was recorded in TBT6 (87.1%) followed by TBT7 (82.2%), whereas the minimum growth inhibition (59.7%) was recorded in *T. longibrachiatum* isolates TBT10 after 7 Days. Chitinase activity was assayed by measuring the release of reducing saccharides from colloidal chitin. Standard curve generated with N-acetyl-β-D-glucosamine (NAGA) was used to determine reducing saccharides concentration. The activity ranged from 5.17 unit/ml to 21.15 unit/ml commercial chitin derived colloidal chitins of the TBT10 and TBT6 isolates respectively; while TBT7 isolate presented 20.12 unit/ml chitinase enzyme activities.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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