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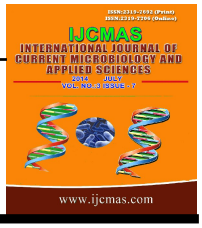

Original Research Article

Molecular characterization of *Trichoderma harzianum* strains from Manipur and their biocontrol potential against *Pythium ultimum*

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A B S T R A C T

Pythium ultimum is one among the most aggressive soilborne pathogens causing seed rot and seedling damping-off of many crops, while some members of the genus *Trichoderma* are good biological control agents against numerous plant fungal diseases. We have characterized 18 *Trichoderma harzianum* strains from Indian region of Indo-Burma Biodiversity hot spot with special reference to Manipur by morphological characteristics and partial ITS-1&4 sequencing data. Cluster analysis among the srtains showed a distinct separation of the isolates and formed two main clades, A and B. All the strains inhibited the growth of *Pythium ultimum* with percentage inhibition of radial growth (PIRG) values ranging from 46% to 81%. Screening of enzymatic activities showed that all the isolates produced Cellulase activity with the zone diameter ranging from 10mm to 48mm and 50mm to 82mm for β-1,3-glucanase activity. Production of volatile and non-volatile antifungal compounds against *P. ultimum* gave PIRG values between 11% to 44% for volatile compounds and 5% to 77% for non-volatile compounds over 6 days. Among all the *T. harzianum* strains, IBSDT86 (Accession no. JX518919) exhibited the strong candidate to fight against *P. ultimum* with the highest production of β-1,3 glucanase and secondary metabolites suitable to develops a potential BCA to be used in the acidic soil of North east India.

Keywords

Biocontrol / Cellulase / β-1,3-glucanases/ non-volatile / volatile

Introduction

Trichoderma spp. have been extensively studied as potential biocontrol agents (Papavizas, 1992). Some of them are economically important as a result of their production of industrial enzymes (cellulases, hemicellulases) and antibiotics and their action as biocontrol agents against plant pathogen fungi based on various mechanisms such as the production of antifungal metabolites, competition for space and nutrients, mycoparaitism etc. (Woo and Lorito, 2007). *T. harzianum* Rifai (Ascomycota, Hypocreales, Hypocreaceae) is an ubiquitous asexual soil fungus and has

been used in the biological control of several plant-pathogenic fungi, e.g. *Fusarium*, *Pythium*, *Rhizoctonia* and *Sclerotinia* species and also used in the different formulations of bio-fertilizers on various agricultural crops (Harman and Kubicek, 1998). The reason is that *T. harzianum* propagules are capable to produce cheap formulations in large quantities and high concentration, whether in liquid (water or oil) or dry (granules, pellets and wettable power), and it can also be strored long period without losing any beneficial effects (Siddiquee et al., 2009). Among the different strains of *Trichoderma*, *T. harzianum* was found to be the most effective antagonists against phytopathogenic fungi (Siddiquee et al., 2009).

Pythium species are among the most aggressive soilborne pathogens, causing seed rot and seedling damping-off in many crops (Whipps and Lumsden, 1991). Despite substantial economic losses associated with the conspicuous and destructive nature of *Pythium ultimum*, management of the diseases caused by these soilborne plant pathogens through fungicidal seed treatments has proved to be impractical, mainly because of increased concern about the fate of synthetic chemicals in the environment and the development of fungicide-resistant *Pythium* strains (Whipps and Lumsden, 1991). In this context, the major task now facing scientists is to develop alternatives to chemicals using a combination of approaches for effective management of crop diseases caused by *Pythium* spp. *Pythium* spp of the class oomycetes are exceptional in that their cell walls contain β-(1,3)-(1,6)-D-glucans and cellulose instead of chitin as major structural components (Blaschek et al., 1992) and it can be speculated that the role played by glucanases and cellulases in the antagonistic processs is crucial. The aim of this study was to characterize from climatic condition of NE India with reference to Manipur for the potential antagonist against *P. ultimum*. *Trichoderma harzianum* is so far unique in *Trichoderma*, as it exhibit a considerably higher intraspecific gene sequence diversity than other species of *Trichoderma* (Gams and Meyer, 1998). So, in a practical biological control situation, differentiation is required to define populations within a single species. A particular strain of *T. harzianum* may be a good or bad biological control agent depending upon the intended target and the functions required. It will therefore be necessary to select the most efficient strain for each individual pathosystem (Sharma et al., 2009).

Materials and Methods

Isolation, Purification and Identification of the test organism

Trichoderma spp were isolated from the soil samples collected from different agricultural fields of Manipur by following the method of Tate, R.L. (Tate, 1995) using *Trichoderma* selective medium (TSM). *P. ultimum* was obtained from the infected ginger rhizome rot sample collected from agricultural fields of Manipur. The plates were incubated at 25±2ºC for 3-7 days. *Trichoderma* spp. isolates were identified to species levels morphologically (Rifai, 1969) as well as by molecular technique following ITS sequence analysis. The cultures were stored in mineral oil and lyophilized form for further used.

Preparation of fungal mycelium and genomic DNA extraction

Trichoderma harzianum and *P. ultimum* isolates were grown in 100 ml of potato dextrose broth for 3 days at room temperature with rotary shaking at 120 rpm.

Mycelia were harvested by filteration through filter paper and washed with distilled water. Genomic DNA were extracted using the method of Raeder and Broda (1985) with minor modifications as described by Hermosa *et al*. 2000. A total of 200 mg of fresh mycelia was ground and extracted with 1.0 ml of lysis buffer. Samples were mixed by vortexing and incubated at 37ºC for 1hr. Then it was centrifuged at 12000 rpm for 15 min by adding 25:24:1 (by volume) phenol-chloroform-isoamyl alcohol at 4ºC. Nucleic acid in aqueous phase was precipitated with 0.25 volume of 7.5 M ammonium acetate and with 2.5 volumes of ice-cold ethanol. The samples were centrifuged at 12000 rpm for 15 min and pellets were washed with 70% ethanol, air dried and suspended in 50µl of TE buffer. The nucleic acid dissolved in TE buffer were stored at -20ºC until use. DNA was quantified by using Nano-drop at 260 nm. DNA was also quantified by means of 1% agarose gel electrophoresis using a 1 kbp DNA ladder as DNA size marker.

DNA amplification

DNAs were amplified using primers ITS1 (5'-TCGGTAGGTGAACCTGCGG-3') and ITS4 (5'-CCTCCGCTTATTGATATGC-3') (White et al., 1990). PCR was performed in a total reaction volume of 50 µl containing 5 µl of 5X buffer with $MgCl_2$ (Promega), 0.7µl of dNTP, 0.8µl Taq DNA polymerase, 0.5µl each of primers ITS1 and ITS4, 39.5 µl Miliq. H_20 and 3µl of genomic DNA (50 ng/ µl). The amplification program included an initial denaturation at 94ºC for 1 min, followed by 30 cycles of denaturation at 94ºC for 1 min, followed by 30 cycles of denaturation at 52ºC for 1min, annealing at 72ºC (90sec), extension at 72ºC in a thermocycler. Following amplification, the PCR products were electrophoresed on 1% agarose gel buffered with 0.5X TBE and photographed.

DNA sequencing and Phylogenetic Analysis

Template DNA (50 µl) was directly prepared from PCR-ITS region and got sequence from Bangalore Genei, India and submitted to NCBI Gene Bank. The patterns were normalized and then processed with pattern analysis software. Isolates were grouped using the cluster analysis UPGMA (Unweighted Pair Group Method with Arithmatic Average) (Nei and Li, 1979). Phylogenetic analysis were completed using the MEGA package. Bootstrap tests with 1,000 replications (Felsenstein, 1985) were conducted to examine the reliability of the interior branches and the validity of the trees obtained.

Dual culture technique for antagonistic activity

All of the 18 *T. harzianum* isolates identified by morphological as well as sequence analysis data were screened for their mycoparasitic ability against the *P. ultimum* using the modified method given by Evans et al (2003) with some modifications. Sterilized PDA media of 20 ml was poured into the sterile petri dishes and each plate was then inoculated with 5mm diameter agar disc cut from the edge side from the actively growing pure culture of *P. ultimum* and placed at one side of the PDA culture plate. After that, a 5mm disc was taken from the edge of a 2 days old pure culture of each *T. harzianum* isolates and placed at the periphery on the opposite side of the same Petri dish. For the control plate, only *P. ultimum* was placed in a similar manner without *T. harzianum*. Results revealed as mean colony growth of the causal pathogen in the presence and absence of the

antagonist. The percentage inhibition of radial growth (PIRG) was calculated using the formula % PIRG = R1-R2/R1 X100 (Skidmore and Dickinson, 1976).

Where, PIRG = percentage inhibition of radial growth; R1= radial growth of *P. ultimum* in the absence of the antagonist (control); R2 = radial growth of *P. ultimum* in the presence of the antagonist.

Production of Enzymatic activities

Production of Cellulase activity

The production of Cellulase activity from the *T. harzianum* isolates were determined via the Gram's iodine method (Kasana et al., 2008). In this method, a 5 mm disc of *Trichoderma* culture was inoculated onto a petridish containing carboxymethyl cellulose (CMC agar and then incubated for 3 days. A control plate without the culture disc was also maintained. After 3–4 days, the CMC agar plates with the isolates and the controls were flooded with Gram's iodine solution (2.0g Kl and 1.0g I, per 300 ml dH_20) and the plates were allowed to sit for 5 minutes until the dye settled into the media to visualize the cellulase activity. The appearance of clear halos around the culture growth confirms cellulase activity. The halo diameters were measured using a scale for a semi-qualitative comparison of cellulase activity among the isolates (Miranda et al., 2011).

Production of β-1,3-glucanases

For screening of β-1,3-glucanases activity, carboxymethyl cellulose agar (CMC agar) medium amended with laminarin was used according to the modified method given by El-Katatny et al 2001. A 6 mm culture disc of *T. harzianum* was placed at the centre of the CMC agar plate. Plates were incubated at 25°C for 3 days. ß-1,3-glucanases activity on the plates was observed by dipping in 0.1% congo red dye for 15–20 min followed by distaining with 1 N NaCl and 1 N NaOH for 15 min. The distaining was repeated twice and activity was recorded with the clearance zone formation

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Production of volatile and non-volatile metabolites

T. harzianum isolates were evaluated for the production of volatile inhibitory substances by following the modified methods of Dennis and Webster (Dennis and Webster, 1971A). The 5-mm disc of *T. harzianum* colony was inoculated centrally on PDA plates in triplicates sealed with parafilm. After 5 days of incubation, the test pathogens (*P. ultimum*) were inoculated on fresh PDA and covered with the lids of the petriplates inoculated with antagonist. The plates were fixed with cellophane-tape and incubated for another 7 days; whereas, control plates were inoculated with pathogen alone. Growth of *P. ultimum* was measured after 5–6 days of incubation and the inhibition zones were recorded. The production of non-volatile substances by the *T. harzianum* isolates against the *P. ultimum* was studied using the modified method described by (Dennis and Webster, 1971B). *Trichoderma* isolates were inoculated in 100 ml sterilized potato dextrose broth (PDB) in 250 ml conical flasks and incubated at 25 ± 1ºC on a rotatory shaker set at 100 rpm for 4-5 days. The mycelium was removed and the broth was centrifuged at 3000rpm for 15 min and the pallet was discarded. The supernatant was concentrated to $1/10^{th}$ times by lyophilizing. Three holes were made by using cork borer in the PDA plate and 0.45 µl of the concentrated broth was added into each holes and a small disc of pathogen was inoculated into each holes and a small disc of pathogen was inoculated at the centre of the plate. The colony diameter of the

pathogen and the inhibition percent of the pathogen were calculated. There were three replicates for each treatment. The inhibition zone of the mycelial growth in relation to growth of the control was recorded.

Statistical analysis

Statistical calculations were performed by using 1-way Anova factor. Comparison among the treatments were means of three replicates. Differences between treatment means were determined using LSD at the 0.05 probability level.

Results and Discussion

Isolation of *Trichoderma* on TSM

Trichoderma spp. were isolated from the soil collected from different agricultural fields of Manipur using TSM. After four days of incubation, *Trichoderma* colonies were seen and these colonies were further transferred into Potato dextrose agar (PDA) slant and kept inside the freeze for further studies. A total of 40 *Trichoderma* colonies were obtained from the soil samples throughout the different sampling sites.

Morphological characterization of *T. harzianum* isolates

Trichoderma colonies were initially observed as white specks on the TSM which then enlarged of 5 to 6 mm within 5-6 days. By this time, the white colony turned into green/yellow/off-white on the medium (Fig.1). The colonies became rapidly and readily developed with typical yellow-green colour, which make their identification easy from other soil-borne fungi. After 7 to 8 days, 50 to 60% of the colonies appeared as dark green with compact conidiophores throughout the 90mm plate (Fig.2). The reverse side of *T. harzianum* colonies on PDA were also appears green or yellowish in colour. Microscopic examinations showed that the conidiophores typically formed branches at 90°C along the length of the main axis from which they arise. Phialides were enlarged in the middle and cylindrical in structure. It forms branches towards the tip and secondary branches tended to be held in the whorls which were typically flask-shaped, densely clustered on the main axis (Fig.3). Among the total 40 *Trichoderma* isolates, 18 isolates were identified as *T. harzianum* morphologically.

Sequence Alignment

The PCR product of the ITS region of all the expected eighteen *T. harzianum* strains amplified with band sizes ranges from 500 to 600bp. These PCR products were directly sequenced and aligned. The priming site of the ITS1 and ITS4 primers were determined in order to confirm that the sequences obtained corresponding to the actual ITS1 region (Table 1) (Kamala et al., 2013). All the eighteen *Trichoderma* isolates were found to be closely related with those *T. harzianum* found in NCBI. A multiple sequence alignment was carried out that includes the ITS1 region, including gaps and the complete sequences align. There were quite a number of gaps that were introduced in the multiple sequence alignment within the ITS1 region which indicates that the sequences are similar and closely related. From the sequence alignment of the eighteen *T. harzianum* isolates, variations were observed between *T. harzianum* isolates.

Phylogenetic Analysis

For phylogenetic analysis of the eighteen isolates of *T. harzianum*, a dendrogram was constructed through the method of UPGMA using Mega 5 software (Fig.4). Considerable variation was observed among some of the

isolates of *T. harzianum*. The combined tree of the *T. harzianum* strains inferred from the sequence analysis split into two strongly supported Clades, A and B. Clade A contained twelve isolates of *T. harzianum* (IBSDT54, IBSDT75, IBSDT40, IBSDT11, IBSDT142, IBSDT100, IBSDT1, IBSDT47, IBSDT74, IBSDT77, IBSDT17 & IBSDT86). Whereas Clade B contained only six strain of *T. harzianum* (IBSDT101, IBSDT70, IBSDT137, IBSDT112, IBSDT12 & IBSDT89). The robustness of the internal branches was assesses with 1000 bootstrap replications.

Antagonistic activities of *T. harzianum* against *P. ultimum*

All of the 18 *T. harzianum* isolates produced different rates of growth inhibitions against *P. ultimum* demonstrating variations in antagonistic action ranging from 46% to 81% PIRG value (Table 2). The data obtained were subjected to ANOVA analysis. Strain IBSD T86 showed the highest PIRG value of 81% against *P. ultimum*. Based on the PIRG values, the 5 best antagonist isolates in decreasing order were rated as T86> T11 > T142 >T89 > T112. Whereas strains IBSD T54 showed the smallest amount of inhibitory effect on the *P. ultimum* mycelial growth with (46.5%) inhibition. The degree of colonizing and penetrating the *P. ultimum* hyphae by *Trichoderma harzianum* thereby killing the pathogen was examined under trinocular microscope and photograph has been taken (Fig. 5).

Screening of *T. harzianum* isolates for enzymatic production

The activity of lytic enzymes production of Cellulases and β-1,3-glucanases activities of *T. harzianum* strains are summarized in Fig.6. All the sixteen isolates of *T. harzianum* exhibited Cellulases activity with zone diameter ranging from 10mm to 48.5mm. IBSD T89 isolate produced the highest Cellulase activity with a zone diameter of 48.5mm, while IBSD T11 isolate produced minimum Cellulase activity with a zone diameter of 10.5mm. All the isolates of *T. harzianum* exhibited the β-1,3-glucanases activities with a zone diameter ranging from 50mm to 82mm. The isolate IBSD T86 produced the maximum β-1,3-glucanases activity (82.5mm) while IBSD T1 produced the least activity (50.5mm).

Inhibition effects of volatile compounds

After screening the relative potency of the eighteen *T. harzianum* isolates for their antagonistic activities as well as the enzymatic activities such as Cellulases and the β-1,3-glucanases activities, they were further screened for volatile and non-volatile metabolite production. Percentage inhibition radial growth (PIRG) of *P. ultimum* in the presence of volatile compounds ranges from 11% to 44% at 6 days of the experimental period (Table 2). The data were subjected to ANOVA analysis. Strain IBSD T89 showed highest PIRG value of 44% (Table 2). On the other hand, isolates IBSD T142 showed the least inhibitory effect on the *P. ultimum* mycelia growth at 11%.

Inhibition effects of non-volatile compounds

All the *T. harzianum* isolates released non-volatile compounds that diffused through the resulting inhibition growth of *P. ultimum*, giving PIRG values that ranges from 5 to 77 % (Table 2). The data obtained were transformed and subjected to ANOVA analysis. The highest PIRG was shown by isolate IBSD T86 (77.5%), whereas the lowest PIRG was shown by isolate IBSD T1 at 5.5%. The *P. ultimum* mycelia smoothly

and freely grew on the control plate, but in the presence of *T. harzianum*, it released some toxic substances to inhibit the *P. ultimum*.

Trichoderma species can act as biocontrol agents through different synergistic mechanisms. However, it is difficult to predict the degree of synergism and the behavior of a BCA in a natural pathosystem. The right selection of BCAs, which begins with a safe characterization of biocontrol strains in the taxonomic schemes of *Trichoderma* is important since, the exact identification of strains to the species level is the first step in utilizing the full potential of fungi in specific applications (Lieckfeldt et al., 1999). A total of 18 strains of *Trichoderma harzianum* were identified at the species level by morphological character using the existing taxonomic criteria and by the analysis of their ITS region gene sequences. Aggregate species of *T. harzianum* can be differentiated on the basis of macroscopic (colour of conidia, sporulation patterns and density and microscopic (structure and arrangement of phialides, conidial size and shape) features. In a similar study, Sariah et al (2005) morphologically identified *T. harzianum*, *T. virens*, *T. koningii* and *T. longibrachiatum* made up 72, 14, 10 and 4%, respectively. But, there is evidence that morphologically defined taxa are polyphyletic e.g., *T. harzianum*; Muthumeenakshi et al (1994) : Fujimori and Okuda (1994). This result suggests that there are not enough morphological and cultural characters to reliably define species. In the recent years, molecular biological techniques have proven to be valuable tools in fungal taxonomy, and their application has led to the reconsideration of several genera. The utility of molecular techniques in indicating interrelations among species and, when combined with phenotypic characters, can lead to a sound taxonomy that is reflective of phylogenetic relationships (Kindermann et al., 1998). ITS region of rDNA of *T. harzianum* were amplified using genus specific ITS-1 and ITS-4 primers. Amplified products of size in the range of 600 bp were produced. These results are in accordance with Mukherjee et al (Mukherjee et al., 2002) who studied the identification and genetic variability of the *Trichoderma* isolates. On the basis of a UPGMA bootstrap tree analysis with 1,000 bootstrap replications demonstrated two main clusters as Clade A and Clade B (Fig. 4).

Antagonistic effect based on the dual culture experiments showed that *T. harzianum* isolates significantly inhibited the mycelia growth of *P. ultimum* ranging from 46 to 81 %. Inhibition zones were visibly observed. Isolate IBSDT86 gave the highest PIRG value of 81 % with an incubation period of 6 days. Here, all isolates gave different degree of PIRG values, even though they belongs to the same species. Naseby et al. (Naseby et al., 2000) reported that *Trichoderma* strains have antagonistic activity towards the pathogen *P. ultimum*, which is related to an improvement in plant production.

The production of Cellulase and the β-1,3-glucanase are a characteristics attributed to wide variety of fungi and majority of fungus secret these two enzymes into the medium. These enzymes play important role in the enzymatic degradation of cell walls of phytopathogenic fungi like *P.ultimum* during mycoparasitic interaction. In our experiment, all the 18 isolates of *T. harzianum* produces Cellulase enzyme with a wide range of diameter ranging from 10mm to 48.5mm with the reduction in the growth of *P. ultimum*. Benhamou and Chet (1997) reported that loosening and even dissolution of the deposits initially formed

between the invaginated plasma membrane and the host cell walls were often seen in areas of *Trichoderma* penetration, as exemplified by the deposits. This obviously indicates that large amounts of cellulytic enzymes are produced. It is clear that such enzymes play a key role in breaching the host cell walls at sites of attempted penetration. They reported that by 7 days after inoculation, *Pythium* cells were completely degraded, and in most cases, only some slightly labeled wall remnants were indicative of the formers presence of a fungal cell.

The production of β-1,3-glucanases enzymes showed the reduction in the growth of *P. ultimum*. Strain IBSDT86 produced the highest β-1,3-glucanases activity with a 82.5% zone diameter while strain T47 produces least amount of this enzyme (50.5%). Benhamou and Chet (1997) reported a significant decrease in the labeling of *Pythium* cell wall while incubated with *T. harzianum.* They also observed that at an advanced stage of the antagonism (6-7days after inoculation), labeling of *Pythium* cell walls with the β-1,3 disappeared and at that time the antagonist sporulated abundantly. Screening for the enzymatic activity among the eighteen isolates of *T. harzianum* showed that IBSDT89 produced the highest Cellulase activity while T86 was the highest producer of β-1,3-glucanase.

The growth of *P. ultimum* was inhibited when exposed to the trapped headspace of volatile compounds produced in the presence of *T. harzianum* isolates. The inhibition started after 24 hours and increased until the end of the experiment (i.e. 7 days). Inhibitory effect in the presence of headspace gases on agar Petri dishes indicated that the unidentified volatile substances suppressed the pathogenic fungus of *P. ultimum*. None of the isolates shown above 70% inhibitory effect on the growth of *P. ultimum* mycelia in the presence of *T. harzianum* isolates. These results are consistent with the study of Doi and Mori 1994. which observed that the volatile compounds from *Trichoderma* spp impeded the hyphal growth of different fungal pathogens on agar plates. The highest PIRG value of isolate IBSDT89 due to volatile compound was observed at 44%. The growth of the *P. ultimum* was also inhibited when exposed to the non-volatile compounds produced by the *T. harzianum* isolates. The highest PIRG value of isolate IBSDT86 with the production of non volatile compound was observed at 77.5%. Kexiang et al (2002) showed that the two strains of T88 (*T. harzianum*) and T95 (*T. atroviride*) produced non-volatile antibiotic substances, which suppressed ability of the growth of *Botryosphaeria berengeriana*.

From this result, it was found IBSDT86 (Acc. No. JX518919) to be the best *T. harzianum* isolate having highest antagonistic activity against *P. ultimum* and produced highest β-1,3-glucanase as well as non-volatile compound when screened in *in vitro* conditions. Thus it can be concluded that the isolate IBSDT86 is ideal for mass production for the control of plant diseases caused by *P. ultimum* as an alternative to the chemical pesticides. *T. harzianum* isolates are very important to explore the synergistic effects of their mechanisms expressing based on *in vitro* dual culture technique, production of enzymatic activities, production of growth suppressive volatile and non-volatile compounds in the presence of pathogenic fungus *P. ultimum*. In conclusion, the biocontrol properties of *T. harzianum* are isolates-specific, not species-specific.

Table.1 *Trichoderma harzianum* strains along with their Accession nos. and Geographical location

SL. No.	**Isolate Codes**	***Trchoderma* strain**	**GenBank Accession No.**	**Sources**	**Geographical location**		
					Longitude	**Latitude**	**Altitude (m)**
1.	IBSD T1	*T.harzianum*	JX518889	Soil	93º56’10.2’’E	24º47’52.1’’N	787
2.	IBSD T11	*T.harzianum*	JX518894	Soil	93º37’46.0’’E	24º49’58.6’’N	785
3.	IBSD T12	*T.harzianum*	JX518895	Soil	93º57’36.1’’E	24º47’09.3’’N	782
4.	IBSD T17	*T.harzianum*	JX518897	Soil	93º56’16.9’’E	24º32’8.7’’N	760
5.	IBSD T40	*T.harzianum*	JX518902	Soil	93º46’03.0’’E	24º38’57.3’’N	792
6.	IBSD T47	*T.harzianum*	JX518904	Soil	93º47’23.2’’E	24º40’36.5’’N	785
7.	IBSD T54	*T.harzianum*	JX465708	Soil	93º45’45.2’’E	24º37’42.1’’N	816
8.	IBSD T70	*T.harzianum*	JX518909	Soil	93º45’45.2’’E	24º37’42.1’’N	816
9.	IBSD T74	*T.harzianum*	JX518911	Soil	93º45’41.9’’E	24º36’54.3’’N	814
10.	IBSD T75	*T.harzianum*	JX518912	Soil	93º45’54.2’’E	24º35’03.01N	791
11.	IBSD T77	*T.harzianum*	JX518913	Soil	93º45’57.4’’E	24º29’49.1’’N	781
12.	IBSD T86	*T.harzianum*	JX518919	Soil	93º45’57.4’’E	24º29’49.1’’N	781
13.	IBSD T89	*T.harzianum*	JX518920	Soil	93º45’57.4’’E	24º29’49.1’’N	781
14.	IBSD T100	*T.harzianum*	JX518924	Soil	94º02’433’’E	25º04’387’’N	823
15.	IBSD T101	*T.harzianum*	JX518925	Soil	94º02’319’’E	25º04’319’’N	820
16.	IBSD T112	*T.harzianum*	JX518927	Soil	94º35’01’’E	24º29’0.1’’N	2150
17.	IBSD T137	*T.harzianum*	JX518930	Soil	93º59’57.7’’E	24º29’33.0’’N	769
18.	IBSD T142	*T.harzianum*	JX518931	Soil	94º01’12.4’’E	24º26’18.0’’N	801

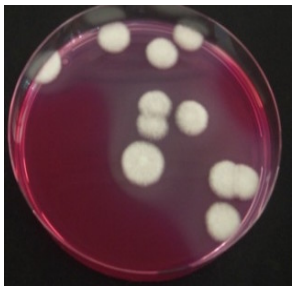

Fig. 1 *Trichoderma* CFU on TSM

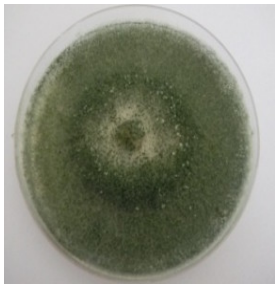

Fig. 2. Colony of *Trichoderma arzianum* on PDA medium

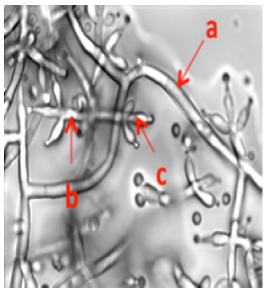

Fig. 3. Microscopic examination of *T. harzianum*. a. Hypha, b. Conidiophore , c. Phialide

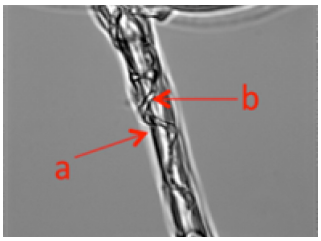

Fig. 5. Coiling and penetration of *P. ultimum* hyphae by *T. harzianum*. a. *P. ultimum*, b. *T. harzianum*.

Table.2 PIRG value of sixteen isolates of *T. harzianum* against *P. ultimum* by dual culture assay and by production of volatile and non-volatile secondary metabolites.

SL.No	*T.harzianum* strains	*T. harzianum* against *P.ultimum* (%)	Production of secondary metabolites	
			Volatile (%)	Nonvolatile (%)
1.	IBSD T1	$70.5^{cde} \pm 0.5$	$41^{b} \pm 1$	$5.5^{g} \pm 1.5$
2.	IBSD T11	$74.5^{bcd} \pm 0.5$	$26.5^{de} \pm 0.5$	$41.5^{i} \pm 1.5$
3.	IBSD T12	$70^{cde} \pm 0.5$	$34^{c} \pm 1$	$69^{de} \pm 1$
4.	IBSD T17	$70.5^{cde} \pm 0.5$	$41^{b} \pm 1$	$33.5^{j} \pm 1.5$
5.	IBSD T40	$64.5^{efgh} \pm 0.5$	$16.5^{hi} \pm 4.5$	$41^{i} \pm 1$
6.	IBSD T47	$67.5^{deg} \pm 2.5$	$15^{hij} \pm 1$	$65.5^{e} \pm 0.5$
7.	IBSD T54	$46.5^{i} \pm 3.5$	$21^{fg} \pm 1$	$26^{l} \pm 1$
8.	IBSD T70	$61^{gh} \pm 1$	$22.5^{ef} \pm 0.5$	$55^{g} \pm 1$
9.	IBSD T74	$57.5^{h} \pm 4.5$	$12.5^{i} \pm 2.5$	$61^{f} \pm 1$
10.	IBSD T75	$71.5^{cde} \pm 6.5$	$43.5^{b} \pm 1.5$	$70.5^{cd} \pm 2.5$
11.	IBSD T77	$71^{cde} \pm 1$	$30.5^{cd} \pm 1.5$	$71.5^{cd} \pm 1.5$
12.	IBSD T86	$81^{ab} \pm 1$	$33.5^{c} \pm 1.5$	$77.5^{b} \pm 2.5$
13.	IBSD T89	$73.5^{bcd} \pm 1.5$	$44^{b} \pm 1$	$70.5^{cd} \pm 2.5$
14.	IBSD T100	$61.5^{fgh} \pm 1.5$	$12^{gi} \pm 1$	$74.5^{bc} \pm 1.5$
15.	IBSD T101	$69^{cdfg} \pm 1$	$14^{hi} \pm 1$	$56^{g} \pm 1$
16.	IBSD T112	$73^{bc} \pm 2$	$19^{fgh} \pm 2$	$47^{h} \pm 1$
17.	IBSD T137	$47.5^{i} \pm 2.5$	$11.5^{ij} \pm 1.5$	$31^{jk} \pm 1$
18.	IBSD T142	$74^{j} \pm 2$	$11^{j} \pm 1$	$29^{kl} \pm 1$
19.	CONTROL	$85^{a} \pm 0$	$80^{a} \pm 0$	$80^{a} \pm 1$
LSD (P=0.05)		0.999	0.003	0.652

*****Values followed by the same letter in each column do not differ significantly.

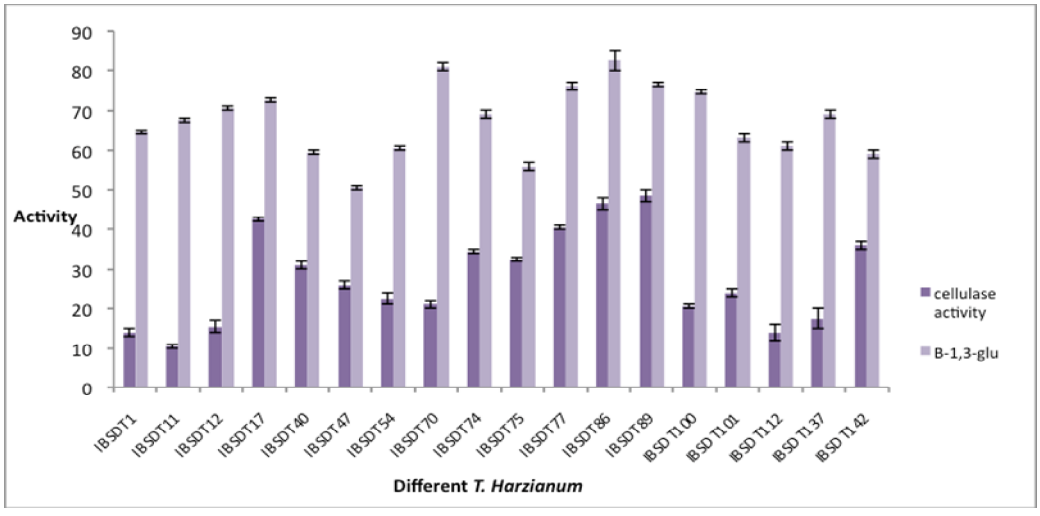

Fig.6 Graph showing the Cellulases and β-1,3 glucanases activities exhibited by the eighteen *T. harzianum strains*

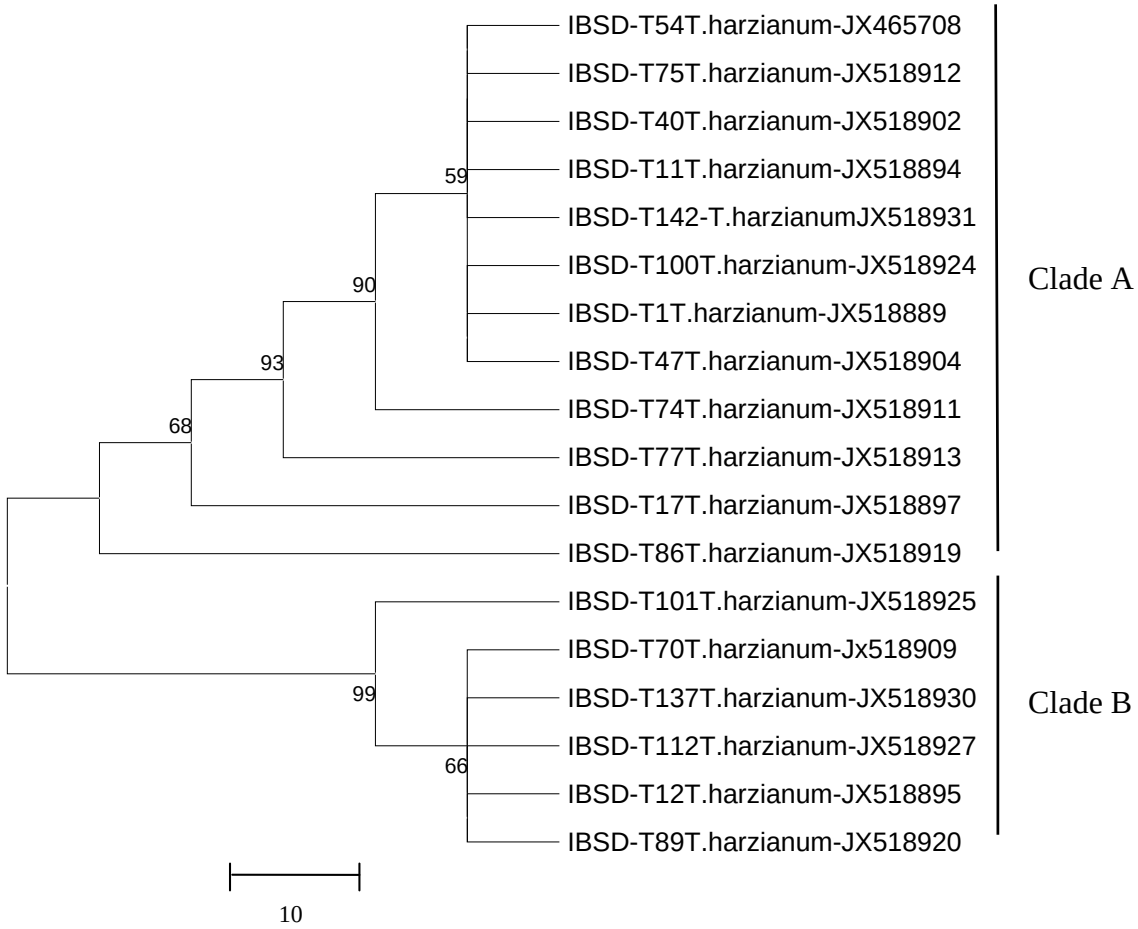

Fig.4 Phylogenetic tree of the 18 *T. harzianum* strains

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