Endophytic Fungi Isolation and Identification from Stem of Tinospora Cordifolia

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Abstract

Endophytes refer to the microorganisms (mostly fungi and bacteria) colonising the intercellular and intracellular regions of healthy plant tissues at a particular time, whose presence is unobtrusive and asymptomatic. Fungal endophytes are believed to be treasure of structurally and biologically active compounds. Sometimes, the medicinal properties of plants result from the endophytes present in the host plant and the type of secondary metabolites produced by those endophytes. The present study was conducted with a culture-dependent based approach in order to isolate, identify endophytic fungi from stem of *Tinospora cordifolia i.e*. common medicinal plant *Giloy*. In this study a total of nine endophytic fungal isolates were obtained. All isolates were primarily identified by morphological characteristics and then were subjected to single spore isolation process where distinguished culture of every individual colony was prepared and phyto-genetically examined by molecular experiments. The endophytic fungi were velvety reddish white or grey, cottony and woolly grey or pink growth with spores touching the lid converted the agar into red, grey, pink colour. The growth characteristics of endophytic fungal isolates in Potato dextrose broth showed mycelia formation at the surface with colouration of the broth as seen on the agar. Molecular characterization was done by Polymerase chain reaction and gene sequencing of the internal transcribed spacer regions of the rDNA gene which revealed that the endophytic fungi isolates from *Tinospora cordifolia* stem belonged to *Phoma sojicola* and *Lasiodiploda theobromae*.

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Introduction

The indiscriminate and inappropriate use of antimicrobials leads to the development of antimicrobial resistance. Hence the medicines become ineffective and infections persist in the body, increasing the risk of transfer of resistance to others (Ruiz 2003). New antimicrobials are in need of development to treat such infections (Martinez-Klimova et al. 2017). The use of medicinal plants as alternate for antimicrobial drugs leads to degradation of environmental, loss of biodiversity, spoilage of land, water and are expensive also. These plants have distinct invading microbiome that has potential to produce unique and divergent bioactive compounds (Tejesvi et al. 2007). Therefore, micro-organisms residing inside the medicinal plants are suitable alternatives in drug discovery process (Porras-Alfaro and Bayman 2011). The term “endophyte” is derived from the Greek word, endon meaning within and phyte means plant. It was first introduced in 1866 by de Bary (Arnold 2008). Endophytes refer to the microorganisms (mostly fungi and bacteria) colonising the intercellular and intracellular regions of healthy plant tissues at a particular time, whose presence is unobtrusive and asymptomatic (Schulz and Boyle 2006). Endophytic fungi are the underexplored group of microorganisms which are believed to be treasure of structurally and biologically active compounds (Tan and Zou 2001). Sometimes, the medicinal properties of plants

result from the endophytes present in the host plant and the type of biologically active secondary metabolites that are produced by endophytes (Schulz and Boyle 2006). Colonization of the endophytic fungi in the host plant contribute to the adaptation to the abiotic and biotic stress factors. Beneficial endophytes help in promoting growth of host plant, increase nutrients uptake, inhibit pathogenic growth inside the host plant and reduce disease severity (Redman et al. 2011).

Tinospora cordifolia, a medicinal plant commonly known by names such as *guduchi*, *giloy* or *amrita* (heavenly elixir) in most Indian languages. It is a dioecious, climber, rarely erect, spreads very quickly and usually grows on the tall trees (Nayak 2018). It is the most commercially exploited plant in pharmaceutical industries. A well-documented plant in *Ayurveda* and is known for its anti-rheumatic, anti-spasmodic, anti-microbial, anti-osteoporotic, anti-inflammatory, anti-arthritic, antiallergic, immunomodulatory and anti-diabetic properties (Kapoor and Saxena 2018). The stem of the plant has been reported to exhibit antibacterial activity against some bacterial strains like *Salmonella* Typhi, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Shigella dysentria* (Singh and Singh 2012).

The present study was conducted with a culture-dependent based approach in order to isolate fungal endophytes from stem of *Tinospora cordifolia* plant collected from three different places *viz*, College of Veterinary Science and Animals Husbandry (N.D.V.S.U.), Jabalpur, Jawaharlal Nehru Krishi Vishwavidyalaya (J.N.K.V.V), Jabalpur and Tropical Forest Research Institute (T.F.R.I.), Jabalpur. Endophytic fungi were identified by morphological and molecular methods.

Materials And Methods

Collection of *Tinospora cordifolia* stem

Healthy stems of *Tinospora cordifolia* plant were collected from College of Veterinary Science and Animals Husbandry (N.D.V.S.U.) Jabalpur, Jawaharlal Nehru Krishi Vishwavidyalaya (J.N.K.V.V) Jabalpur and Tropical Forest Research Institute (T.F.R.I.) Jabalpur. Mature healthy plant stem samples from each area, from two different plants were taken, brought to the laboratory in bags and further processed for isolation of endophytic fungi.

Inoculation of stem and isolation of endophytic fungi

The sterilization of collected samples of *Tinospora cordifolia* stem was done according to Madhu priya and Theoder (2018) by first washing under running tap water then immersed in 0.1 per cent sodium hypochlorite for five minutes. Later immersed in 0.01 per cent bavistin then were kept in double distilled water for 5 minutes then exposed to 0.05 per cent streptomycin followed by treatment with double distilled water for 5 minutes. Further exposed to 70 per cent ethanol and again were kept in double distilled water for 5 minutes. To verify and confirm the efficiency of surface sterilization procedure, sterility check test was performed by pouring 50 µl of the distilled water which was used in the final rinse of surface sterilization procedure of the stem samples in nutrient agar medium (NA) and incubated at 37°C for 24 h (De souza Ferriera et al. 2017).

The potato dextrose agar (PDA) medium supplemented with antibacterial chloramphenicol (100 µg.ml^{-1}) to suppress the growth of bacteria was poured into sterile petri plates (Prasher and Dhanda, 2017). The sterilized stem samples after drying were cut into two halves and inoculated on PDA plates with inner tissue towards agar. The plates were sealed with parafilm and incubated at 25°C for 5–7 days (Fig. 01). The hypheal tips of 1 cm^2 of primary culture of endophytic fungi growing out of the plant tissue were transferred to the centre of fresh potato dextrose agar. After incubation at 25°C for 7 to 14 days purity of the cultures was determined by colony morphology. Sub-culturing of endophytic fungi was done periodically and cultures were stored as stock.

Pathogenicity of endophytic fungi

Fungus commonly does not cause disease in healthy immune-competent hosts. Disease occurs as a result of accidental penetration of host barriers by fungus or when immunologic defects or other debilitating condition exist that favour fungal entry and growth. Fungus often develops both virulence mechanisms (*e.g.*, capsule and ability to grow at 37°C) and morphologic forms (*e.g.*, yeasts, hyphae, spherules, and sclerotic bodies) that facilitate their multiplication within the host. The endophytic fungi growing out of the stem tissue of *Tinospora cordifolia* plant were grown at 37°C to check the virulence of fungi. The presence of capsule, enzymes such as keratinase, the ability to grow at 37°C and dimorphism *etc* contribute to fungal pathogenesis which involves a complex interplay of many fungal and host factors (Kobayashi 1996).

Morphological based identification of fungi

Endophytic fungi were identified on the basis of morphological characteristics such as colony morphology and by wet mount method and slide culture technique (Markey et al. 2013). The cultures were further selected for molecular identification.

Molecular identification of fungi

Molecular characterization of isolated endophytic fungi was done by the method based on sequencing of the internal transcribed spacer regions of the rDNA gene. Blast analysis was done for obtained sequence in NCBI and on the basis of genetic similarity; phylogenic tree was prepared to characterize the endophytic fungi.

Pure cultures were selected for DNA isolation. Universal fungal primers ITS1 and ITS4 (Prasher and Dhanda 2017) were used to amplify ribosomal internal transcribed spacer (ITS). 5 µl of isolated DNA in 25 µl of PCR reaction solution were added (1.5 µl of Forward Primer and Reverse Primer, 5 µl of deionized water, and 12 µl of Taq Master Mix). PCR was performed under thermal cycling conditions: Initial denaturation at 95°C for 2 min, followed by 25 cycles of denaturation at 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min; and finally 72°C for 10 min. Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems). The sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of

query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment.

Results

Morphological characteristics of endophytic fungi isolated from *Tinospora cordifolia*

No microorganism appeared on the culture media containing last washing water affirmed the effectiveness of the surface sterilization in removing and destroying epiphytes. Primary endophytic fungal growths are shown in Fig. 02. A total of nine isolates were identified and purified from stem of *Tinospora cordifolia* plant (pure cultures are shown in Fig. 03) designated as TN-1 to TN-4 (N.D.V.S.U.), TJ-1 and TJ-2 (J.N.K.V.V.) and TT-1 to TT-3 (T.F.R.I.). They were characterized as endophytic fungi basically into two groups namely *Phoma* spp. and *Lasiodiploda* spp. as depicted in Table 01.

Table 01
Growth of endophytic fungi isolated from stem of *Tinospora cordifolia*

Isolate Name	Macroscopic characteristics		Microscopic characteristics	Fungal type
	Obverse	Reverse		
TN	Velvety reddish white growth	Entire agar is of red colour	Round and oval conidia, branched hyphae	*Phoma* spp.
TJ	Black crest like growth, segmented	Black with serrating segments	Straight sporangiophores which terminated with black sporangium containing a columella root like hyphae. Sporangiophores were present in clusters.	*Phoma* spp.
TT	Wooly growth initially blackish brown then off white	Entire agar is grayish brown	Brown coloures straight or septate hyphae	*Lasiodiploda* spp.

The surfaces of fungi TN-1 to TN-4, TJ-1 and TJ-2 on PDA plates were rosy buff to greenish olivaceous, grey olivaceous, olivaceous grey in centre, sometimes with dark herbage green sectors; aerial mycelium felty to finely floccose, pale olivaceous grey; reverse similar, leaden grey to iron grey in centre grayish pink and whitish green and showed velvety growth. This kind of morphological characteristics were quite similar to *Phoma* spp. After visualizing the surfaces of the isolated fungi TT-1 to TT-3 on the PDA plates, woolly mycelium, thick spreading colonies, greyish white and greyish pink with reverse blue, brown and pink pigmentation that turned dark with age were found. All these characteristic features were quite similar to *Lasiodiploda* spp. (Fig. 03). Since the *Tinospora cordifolia* plants used in our study appeared healthy and *Phoma* spp. and *Lasiodiploda* spp. are well known pathogens of the plants. It appears that these fungal isolates are avirulent, hypovirulent or virulent but in a latent phase, so they do not harm the plant. The endophytic fungi grown out of stem tissue of *Tinospora cordifolia* plant were grown at 37°C

for pathogenicity testing. No visible growth of fungus was seen after incubation at 37°C for 7 days. The results of staining are mentioned in Table 01. Staining of endophytic fungal isolates is shown in Fig. 04.

Molecular characterization of isolated endophytic fungi

The amplified PCR products were run on agarose gel electrophoresis, where the fragments with 600 bp were observed (Fig. 05). Endophytic fungi were identified by comparing ITS gene sequences obtained from fungal isolates and those deposited in (NCBI) GenBank database using BLAST tool to obtain the sequences that displayed maximum similarity (Figs. 6 and 7) and phylogenic tree was constructed (Figs. 8 and 9). Based on the rDNA sequence analysis, *Phoma sojiclola and Lasiodiploda theobromae* fungi were identified as the endophytic fungi from stem of *Tinospora cordifolia* plant.

Endophytic fungus I Phoma sojicola:

Phoma sojicola was isolated from stem of *Tinospora cordifolia* bought from N.D.V.S.U. and J.N.K.V.V., Jabalpur This fungus produces velvety greyish pink, greenish white colonies with white edges on PDA plates. Reverse side of the colonies were red. Conidiophores were pale brown, simple or branched, bearing catenulate conidia at the apex and apical fertile parts. Conidia were catenulate, mostly in a chain, often branched, cylindrical or spindle-shaped, often with cylindrical beaks. Chlamydospores were unicellular, single or in chains, sometimes clustered. This strain belonged to *Phoma* and was affiliated to *sojicola* spp.

Endophytic fungus II *Lasiodiploda theobromae*:

Lasiodiploda theobromae was isolated from stem of *Tinospora cordifolia* bought from T.F.R.I., Jabalpur. On PDA woolly mycelium, thick spreading colonies, grayish white and grayish pink with reverse blue, brown and pink pigmentation that turns dark with age. Microscopy revealed hyphae that were hyaline, simple, sometimes septate, branched cylindrical, arising from the inner layers of cells lining the pycnidial cavity were observed. This fungus was affiliated to *Lasiodiploda theobromae* belonging to family Botryosphaeriaceae and the taxonomic characterization was confirmed by ITS sequencing.

Discussion

Different parts of *Tinospora cordifolia* plant have been studied for isolation of endophytic fungi by many researchers, Mishra et al. (2012) isolated endophytic fungi (*Penicillium* spp., *Colletotrichum* spp., *Cladosporium* spp., *Chaetomium globosum*, *Curvularia* spp. and *Alternaria alternate*) from stem, leaf, petiole, and root of *Tinospora cordifolia*. Uzma et al. (2016) isolated twenty five endophytic fungi from different parts of *Tinospora cordifolia* plant. Mishra et al. (2018) isolated *Pseudofusicoccum adansoniae* from roots of *Tinospora cordifolia*. Nayak (2018) identified endophytic fungal species from *Tinospora cordifolia* plant namely *Alternaria alternata*, *Aspergillus niger*, *Cladosporium herbarum*, *Penicillium chrysogenum, Penicillium citrinum, Fusarium* sp., *Bispora* sp., *Curvularia lunata* and *Eremascus albus*. Sushma et al. (2018) isolated endophytic fungi from *Tinospora cordifolia* and identified as *Stem-phylium*

lycopersici, Epicoccum nigrum, Leptosphaerulina arachidicola and *Phomopsis azadirachtae*. Kapoor and Saxena (2018) identified fungal endophytes (*Fusarium Alternaria, Curvularia* and *Colletotrichum*) from stem of *Tinospora cordifolia.* Yadav et al. (2020) identified an endophytic fungus, Alternaria GFAV15 with antimicrobial potential from the green unripe fruit of *Tinospora cordifolia*. Habbu et al. (2021) identified *Trichoderma longibrachiatum* and *Aspergillus versicolor* as endophytic fungi from leaves of *Tinospora cordifolia* plant.

Endophytic fungus I Phoma sojicola:

Phoma sojicola produces velvety greyish pink, greenish white colonies on PDA (Rajak et al., 1984). Catenulate conidia occuring mostly in a chain, often branched and unicellular chlamydospores, single or in chains, sometimes clustered as also described by Kovics et al. (1999).

Phoma species are ubiquitous and are common inhabitants of soil (Rai and Tiwari 2014). *Phoma* as the most prominent endophytic fungus has been previously reported by Khan et al. (2007) in a study of endophytic fungi isolation from *Calotropis procera*. *Phoma sojicola* as an endophytic fungus also has been reported by Bhagat et al. (2011) where sixty three endophytic fungal isolates from two traditional medicinal plants, *Ocimum sanctum* and *Sapindus detergens* were isolated. The fungal endophytes belonged to order Pleosporales (*Alternaria* spp., *Phoma sojicola* and *Exserohilum* spp.). Similarly *Phoma* spp. as an endophytic fungus isolated from leaves and petioles of *Piprum nigrum* in a study conducted by Uzma et al. (2016).

Lasiodiploda theobromae represents the asexual (= anamorphic) state of *Botryosphaeria rhodina*, an important plant pathogenic fungus for both tropical and subtropical regions, causing leaf spots, necrosis, gummosis and even the death of many plants (Orlandelli et al. 2012). Some studies have shown its endophytic association with the host plant (Mohali et al. 2005; Slippers and Wingfield 2007). This fungus has been reported to produce biological compounds like taxol (anticancer drug) as studied by Pandi et al. (2011). *Lasiodiploda theobromae* is a cosmopolitan fungus with a worldwide distribution in the tropic and sub tropic regions. *Lasiodiploda theobromae* can also be considered as a latent pathogen capable of endophytic infection.

Tinospora cordifolia plant and its parts have been studied extensively but the reports on its endophyte researches are still modest. *Lasiodiploda theobromae* had been isolated by other researcher from different plants like *Terminalia arjuna*, *Acanthus ilicifolius*, *Solanum nigrum* and *Boswellia ovalifoliolata*. This is the first report of isolation of *Lasiodiploda theobromae* from *Tinospora cordifolia* plant. This is a cosmopolitan fungus with a worldwide distribution in the tropic and sub tropic regions and there is no evidence of host specificity for this fungus.

Lasiodiploda theobromae was isolated from the medicinal plant *Morinda citrifolia* and is an excellent candidate for an alternate source of taxol in a study conducted by Pandi et al. (2011) from *Costus igneus* in a study conducted by Amirita et al. (2012), from leaves of the marine mangrove *Acanthus ilicifolius* by Chen et al. (2016), from stem of *Piper nigrum* by Uzma et al. (2016), from fruits of *Solanum nigrum* by El-

Hawary et al. (2017), from *Teucrium polium* by Balbool et al. (2018) and Ujam et al. (2020) isolated the similar endophytic fungus *Lasiodiplodia* from stem of *Psiduim guajava*.

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Figures

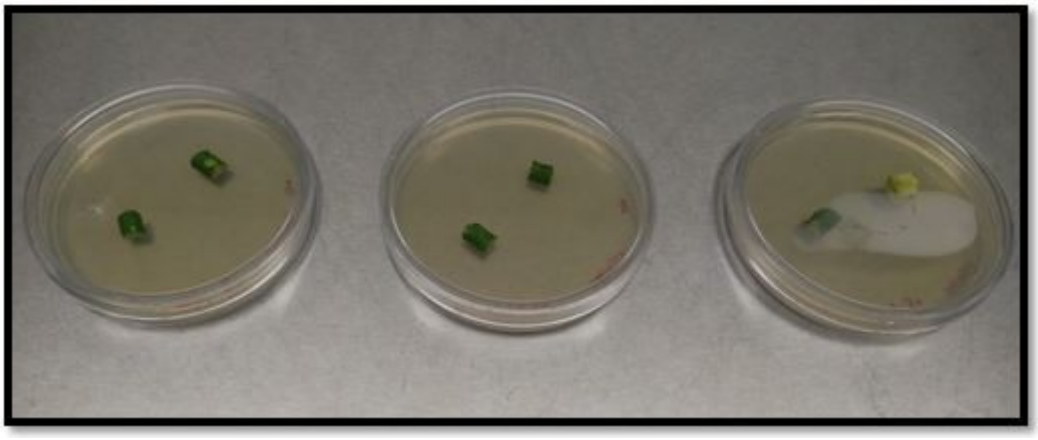

Figure 1

Inoculation of stem samples of *Tinospora cordifolia* on PDA

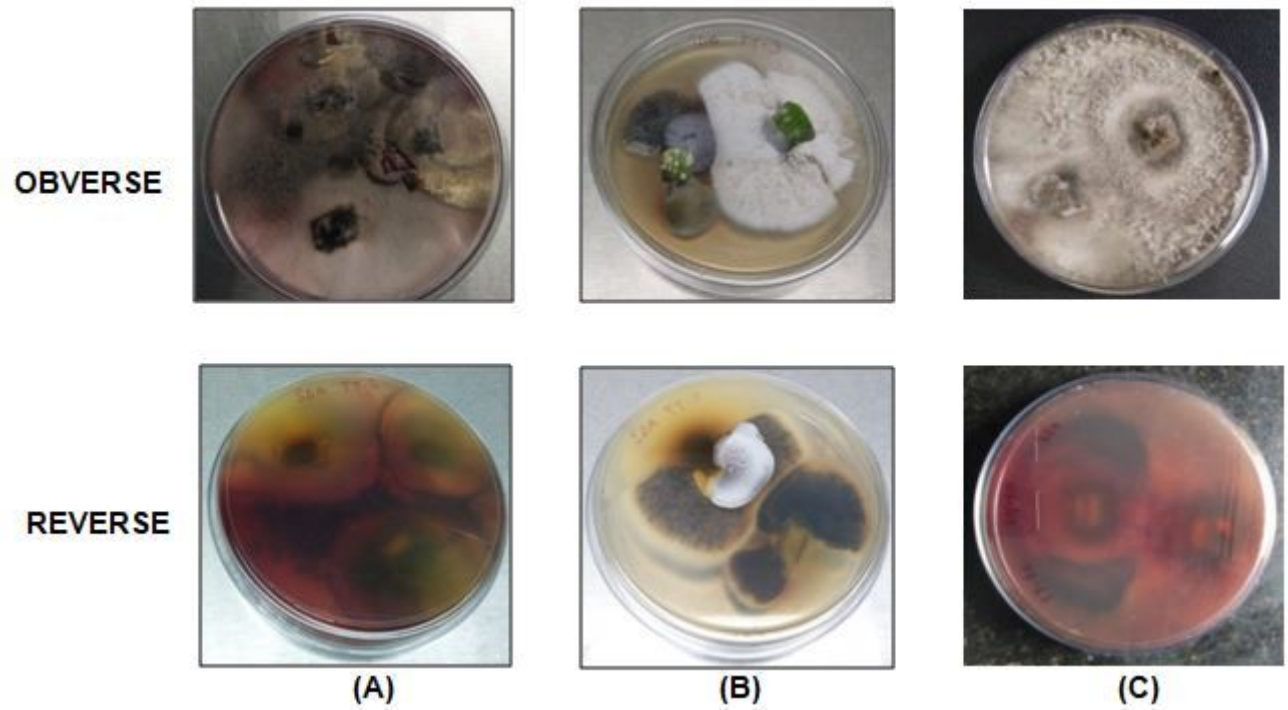

Figure 2

Primary growth of endophytic fungi from stem of *Tinospora cordifolia*

Growth from samples bought from College of Veterinary Science and Animals Husbandry (N.D.V.S.U.) Jabalpur (A), Growth from samples bought from Jawaharlal Nehru Krishi Vishwavidyalaya (J.N.K.V.V.) Jabalpur (B) and Growth from samples bought from Tropical Forest Research Institute (T.F.R.I.) Jabalpur (C)

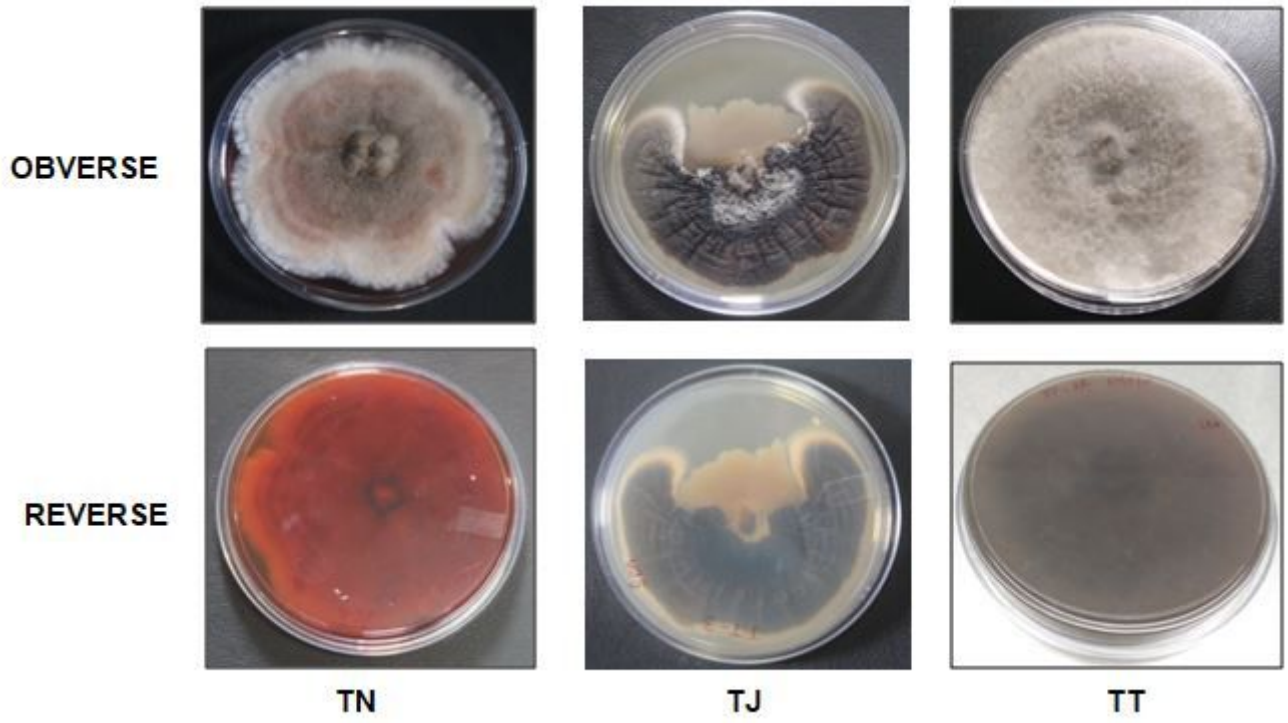

Figure 3

Pure cultures of endophytic fungal isolates followed by subculturing of primary cultures of endophytic fungi

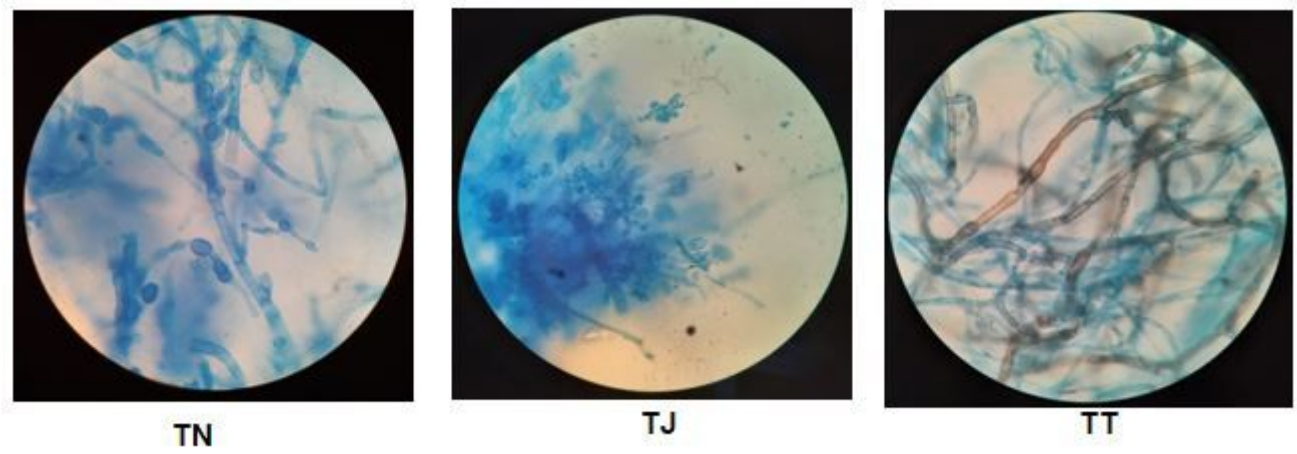

Figure 4

Staining of endophytic fungal isolates by Lactophenol cotton blue stain

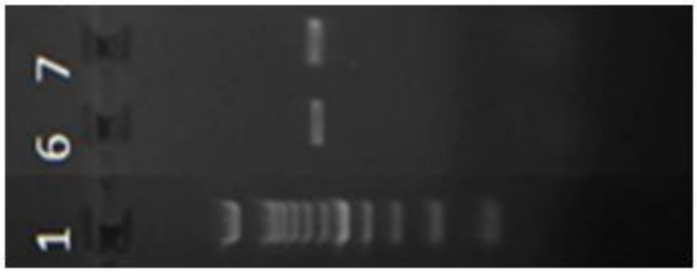

Figure 5

Agarose gel electrophoresis of rDNA gene PCR product of endophytic fungi isolated from *Tinospora cordifolia* stem

Lane 1: 100 bp ladder

Lane 6: 600 bp product I (TN)

Lane 7: 600 bp product II (TT)

> Contig - TS229

TTTCCGTAGGTGAACCTGCGGAAGGATCATTACCTAGAGTTGCGGGCTTTGCCTGCCATCTCTTACCCATGTCTTTTGAGTACC
TTCGTTTCCTCGGCGGGTCCGCCCGCCGATTGGACAAAACTTAAACCCTTTGTAATTGAAATCAGCGTCTGAAAAAACTTAATAG
TTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATT
CAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTC
AAGCTTTGCTTGGTGTTGGGTGTTTGTCTCGCCTCTGCGCGCAGACTCGCCTCAAAACGATTGGCAGCCGGCGTATTGATTTCG
GAGCGCAGTACATCTCGCGCTTTGTAGTCTCAACGACGACGTCCAAAAAGTACTTTTTTCACTCTTGACCTCGGATCAGGTAGG
GATACCCGCTGAACTTAA

Result: *Phoma sojicola*

Figure 6

Nucleotide sequence of isolated PCR product I from stem of *Tinospora cordifolia*

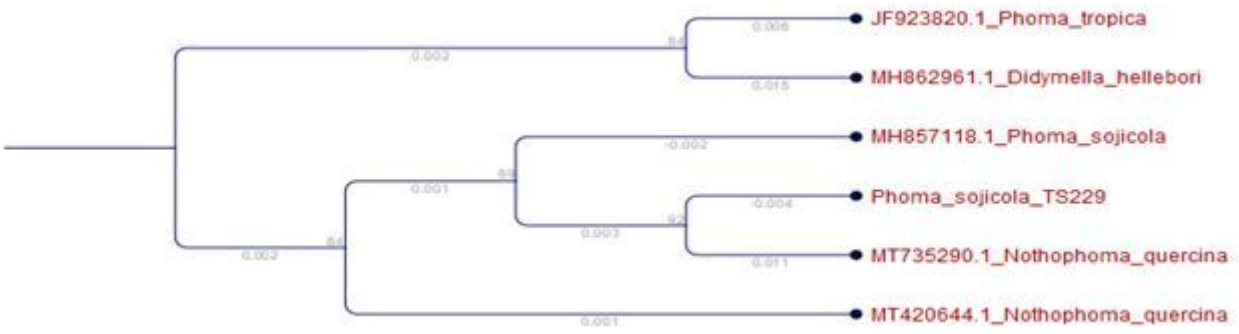

Figure 7

Construction of phylogenetic tree of isolated endophytic fungi from stem of *Tinospora cordifolia*

>CONTIG TS 256

GATGCCAGAACCCAAAGGTTCCGTGTGAAAGTTTTAGTTTTTTAACTTGTTTATTCGAAGTTTGCGTTTAGCGACCGAGTTTGAA
GGTCCTTGGCGCCGAAGCCGCCAAAGCAAGAGAGGTACGTTCACAAAGGTGGAGAGTCGAGCGAAGCTCGAAAACTCGGTAA
TGATCTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTTTTCGAGCTTCGGCTCGACTCTCCCACCCTTTGTGAACGTAC
CTCTGTTGCTTTGGCGGCTTCGGCCGCCAAAGGACCTTCAAACTCCAGTCAGTAAACGCAGACGTCTGATAAACAAGTTAATAA
ACTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAAT
TCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTACAACCCT
CAAGCTCTGCTTGGAATTGGGCACCGTCCTCACTGCGGACGCGCCTCAAAGACCTCGGCGGTGGCTGTTCAGCCCTCAAGCG
TAGTAGAATACACCTCGCTTTGGAGCGGTTGGCGTCGCCCGCCGGACGAACCTTCTGAACTTTTCTCAAGGTTGACCTCGGAT
CAGGTAGGAACCAAT

RESULT: *Lasiodiplodia theobromae*

Figure 8

Nucleotide sequence of isolated PCR product II from stem of *Tinospora cordifolia*

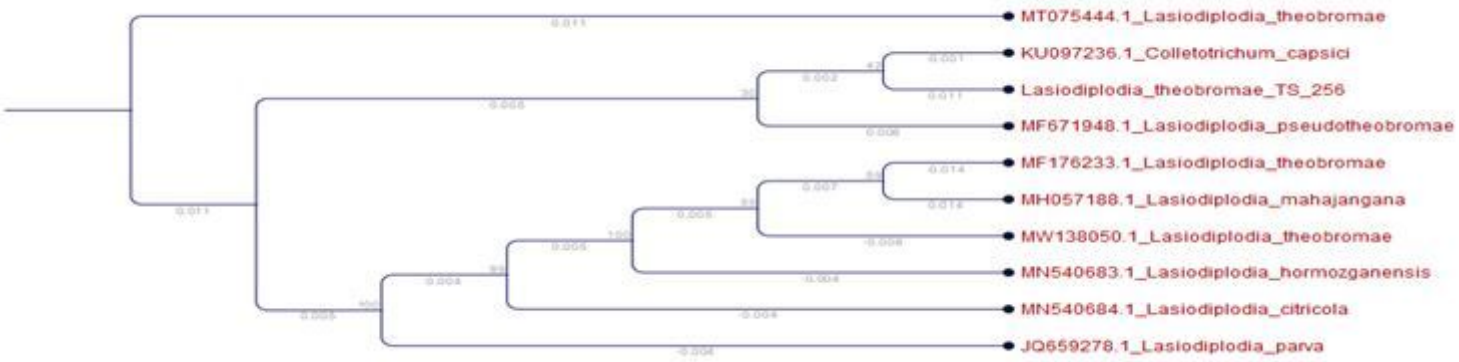

Figure 9

Construction of phylogenetic tree of isolated endophytic fungi from stem of *Tinospora cordifolia*