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ISOLATION, MORPHOLOGICAL CHARACTERIZATION, MOLECULAR DETECTION OF NATIVE *TRICHODERMA* SPP. FROM SORGHUM RHIZOSPHERE AND THEIR EFFICACY AGAINST *MACROPHOMINA PHASEOLINA* (TASSI, GOID) CAUSAL AGENT OF CHARCOAL ROT DISEASE

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ABSTRACT

The aim of present investigation was to assess morphological characters, molecular detection and *in vitro* efficacy of *Trichoderma* spp. of sorghum rhizospheric soils from North Karnataka against charcoal rot disease. Among the native isolates tested, PT-3 (Chikkahesaruru) isolate significantly reduced the mycelial growth of *Macrophomina phaseolina* with 47.73% which was superior over PT-7 (Rupungudi-S_1) and PT-6 (Ballichakra) with 46.00 and 43.40 % respectively. Minimum inhibition was observed in PT-1 (Joladarasi) isolate with 34.10%. All these isolates exhibited variation in their morphological characters like mycelia form (floccose to arachnoid, compact and cottony growth) and width (4.8-3.14µm), conidia colour (Green to pale green) and shape (oval to globose), conidium length (6.87-3.55µm) and width (5.3-3.05µm), conidiophore branching (highly branched regular to irregular branches and repeatedly paired branches), phialide arrangement (pin shaped, broad base narrow to pointed at tip, broad base narrow bent at tip and narrow base pointed at tip), chlamydospore (intercalary and terminal) length (9.97-5.28µm) and width (9.64-5.12µm). Molecular detection of these isolates was carried out by using ITS-1 and ITS-4 universal primers for amplification of 28S rRNA fragment through PCR technique.

Keywords **:** *Macrophomina phaseolina, Trichoderma,* characterization, 28S r RNA

Introduction

Sorghum (*Sorghum bicolor* (L.) Moench), is grown both during south-west monsoon (*Kharif*) and post monsoon (*Rabi*) seasons which belongs to Poaceae family (Nagara (2017). It is nutritionally superior to rice which contains starch (60-75%), proteins (7-15%), non-starch polysaccharides (2-7%) and fat (1.5-6%). The average energetic value of whole sorghum grain flour is 356 k cal/100gm (Dicko *et al*., 20066). *Rabi* crop provides high quality grains as they mature during winter season under clean dry and rain free climate. However, decline in productivity of *Rabi* sorghum is due to drought and aggravated by charcoal rot disease which usually appears at grain maturity stage and causes severe lodging. It has been estimated up to 48.6 per cent loss in seed weight at Dharwad (Anahosur, *et al.*, 1977). Depending upon the cultivars, weather conditions and disease severity, yield losses ranged from 15.18 to 54.59% (Anahosur and Patil, 1982).

The pathogen *M. phaseolina* has a wide host range and associated with high soil temperature from 30 to 40 ºC and low soil moisture content (Arora and Pareek (2013). Microsclerotia are formed from the

aggregation of hyphae with 50 to 200 individual cells coupled by a melanin pigment (Kaur *et al*., 2012). Hence, it is difficult to be managed by virtue of its long survival mechanism, vast distribution in soil and wider host range. Keeping this point in view, present investigations are aim to exploit the *Trichoderma* spp. associated with sorghum rhizosphere across major sorghum growing areas and their efficacy on charcoal rot disease would help in identification of able bio-candidate for inhibition of infective propagule.

Trichoderma species are used as biocontrol agents, which is asexually reproducing saprophytic fungi, frequently present in all temperate and tropical soils, decaying plant tissues and root ecosystems. Their strains are strong opportunistic invaders, fast growing, prolific producers of spores and powerful antibiotic producers. Since, microorganisms which are isolated from the rhizosphere of a specific crop, provides an effective control of diseases compared to the organisms isolated from other crops. Such microbes serve as better biocontrol agents as they are well adopted to plant and environmental conditions where they supposed to function (Cook and Baker, 1983).

Materials and Methods

Isolation of the pathogen

Macrophomina phaseolina was isolated from infected sorghum stalks by using tissue isolation technique, which were collected from severely infected field (Hagari). The 7 days old culture of the pathogen was maintained on PDA medium in slants at 4 ºC.

Isolation of *Trichoderma* spp.

Twenty-four sorghum rhizospheric soil samples were collected from Ballari, Vijayanagara, Vijayapura, Yadgiri, Kalaburgi, Koppal and Raichur districts of Karnataka. Ten grams of soil was taken separately and suspended in 90 ml of sterile water and stirred well to get 1:10 (10^{-1}). One ml from this was transferred to test tubes containing 9 ml of distilled water to get 1:100 (10^{-2}) dilution. Likewise, the dilution was made up to 1:10000 (10^{-4}). One ml of the final dilution of each sample was aseptically transferred into Petri plates containing potato dextrose agar medium, the plates were incubated at 25± ºC for 5 to 10 days. Fungus was sub cultured on PDA slants and allowed to grow at 25 ± 2 ºC for 15 days and preserved in a refrigerator at 4 ºC.

Efficacy of native *Trichoderma* spp. against *M. phaseolina*

Dual culture method was used to assess the inhibition of radial growth of the pathogen by the antagonists. Approximately 20 ml molten PDA was poured into each of 90 mm diameter sterilized Petri plates. Following solidification, 5 mm bit of the pathogen and antagonist *Trichoderma* isolates were placed on PDA surface at equidistant from each other (≥ 2 cm apart). The control plates were inoculated by placing one bit of the pathogen in center. The plates were incubated at 28 ± 2 °C. The radial growth of the pathogen was recorded after 7 days of inoculation. Per cent inhibition over control (Vincent, 1947) was calculated by using the formula;

$$I - \frac{C\text{-}T}{C} \times 100$$

Where,

I = Per cent inhibition of mycelium

C = Growth of mycelium in control

T = Growth of mycelium in treatment

Morphological characterization

A week-old culture of these isolates grown on PDA at 25 ºC were used for the study. The morphological characteristics of each isolate such as colony color, mycelium form and septation, shape of the conidia, conidiophores, formation of phialides, arrangement of chlamydospores and measurements of mycelia width, conidia length and width, chlamydospore length and width were recorded by using Olympus binocular microscope and were photographed digitally.

Molecular characterization

The genomic DNA of each isolate was isolated using cetyl trimethyl ammonium bromide (CTAB) method. Amplification of ITS-1 and ITS-4 regions from the isolates of genomic DNA was done by using universal primer for the amplification of 28S rRNA conserved gene through polymerase chain reaction (PCR) technique at division of Plant Pathology IIHR, Hassaraghatta Bengaluru.

Results and Discussion

Isolation of *Trichoderma* spp.

Twenty-four isolates were isolated on PDA medium by using dilution plate technique. Among them only 10 rhizospheric soil samples *i.e*., Joladarasi, Gajapura, Chikkahesaruru, Hiresindogi (S_2), Jola, Ballichakra, Rupungudi (S_1), Hullihala, Raichur (MARS) and Hagari (S_1) had the population of *Trichoderma* spp (Plate 1). Pure cultures of all the isolates were preserved at 4 ºC.

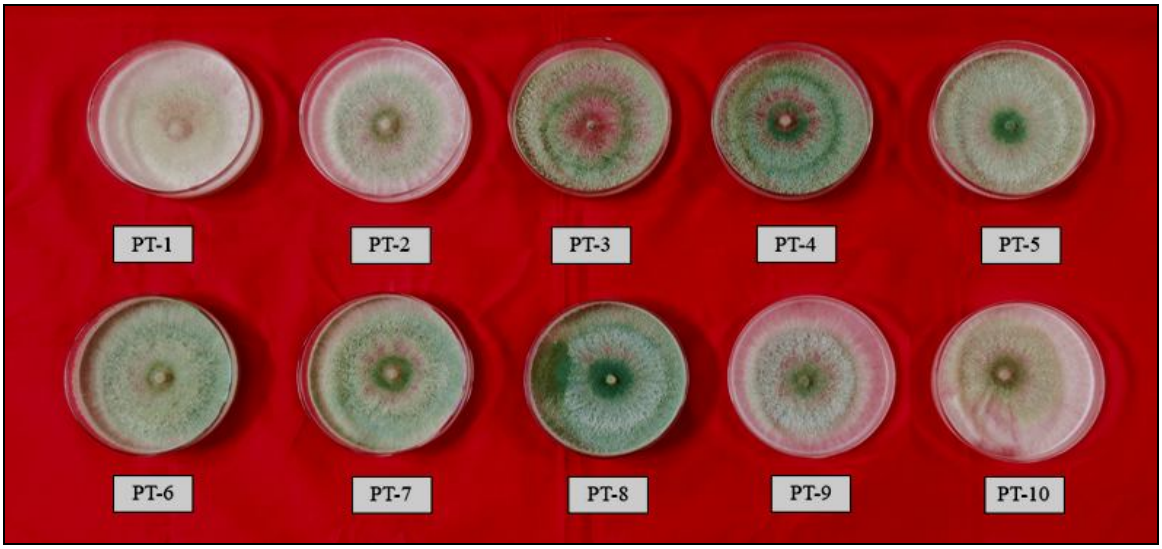

Plate 1 : Cultural characterization of *Trichoderma* spp.

Sixteen soil samples were collected from tomato rhizosphere of eight districts of Marathwada region and isolated 10 *Trichoderma* spp. on PDA medium by using serial dilution plating technique (Kale *et al*., 2018).

Three *Trichoderma* sp. isolated from the maize rhizospheric soil samples collected from Maros, Takalar and Jeneponto regencies of Indonesia to know the potentiality of indigenous *Trichoderma* isolates as an agent of plant growth (Katriani *et al*., 2015). *T. harzianum* 8.4, *T. asperellum* 12-2, *T. asperellum* BP60 were isolated from sandy soils collected from the north west Mexico region (Montoya *et al.*, 2016). Several authors have also isolated *Trichoderma* spp. from rhizosphere of different crop ecosystems (Barari, 2016; Nagamani and Bhagat, 2015 and Sreedevi *et al*., 2011).

Efficacy of *Trichoderma* spp. against *M. phaseolina*

All the ten native isolates of *Trichoderma* spp. were tested against *M. phaseolina* by dual culture technique (*in vitro*). Maximum inhibition was observed in PT-3 (Chikkahesaruru) isolate with 47.73%, which was significantly superior over PT-7 (Rupungudi-S_1) and PT-6 (Ballichakra) with 46.00 and 43.40%, respectively. The isolates PT-5 (Jola), PT-8 (Hullihala), PT-2 (Gajapura) and PT-4 (Hiresindogi-S_2) were on par with each other (41.91, 41.78, 41.04 and 40.67%, respectively). PT-4 (Hiresindogi) with 40.67% was also on par with PT-10 (Hagari) isolate (40.30%).

PT-9 (MARS, Raichur) isolate with 35.72 per cent was found to be less effective antagonist and the least inhibition was recorded in PT-1 (Joladarasi) isolate with 34.10 per cent (Table 1 and Plate 2). This antagonist activity of *Trichioderma* spp. against the phytopathogens are due to antibiosis, lysis competition, hyperparasitism and mycoparasitism (Naik *et al*., 2009). The efficacy of four *T. harzianum* isolates were tested against *M. phaseolina* of mungbean. Th-Raichur (76.96%) isolate was found to be most effective in inhibiting the mycelial growth of the pathogen (Srivastava *et al*., 2012). Seven species of *Trichoderma* isolates were tested for antagonism with *M. phaseolina*. Maximum inhibition was recorded in MTCC 796 (74.3%) followed by Th 1 (61.4%), Tha-1 (60.8%), Tv 23(55.5%), Tvs 12 (45.2%). MTCC 2048 and *T. harzianum* local (< 40%) were least effective in inhibiting the pathogen. The antagonistic activity of *Trichoderma* isolates was mainly attributed to the induction of higher chitinase, protease and β-1, 3 glucanase activity (Patel *et al*., 2012).

Table 1 : Efficacy of *Trichoderma* spp. isolates against *M. phaseolina*

Sl. No.	Isolate	Place	Radial mycelial growth (mm)*	Mycelial inhibition* (%)
1	PT-1	Joladarasi	59.31(50.35)	34.10(35.71)
2	PT-2	Gajapura	53.06(46.74)	41.04(39.82)
3	PT-3	Chikkahesaruru	47.04(43.29)	47.73(43.68)
4	PT-4	Hiresindogi (S_2)	53.39(46.93)	40.67(39.61)

5	PT-5	Jola	52.28(46.29)	41.91(40.33)
6	PT-6	Ballichakra	50.94(45.52)	43.40(41.19)
7	PT-7	Rupungudi (S_1)	48.60(44.18)	46.00(42.69)
8	PT-8	Hullihala	52.39(46.35)	41.78(40.25)
9	PT-9	MARS, Raichur	57.86(49.50)	35.72(36.68)
10	PT-10	Hagari (S_1)	53.73(47.12)	40.30(39.39)
11	Control		90.00(71.54)	-
S. Em. ±			**0.42**	**0.48**
CD @ 1%			**1.23**	**1.39**

PT = Potential *Trichoderma* spp. S_1 = Sample 1 S_2 = Sample 2 * Mean of three replications

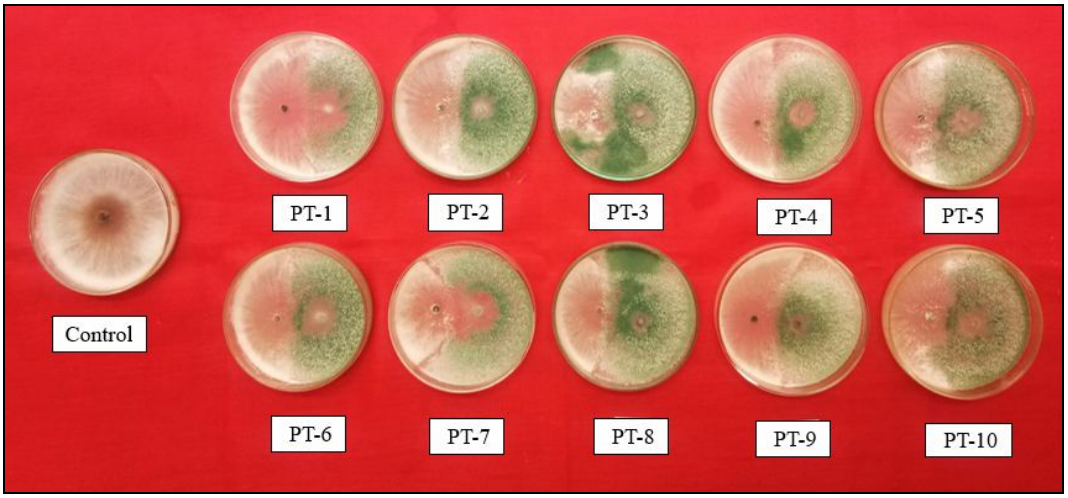

Plate 2 : Efficacy of *Trichoderma* spp. against *M. phaseolina*

Morphological characterization

All then ative isolates of *Trichoderma* sp. were cultured on PDA medium for 7 days and selected for the morphological studies. The 12 morphological characters such as mycelial form, colour, type and width, conidial colour, shape, length and width, conidiophore branching, phialide arrangement, chlamydospore length, width and its nature of production were recorded (Table 2 and Plate 3) and recapitulated as follows.

The mycelial form of *Trichoderma* isolates on PDA (Plate 2) varied with compact and cottony (2 isolates), floccose (4 isolates) and floccose to arachnoid (4 isolates). Whereas, all the isolates exhibited hyaline and septate type of mycelium. The highest mycelial width was observed in isolate PT-1 (4.8 µm) and PT-9 has lowest mycelial width (3.14 µm).

Colour of the conidia varied among the isolates as green (3 isolates), pale green (3 isolates) and light green (4 isolates) colour. Four isolates exhibited globose to oval shaped conidia, 5 isolates were oval shaped and only 1 isolate has globose shaped conidia. The highest length of conidia was observed in PT-4 (6.87 µm) and lowest was observed in PT-2 (3.55 µm). On the contrary, the maximum width was observed in the isolate PT-8 (5.3 µm), and minimum width was observed in PT-9 (3.05 µm).

Characteristic feature of conidiophore is also viewed between the isolates as highly branched regular conidiophore (7 isolates). One exhibited irregular branch and remaining 2 isolates expressed repeatedly paired branches.

The arrangement of phialides on conidiophore was also varied from broad base and pointed tip (3 isolates), narrow base and pointed tip (4 isolates) PT-2 and PT-5 isolates showed pin shaped and broad base, narrow and bent at tip, respectively. PT-4 isolate exhibited broad base and narrow at tip.

All the isolates produced intercalary and terminal chlamydospores. The maximum length and width were observed in PT-4 (9.97 and 9.64 µm). On the contrary minimum length and width were observed in PT-9 (5.28 and 5.12 µm). The morphological characters of 12 isolates of *Trichoderma* sp. were studied, among them 5 isolates belong to *T. harzianum* and 7 isolates to *T. viride* (Muthu and Sharma, 2016).

Four isolates of *T. viride* (Tv_1 to Tv_4) exhibited variation in their colony character *viz.*, initially moderate white and later become as fluffy colony and

green. Length and breadth of conidia was ranged from 3.05-3.70 µm and 2.25-3.30 µm, respectively (Rajamohan *et al*., 2020). Five *Trichoderma* spp. isolates were collected from the rhizosphere soil of healthy groundnut plants and are identified using morphological and microscopic characteristics (Seaby, 1987). Similarly, earlier workers also studied the morphological characters such as colony pigmentation, conidia colour, conidium length and width, phialide length and chlamydospore presence of 12 isolates (Srivastava *et al*., 2012). Several authors also studied the morphology of *Trichoderma* sp. (Rekha, 2010, Rifai, 1969 and Seaby, 1987).

Molecular detection

Genomic DNA of ten *Trichoderma* spp. was extracted from culture filtrate and amplified by using ITS-1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS-4 (5'-TCC TCC GCT TAT TGA TAT GC-3') universal primers for the amplification of 28S rRNA gene fragment. The PCR results confirmed the presence of amplified DNA fragment at 720 bp from the tested samples on 1.4 per cent agarose gel (Plate 4). A new strain was identified as *T. longibrachiatum* 21PP by using ITS-1 and ITS4 primers for the amplification of 28S rRNA gene fragment that produced a sharp band of about 700 bp on the gel (Srivastava *et al.*, 2012).

The results are in tuning with previous work (Jaisani, and Pandey, 2017), who studied the phylogeny of isolates by sequencing the ITS region of ribosomal DNA using specific universal primers ITS 1 and ITS 4 and reported that, *T. harzianum* from tomato and tobacco which showed 97 and 83% similarity. *T. viride* isolates from brinjal and groundnut showed 84 and 95% similarity and *T. virens* was similar with *T. asperellum* from sorghum by 97%. Amplification of genomic DNA with TEF728 and TEF1 primers, 651, 628, 635, and 608 bp amplicons were obtained from the four representative *Trichoderma* isolates *viz*., DUCC001, DUCC003, DUCC005, and DUCC007 which were identified as *T. citrinoviride, T. harzianum, T. atroviride* and *T. deliquescens*, respectively by using BLAST programs (Jun *et al.*, 2016).

Table 2 : Morphological characterization of *Trichoderma* spp. isolated from sorghum rhizosphere

Sl. No.	Isolates	Mycelium			Conidia				Conidiophore	Phialide Arrangement	Chlamydospore		
		Colour, Type	Form	Width (µm)	Shape	Colour	Length (µm)	Width (µm)			Nature of Arrangement	Length (µm)	Width (µm)
1	PT-1	Hyaline, Septate	Compact and cottony	4.80	Globose to oval	Green	3.92	3.58	Highly branched regular	Broad base, pointed at tip	Intercalary and terminal	8.08	8.06
2	PT-2	Hyaline, Septate	Floccose to arachnoid	4.51	Globose	Green	3.55	3.49	Highly branched regular	Pin shape	Intercalary and terminal	7.18	7.08
3	PT-3	Hyaline, Septate	Floccose to arachnoid	3.32	Oval	Pale green	3.91	3.47	Irregular branched	Narrow at base, pointed at tip	Intercalary and terminal	7.02	6.56
4	PT-4	Hyaline, Septate	Floccose	3.64	Oval	Pale green	6.87	4.19	Repeated paired branched	Broad base, narrow at tip	Intercalary and terminal	9.97	9.64
5	PT-5	Hyaline, Septate	Floccose	3.94	Globose to oval	Pale green	4.61	3.08	Highly branched regular	Broad base, narrow, bent at tip	Intercalary and terminal	9.59	7.24
6	PT-6	Hyaline, Septate	Floccose	3.21	Globose to oval	Green	4.81	3.63	Highly branched regular	Broad base, pointed at tip	Intercalary and terminal	7.28	7.20
7	PT-7	Hyaline, Septate	Floccose to arachnoid	3.80	Globose to oval	Light green	3.80	3.30	Highly branched regular	Narrow at base, pointed at tip	Intercalary and terminal	9.82	6.76
8	PT-8	Hyaline, Septate	Floccose	4.20	Oval	Light green	5.60	5.3	Highly branched regular	Narrow at base, pointed at tip	Intercalary and terminal	7.74	6.78
9	PT-9	Hyaline, Septate	Floccose to arachnoid	3.14	Oval	Light green	3.70	3.05	Repeated paired branch	Broad base, pointed at tip	Intercalary and terminal	5.28	5.12
10	PT-10	Hyaline, Septate	Compact and cottony	4.56	Oval	Light green	3.84	3.35	Highly branched regular	Narrow at base, pointed at tip	Intercalary and terminal	8.35	6.56

PT = Potential *Trichoderma* spp.

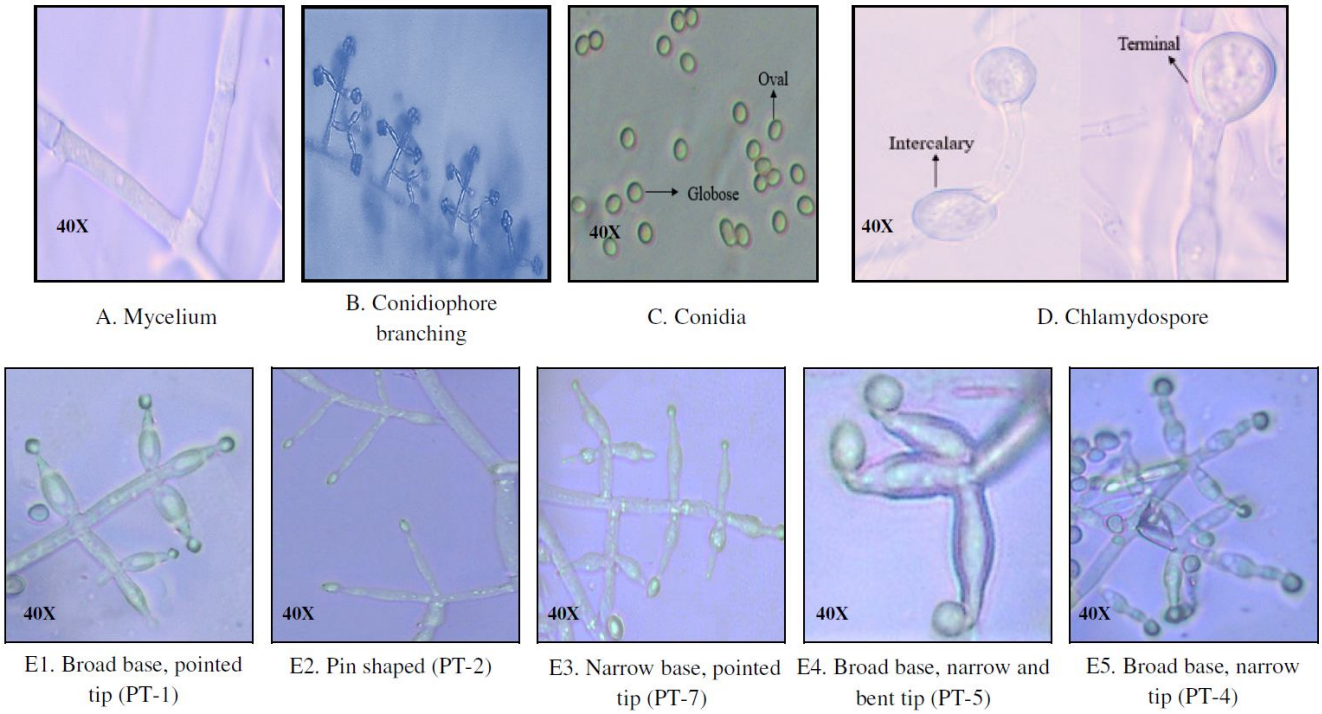

Plate 3 : Morphology of different isolates of *Trichoderma* spp.

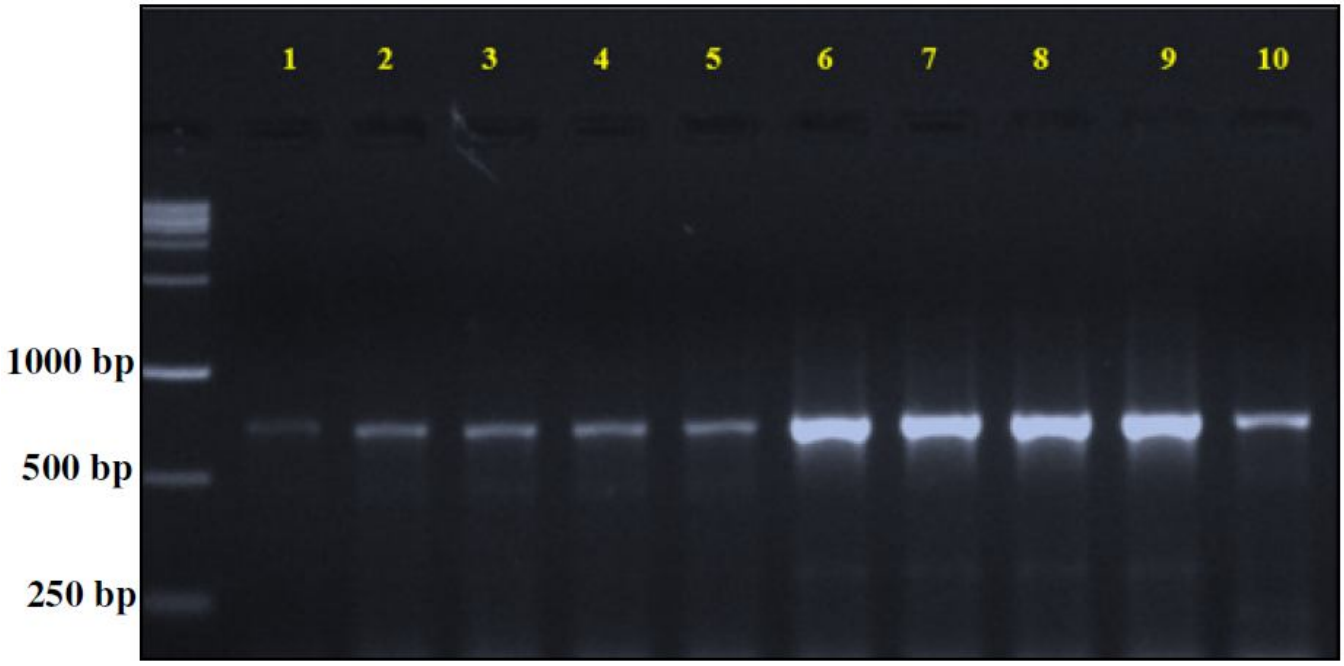

Plate 4 : Detection of 28S rRNA product at 720 bp in native isolates of *Trichoderma* spp.
Lane 1: PT-1; Lane 2: PT-2; Lane 3: PT-3; Lane 4: PT-4; Lane 5: PT-5
Lane 6: PT-6; Lane 7: PT-7; Lane 8: PT-8; Lane 9: PT-9; Lane 10: PT-10

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