Isolation and identification of endophytic fungi from *Tinospora cordifolia*

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Abstract
Endophytic fungi are the most promising reservoir of bioactive compounds with large chemical multiplicity. These effective bioactive agents have been optimized by nature through evolutionary, ecological and environmental factors. Endophytes were found to produce essential bioactive metabolites which were originally produced by their host plant. It raises the prospect of using these microbial endophytes as an alternative source of phytocompounds. In the present study endophytic fungal isolates were screened from leaf and stem tissue of Tinospora cordifolia (Willd) Miers [Synonym: T. sinensis (Lour.) Merr] plant using Azadirachta indica tree as support during summer and winter season. The small segments of surface sterilized leaf and stem tissue were inoculated on Potato Dextrose Agar (PDA) medium incubated at 26°C for 10 days and were checked regularly for the growth of endophytic fungal colonies.

Pure isolates were obtained by picking individual fungal hyphal tips from the PDA plates and subcultured on fresh PDA plates subsequently for 10 days. A total no. of 36 different endophytic fungi were screened from leaf and stem portion of T. cordifolia. These isolated species were identified via cultural characteristics and morphology of spores and fruiting structure using standard taxonomic manuals. Out of 36, 20 isolates (EF 1- EF 20) were isolated from leaf tissue and 16 isolates (EF 21- EF 36) were from stem part. The isolated fungal species were from different generas i.e. Alternaria, Curvularia, Cladosporium, Penicillium, Aspergillus, Fusarium, Colletotrichum, Rhizopus, Helminthosporium, Pleurothecium, Aureobasidium, Cercospora and among these Alternaria, Curvularia, Penicillium and Aspergillus were most abundant species in both (leaf and stem) tissues. The leaf tissue harbors more endophytic species as compared to its stem counterpart.

Keywords: Endophytic Fungi, *Tinospora cordifolia*, PDA Media, Lactophenol Cotton Blue, Conidia.

Introduction

Microorganisms and plants have been known as the prolific producers of biologically active metabolites that are extensively used as agrochemicals, antibiotics, immunosuppressant and anticancer agents. Nowadays, the search for new bioactive compounds has become more intense and demanding due to numerous drug resistance capability of pathogenic microorganisms towards currently available antibiotics in clinics, ineffectiveness of available herbicides and insecticides for agricultural usage and continuous deterioration of environment and human health. Traditionally, bioactive compounds isolated from plants are the major sources of drug discovery and development. But nowadays, several factors such as complications of chemical constituents, seasonal and geographical specificity need for cultivation land and indiscriminate misuse of medicinal plants for extraction purposes may limit the prospective uses of medicinal plants.

The findings that endophytes, microorganisms that colonize healthy plant tissues and could serve as substitute for plant's metabolites, attract more researchers to focus on these organisms for searching novel bioactive compounds that have medicinal and agricultural importance with minor environmental impacts. Endophytic fungi are the prevalent source of the biologically compatible metabolites[1].

Endophytic fungi are crucial for the wellbeing of each terrestrial ecosystem and also for their sustainability[2, 3]. It was also appreciated by evolutionary biologists that fungal endophytes are associated with medicinal plants. There are several evidences which proved the fungi-plant association inside plants which were present into early harsh terrestrial environmental conditions with poor nutrients, steady desiccation and also weakly shaded[4].

For their survival inside plants, fungal endophytes maintain a balance of antagonism not only with their host but also with microbial competitors such as microbial endophytes and pathogenic microbes. Secondary bioactive constituents are essential factors which maintain this equilibrium which resulted in a compatible, multipartite symbiosis and a healthy plant[5].

Tinospora cordifolia (Willd) Miers [Synonym: *T. sinensis* (Lour.) Merr] belongs to the family Menispermaceae commonly named as Giloy, is a genetically diverse, climbing shrub found in tropical Indian subcontinent. The plant is considered as one of the most divine herbs in Ayurveda for its immense therapeutic properties and drawing attention of researchers because of its scientifically reported multi-medicinal properties like antispasmodic, antiinflammatory, antiarthritic, antioxidant, antiallergic, antistress, antileprotic, antimalarial, hepatoprotective, immunomodulatory and antineoplastic activities. A variety of bioactive components

like steroids, diterpenoid lactones, aliphatics glycosides and alkaloids including berberine[6] which attribute medicinal capabilities to this plant, have been isolated.

Microbial endophytes may produce same or similar chemical substances which are also produced by their host plant[7,8]. An effective anti-breast cancer drug i.e. fungal taxol was produced by host plant and also by its endophytic fungus - *Taxus brevifolia- Taxomyces andreanae*[9]. Due to relationship of endophytes with their host plants, these produce a large variety of metabolites such as antibiotics, antioxidants, antimicrobial and anticancerous and immunosuppressive compounds[10].

Thus, to learn if any fungal endophytes may be produced from their host plant *T. cordifolia*, it is essential to do a complete research on the biology and distribution of endophytic fungi living inside this plant from various localities. Ultimately, a compilation of the endophytic microbes would then serve as a library for doing complete screening and characterization studies.

Limited research work has been done on fungal endophytes of *T. cordifolia*, an important Indian medicinal plant. Moreover, endophytic fungi are often tissue and season specific[11-15]. Geographical locations further affect pattern of distribution of endophytes[16]. Hence, in the present study we focus on the isolation and recognition of fungal endophytes from different parts of *T. cordifolia* plant located in Kurukshetra, Haryana, India, during different seasons.

Materials and Methods

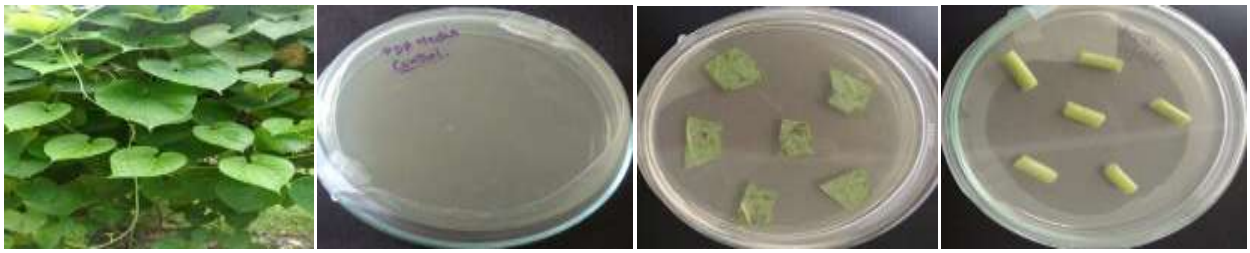
Study area and collection of samples: Leaves and stems were collected during different seasons- March 2017 to August 2017 and November 2017 to February 2018 of the period of one year (March 2017- February 2018) from *T. cordifolia* plant located in Botanical Garden of Kurukshetra University, Kurukshetra, Haryana, India. The plant material was identified by Prof. B. D. Vashistha of Department of Botany, Kurukshetra University, Kurukshetra. A voucher specimen (Herbarium/Bot.K.U./Biotech.-3-2017) of the plant was deposited at herbarium, Department of Botany, Kurukshetra University Kurukshetra. Since, *T. cordifolia* is a vine plant and the plant examined in this study used *Azadirachta indica* plant as a support. The sample explants were taken inside laboratory in sealed plastic bag and kept at 4°C.

Screening of Endophytic Fungi: All plant samples were washed appropriately under tap water followed by mild detergent (Tween-20) and subsequently by double distilled water before processing. Initially, the explants were surface treated for removing epiphytic microorganisms. The explants were immersed in ethanol (70%) for 2 minutes and then in aqueous sodium hypochlorite (4% available chlorine) for 3 minutes, after this, samples were rinsed with ethanol (70%) for 4 seconds and finally rinsed with sterilized water. Leaf and stems were cut into very small pieces, dried and then inoculated on potato dextrose agar media with pH 5.6 and containing streptomycin (30µg/ml) to suppress bacterial growth (Fig. 1 A-D). These Petri plates were sealed and incubated at 26°C for 25 days[17]. The plates were checked for fungal growth and the hyphal tips of the fungus were then subcultured.

Preparation of conidial suspension of fungal strains to study spore morphology: Different strains of fungus were grown on Potato dextrose Agar (PDA) media at 26°C for 10 days. Spores of these fungal strains were harvested by flooding media surface with distilled water and then properly mixed with the help of a glass rod to dislodge the fungal spores. After filtration of suspension, the concentration of conidia was adjusted to 5×10^5 conidia/ ml with the help of sterile water using haemocytometre[18, 19].

Identification of Endophytic Fungi: The fungal endophytes which were screened from *T. cordifolia* were identified via macro and microscopic characteristics such as spore morphology, morphology of fruiting bodies with lactophenol cotton blue stain under low and high power. For the final identification of the fungal genera, standard taxonomic manuals of Barnett and Hunter[20] and Ellis et al[21] were used. Specific code numbers (EF1 to EF 36) were assigned for all the isolated and identified fungal endophytes.

Preservation of Endophytic Fungi: The identified fungal endophytes were preserved in cryovials by using 15% v/v glycerol layered on PDA media at -20°C and and also in lyophilized form and deposited in the Department of Biotechnology, Kurukshetra University, Kurukshetra, Haryana, India.



(A) (B) (C) (D)

Figure 1: (A) *Tinospora cordifolia* (medicinal plant) (B) Control plate (C-D) Explants (leaf and stem tissue) on PDA Media

Results

Different tissues i.e. leaf and stem of *T. cordifolia* were used for the isolation and identification of fungal endophytes. These endophytes were recognized via their microscopic characteristics such as spore size and shape and phenotypic characteristics viz color and morphology of colonies, surface texture etc. using standard key for their identification.

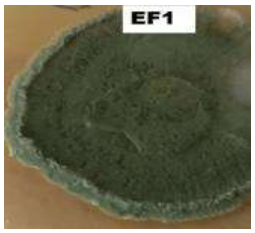
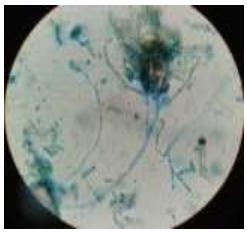
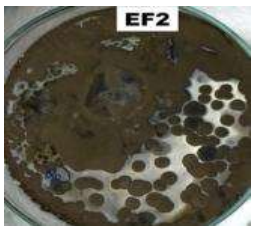
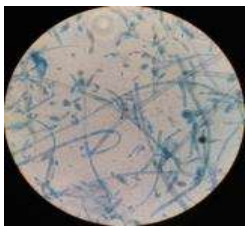
Overall, 36 endophytic fungi were isolated from both part of *T. cordifolia*. Different species of *Alternaria, Curvularia, Cladosporium, Penicillium, Aspergillus, Fusarium, Colletotrichum, Rhizopus, Helminthosporium, Aureobasidium* and *Pleurothecium* were isolated. Out of 36, 20 isolates (EF1- EF20) were from leaf tissue and 16 isolates (EF21-EF36) were from stem part (Table1). The leaf tissue showed the higher presence of fungal endophytes than the stem part. In the isolated fungal endophytes, *Alternaria, Curvularia, Penicillium and Aspergillus* were more abundant species in both (leaf and stem) explants. The *Cladosporium, Aureobasidium pullulans, Curvularia lunata* and *Curvularia hawaiihensis* were isolated from leaf portion and *Cercospora, Colletotrichum gloeosporioide, Curvularia geniculata* were from stem tissue only.

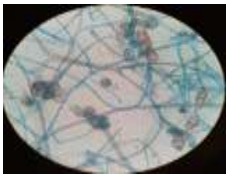
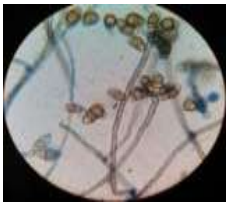
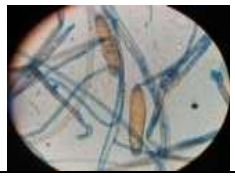
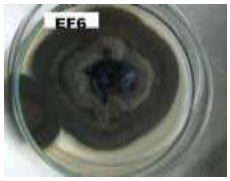
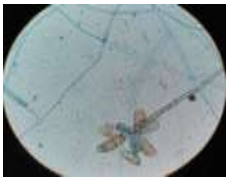
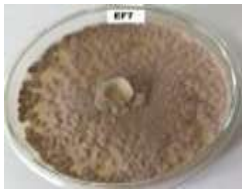
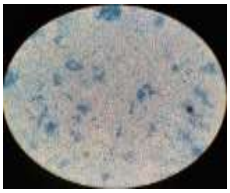
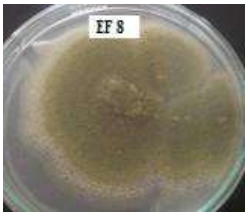
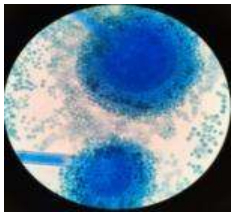
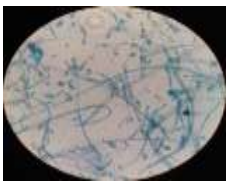
Among the isolated generas, *Alternaria* was most copious. Seven different species of *Alternaria* were screened from the study explants of *T. cordifolia* among which two species were well identified. The identified species were *Alternaria alternata* and *Alternaria atra.* But other five species of *Alternaria* genus could not be well characterized. The *Alternaria atra* was present only in leaf tissue and missing in stem parts. The *Alternaria alternata* showed its presence in both (leaf and stem) explants. The *Curvularia* was second most profused species after *Alternaria* in *T. cordifolia*.

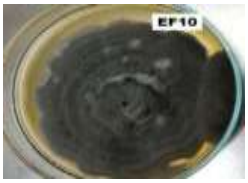
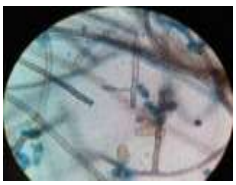
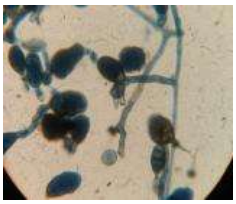
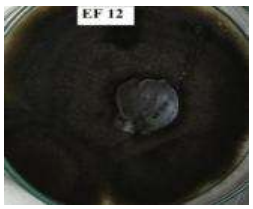
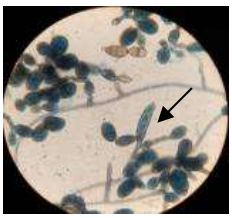
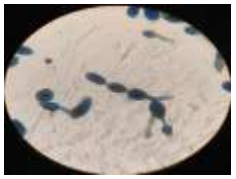
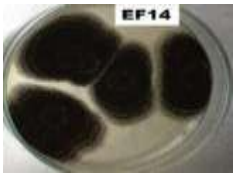
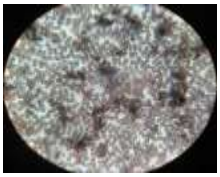
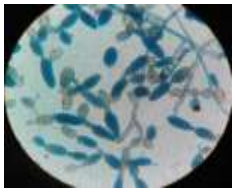
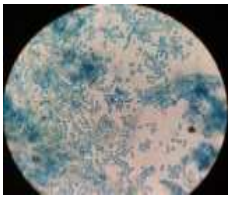
Seven different species of genera *Curvularia* were isolated from leaves and stems. *Curvularia lunata*, *Curvularia eragrostidis* were common in both tissues. *Curvularia geniculata* was there in stem parts and *C. hawaiiensis* showed its presence only in leaf portions. Two different isolated species of *Cladosporium* also showed their presence only in leaf tissues i.e. *Cladosporium fulvum* and *Cladosporium cladosporioide*.

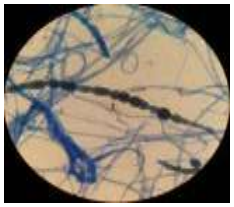
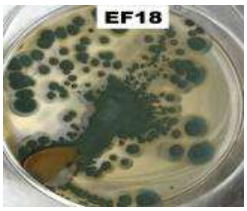
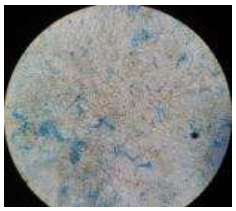
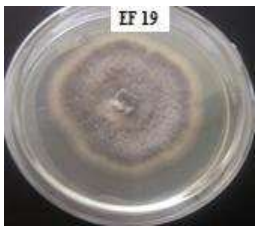
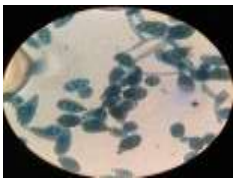
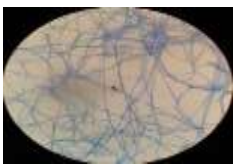
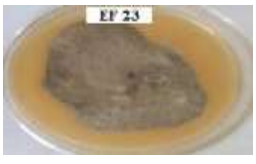
Four different species of *Aspergillus* i.e. *Aspergillus niger*, *Aspergillus flavus, Aspergillus terreus* and *Aspergillus fumigatus* were also screened from stems and leaves explants of *T. cordifolia.* The *Aspergillus niger* was present equally in both leaf and stem portions but other three species were extracted only from leaf tissues. More, two species each of genus *Helminthosporium, Rhizopus*, *Colletotrichum* and *Bipolaris* were present in both explants. Other species of genus *Aureobasidium pullulans* and *Pleurothecium* were isolated only from leaf tissues while *Cercospora, Fusarium oxysporum, Colletotrichum gloeosporioide* registered their presence only in stem parts. Four different *Penicillium* species were also screened from explants of *T. cordifolia.*

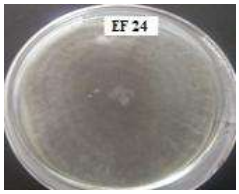
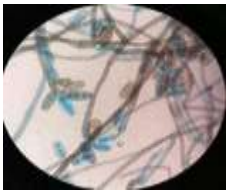
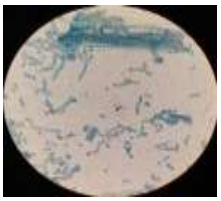
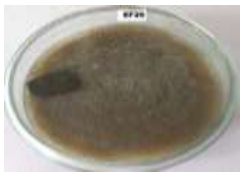
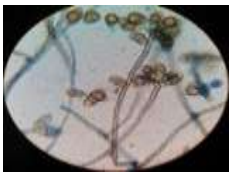
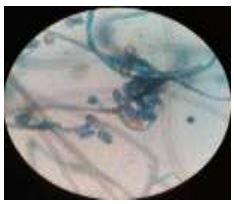
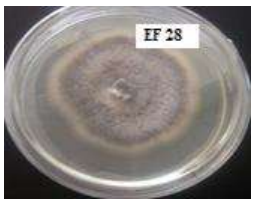
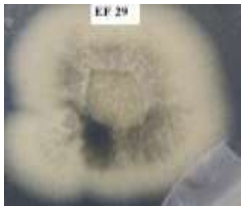
Table 1
Endophytic Fungi isolated from different parts (Leaf and Stem) of *Tinospora cordifolia* with their macroscopic and microscopic characteristics

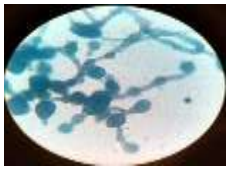
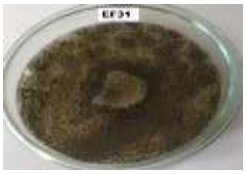
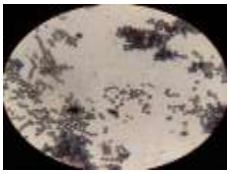
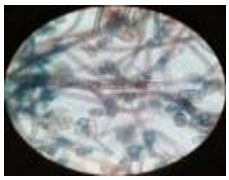
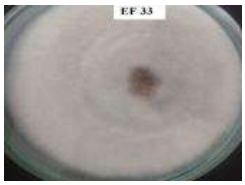
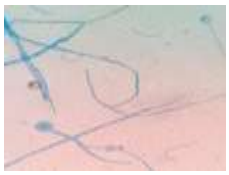
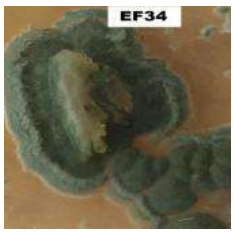
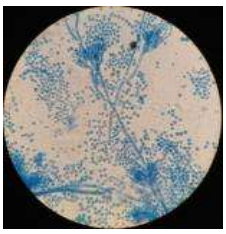
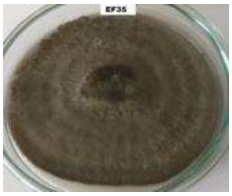
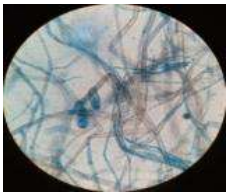
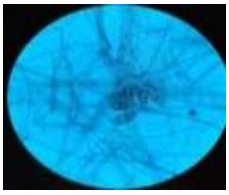
Isolates No.	Source of Explant	Endophytic Fungi	Colony Appearance	Microscopic View	Characteristics
EF1	Leaf	*Penicillium* spp.			The colonies were initially white & become green, visualized as globose shaped cells, fast growing, flat, filamentous, cottony in texture. Hyphae appeared septate and hyaline. Conidiophores were branched with round shaped conidia which were existed in chains. Phialides were presented at the end of conidiophores and grouped in brush like clusters.
EF 2	Leaf	*Cladosporium fulvum*			Colonies were olivaceous brown, powdery in texture due to abundant conidia production, slow growing. Conidiophores were erect straight and unbranched. Conidia were produced in branched acropetal chains, 1-4 cells, have distinct dark hilum. The youngest conidium was presented at the apical/ distal end of the chain.

EF 3	Leaf	*Curvularia eragrostidis*			Colonies were grey to black spreading cottony, velvety with or without zonation. Conidiophores were appeared dark, short/elongate; typically bearing a branched chain and short conidia was septate (three to four).
EF 4	Leaf	*Curvularia lunata*			Colonies were blackish brown. Conidiophores were erect, unbranched, septate with brown scars. Conidia were obvoidal to broadly clavate which were curved at the sub-terminal cell, 3-5 celled with central cell swollen and larger than the other cells.
EF 5	Leaf	*Bipolaris*			Colonies were blackish brown with a black reverse, fast growing. Pseudoseptate conidia were present on a geniculate rachis. Conidia were curved, obclavate, several celled and the germ tube was only on one end.
EF 6	Leaf	*Curvularia hawaiiensis*			Colonies were black and powdery. Conidiophores appeared erect, un-branched, septate, flat conidial scars were presented on the edges. Conidia were smooth, cylindrical, thick-walled and brown in color, with 3 distosepta.
EF 7	Leaf	*Aspergillus terreus*			Colonies were sand-brown in colour with yellow colored in reverse. Conidial heads were compact, columnar and also biseriate. Metulae were as long as the phialides. Conidiophore stipes was hyaline and smooth-walled. Conidia were globose in shape and smooth-walled.
EF 8	Leaf	*Aspergillus flavus*			Colonies were yellow at first but quickly becoming yellowish green with age, granular, flat, with radial grooves. Conidial heads were typically radiate, biseriate, having some heads with phialides which borne directly on the vesicles (uniseriate). Conidiophore stipes was hyaline and coarsely roughened and noticed near the vesicle. Conidia were globose and pale green in color.
EF 9	Leaf	*Cladosporium cladosporioide*			Colonies were slow growing, olivaceous-brown and powdery due to abundant conidia on the surface. Conidiophores were much shorter, acropleurogenous branches that bear numerous conidial chains arising below septa, with sympodial elongations. Conidia were smooth-walled, ellipsoidal, olivaceous-brown and one-celled.

EF 10	Leaf	*Helminthosporium* sp.			Colonies were grey. Mycelium was inter-cellular septate, light brown in color. Conidiophores were present single & also clustered, tall, erect and brown. Conidia were erect, septate and dark brown, born at the tip of conidiophores.
EF 11	Leaf	*Alternaria atra*			Colonies were black spreading velvety with zonation. Conidiophores were short, geniculate, sympodial proliferations. Conidia were obvoid, non-beaked with a narrow base and round apex with vertical and horizontal septa, protuberance from one end.
EF 12	Leaf	*Pleurothecium* sp.			Colonies were brownish black. Conidiophores were single/ in loose clusters, simple, dark, narrower and palerat apex, new growing points produce sympodially and producing new conidia. Sporogenous area recovered to produce a curved cyme; conidia present in moist heads were hyaline, typically 4-celled, ellipsoid/ slightly curved.
EF 13	Leaf	*Alternaria alternata*			Colonies were greyish black spreading cottony, velvety with zonation. Conidiophores were dark, elongate, typically bearing branched chain of conidia. Conidia were dark Obpyriform with 1-8 transverse and 0-3 oblique septa.
EF 14	Leaf	*Aspergillus niger*			Colonies consist of black conidial heads. Conidial heads were bi-seriate with phialides which borne on brown, septate metulae. Conidia were globose in shape, black and rough-walled.
EF 15	Leaf	*Alternaria* spp.			Colonies were grey to black spreading cottony, velvety with zonation. Conidiophores were dark, elongate, bearing a branched chain of conidia. Conidia appeared dark Obpyriform and septate.
EF 16	Leaf	*Penicillium* spp.			The colonies were initially white & become blue green, visualized as globose shaped cells, rapid grown, flat, filamentous, powdery in texture. Hyphae were septate and hyaline. Conidiophores were simple and branched. Phialides were grouped in a brush like clusters at the end of conidiophores. Conidia were round shaped and in chains.

EF 17	Leaf	*Auereobasidium pullulans*			Colonies were cream to brown, fast growing and smooth. Hyphae appeared hyaline and septate, forming chains of 1-2 celled, thick-walled, darkly pigmented arthroconidia. Conidia were hyaline, smooth-walled, single-celled, ellipsoidal and often with an indistinct hilum
EF 18	Leaf	*Aspergillus fumigatus*			Colonies were blue-green in color. Conidial heads were columnar and uniseriate. Conidiophore stipes was short, smooth-walled and has conical-shaped terminal vesicles. Conidia were produced in basipetal succession which formed long chains and were subglobose and green.
EF 19	Leaf	*Colletotrichum capsici*			Colonies were dark greyish & smooth circular. Margin of the colonies were irregular with fluffy mycelium. The *C. capsici*produced greyish white scattered falcate conidia with black acervuli and non-uniform shape of mycelium. All the isolates of *colletotrichum capsici* showed sporulation on PDA media after 10 days.
EF 20	Leaf	*Rhizopus oryzae*			Colonies were white coloured, mycelium was aerial, cottony and non-septate produced straight sporangiophores which terminated with black sporangium containing a columella, root like hyphae. Sporangiophores were present in clusters.
EF 21	Stem	*Alternaria alternata*			Colonies were greyish black, spreading cottony, velvety with zonation. Conidiophores were dark, elongate, beared a simple and branched chain of conidia which were dark Obpyriform with 1-8 transverse and 0-3 oblique septa.
EF 22	Stem	*Cercospora spp.*			Colony cottony, velvety with zonation. Conidiophores were olivaceous to brown, mildly curved, geniculate with 1 to 5 septa. Conidia were hyaline, obtuse at the apex, obconically truncate at the base, had 3 to 20 septa.
EF 23	Stem	*Colletotrichum gloeosporoide*			Colonies were white to grey, grew very quickly. Conidiophores simple and elongate, Conidia were Straight, cylindrical, apex obtuse, base truncate they showed profuse sporulation.

EF 24	Stem	*Helminthospor-ium nodulosum*			Colonies were greyish black. Mycelium is intra and inter-cellular septate, light brown in color. Mycelium often in substrate, stromata often present, conidiophores clustered, tall, erect and brown. Conidia were erect and septate and dark brown, born at the tip of conidiophores.
EF 25	Stem	*Penicillium cheresanum*			Colonies were initially white & become blue green, visualized as globose, rapid grown, flat, filamentous, wooly or cottony in texture. Hyphae septate, hyaline. Conidiophore had long chains of single-celled conidia which were round shaped and presented in chains.
EF 26	Stem	*Curvularia lunata*			Colonies were blackish brown. Conidiophores were erect, unbranched, septate with dark brown scars. Conidia were smooth walled, obovoidal, curved at the sub-terminal cells, 3-5 celled with central cell swollen and larger than the other remaining cells.
EF 27	Stem	*Bipolaris*			Colonies were fast growing, blackish brown with a black reverse. Miroscopic morphology showed sympodial development of hyaline, pseudoseptate conidia were present on geniculate rachis. Conidia appeared curved, obclavate, rarely straight 2-14 pseudoseptate, germinating only from the ends (bipolar).
EF 28	Stem	*Colletotrichum capsici*			*Colletotrichum capsici* formed smooth circular and grey to dark greyish colonies. Margin of the colonies were irregular with fluffy mycelium. The *C. capsici*produced greyish white scattered falcate conidia with black acervuli and non-uniform shape of mycelium.
EF 29	Stem	*Fusarium oxysporum*			Colonies were wooly, white changing color to yellowish. Mycelium extensive and cottony with yellowish tinge inside mycelium; conidiophores were slender shaped, simple, branched & bearing phialides, grouped into sporodochia. Conidia appeared crescent- shaped, attached to conidiophores which were arising from a septate mycelium.

EF 30	Stem	*Alternaria* spp.			Grey to Black colonies. Colonies were spreading cottony, velvety with or without zonation. Conidiophores were dark, short, bearing a branched chain of conidia. Conidia were dark Obpyriform with 1-8 transverse and 0-3 oblique septa.
EF 31	Stem	*Aspergillus niger*			Colonies were black. Conidiophores upright, simple, arise from a septate mycelium and terminating in a globose swelling, bearing phialides at the apex. Conidia were present in chains developed at the end of sterigma.
EF 32	Stem	*Curvularia* spp.			Colonies were brown colored with reverse black and fast growing. Conidiophores short, erect, straight and septate. Conidia were ellipsoidal and curved, rounded at the ends, 2-3septa.
EF 33	Stem	*Rhizopus oryzae*			Colonies were white in color, mycelium appeared aerial, cottony and non-septate produced straight sporangiophores which terminated with black sporangium containing a columella, root like hyphae. Sporangiophores were present in clusters.
EF 34	Stem	*Penicillium verrucosum*			The colonies were flat, filamentous and cottony in texture, initially white become blue green, visualized as globose sausage shaped cells. Hyphae septate and hyaline. Conidiophores of *P. verrucosum* showed two-stage branching. Phialides were grouped in brush like clusters at the end of conidiophores. Conidia appeared round shaped and in chains.
EF 35	Stem	*Curvularia eragrostidis*			Colonies were grey to black, spreading cottony, velvety with zonation. Conidiophores were dark, short and elongate, typically bearing simple, short conidia, three to four septate.
EF 36	Stem	*Curvularia geniculata*			Colonies were cottony, fast growing, brown colored. Conidiophores were erect and septate. Conidia were curved, rounded at the ends, pale brown, short, 3-4 septa. Central cell was large and swollen than others.

Discussion

Various bioactive metabolites valuable in agriculture, pharmaceutical and industries have been produced by fungal endophytes. It is estimated that 70,000 to 80,000 fungal species exist on the planet[22]. A total number of 224 fungal isolates were isolated from diverse tissues of *Dysosma versipellis* by Tan et al[23] in 2018 who further classified and characterized by culture characteristics and also by sequence analyses of the internal transcribed spacer (ITS) region of the rRNA gene.

Different sp. of these fungal endophytes could be exploited as natural antimicrobial or anticancer agents. 28 fungal endophytes belonging to 11 different genera of Ascomycota phylum has been identified by Katoch et al[24] from *Monarda citriodora L*. Endophytic fungi belong to the phylum Ascomycota, Basidiomycota, Zygomycota and Oomycota which are an imperative source for novel and active metabolites which are helpful for the host plants for defending against exterior biotic and abiotic stress[25].

Considering the endophytic fungal isolates in our study, the fungal community of *Tinospora cordifolia* plant comprised of *Alternaria, Curvularia, Cladosporium, Penicillium, Aspergillus, Fusarium, Colletotrichum, Rhizopus, Helminthosporium* and *Pleurothecium*. In previous study, it was observed that some endophytic sp. were restricted to specific plant tissue. Tissue type has a protuberant consequence on species diversity and colonization frequency of endophytic fungal community. Our finding suggests that leaf tissue displayed higher presence of fungal sp. than its counterpart i.e. stems part.

Out of 36 fungal isolates, 20 different species types of fungal endophytes were identified from leaf tissue and 16 from stem portion. Seven different species of *Alternaria* were screened from both leaf and stem tissue. Four different species of *Aspergillus* were also identified; among these *Aspergillus niger* was present in both explants but three species (*Aspergillus flavus, Aspergillus terreus* and *Aspergillus fumigatus*) were found only in leaf tissues. The *Pleurothecium* and *Aureobasidium pullulans* were also present only in leaves and absent in stem portions. During the study of isolation of fungal endophytes from leaf and stem tissue of *T. cordifolia*, it was found that our results were in accordance with some previous study related to the isolation of fungal endophytes from various medicinal plants.

Colonization frequency was higher inside leaf tissue due to highest canopy, greater surface area and less antimicrobial activity than other plant parts[11,26-28]. In addition, De Siqueira et al[29] studied the species composition of fungal endophytes from *Lippia sidoides* and observed that colonization of leaves (50.41%) was superior to stem tissue (35.40%). Gond et al[30] also observed the higher species richness of fungal endophytes in leaves than in stems during their study on medicinal plants of India.

The season for isolation of fungal endophytes also affected their distribution. The colonization frequency was highest during rainy season[11,13]. Tejesvi et al[31] found only five species in winter season and 19 species during monsoon season in the bark portion of *Terminalia arjuna.* In our study, we observed that more number of fungal species were isolated in summer season than winter. Fungal species i.e. *Alternaria, Colletotrichum, Aspergillus, Helminthosporium, Rhizopus, Fusarium, Penicillium* and *Bipolaris* were isolated during summer season (March 2017 to August 2017) and these species were dominated endophytes in both leaves and stems in summer season in *Tinospora cordifolia.* The species such as *Cladosporium, Curvularia* and *Pleurothecium* were isolated in winter season (November 2017 to February 2018).

We also found the difference in endophytes colonization among various locations from which sample explants were taken. In a study by Mishra et al,[28] different endophytes i.e. *F. oxysporum, C. dematium, C. linicola, A. flavus, A. niger, C. cladosporioides, Humicola sp., Nigrospora oryzae, Penicillium sp., T. viride sp., A. sydowii* and *P. violaceum* sp. were identified from leaf tissue of *T. cordifolia* in summer season from Ramanagar, Banaras Hindu University, Maruadih. In our study, the plant material which was screened for endophytic microbes was collected from Kurukshetra is a religious city of north Indian state of Haryana. In our report, the geographical conditions were different from the Ramanagar, Banaras Hindu University, Maruadih. Environmental factors such as temperature, humidity, rainfall and soil profile of that particular location may affect endophytes presence and distribution and also have a determinant role in spread of fungal endophytic spores[32]. The region has sub-tropical continental climate. The Kurukshetra district lies between 29º- 34’15” and 30º-15’15” north latitude and 76º to 10” and 77º-17.5” east longitude. Environmental conditions of this location also affect the distribution of endophytes. The rainfall distribution annually is comparatively satisfactory i.e. 290.5 mm. Saraswati, Markanda and Ghaggar are the central rivers of this region and the soil is commonly alluvial loam and clay.

It is one of the affluent districts from agricultural point of view. So, the conditions prevail which favor prosperous flora and in turn affect the distribution of fungal endophytes. The dominating isolates of *T. cordifolia* during different seasons of this geographical location were from genera *Alternaria, Penicillium, Rhizopus, Fusarium, Helminthosporium, Bipolaris, Aspergillus* (summer season), *Cladosporium, Curvularia* and *Pleurothecium* (winter season).

The flora of this region is not well characterized and documented. Moreover, endophytic microbes are the underexplored group of microorganisms for bioactive metabolites. There is no comprehensive work done for the isolation of endophytic fungi from the *Tinospora cordifolia* plant of Kurukshetra district. Moreover, *T. cordifolia* is a

vine plant. The study by Mishra et al[28] for isolation of fungal endophytes from *T. cordifolia* used mango trees (*Mangifera indica*) as a support but neem (*Azadirachta indica*) plant was used as the supporting plant in our study. The supporting plants like neem, mango may also affect the distribution of endophytes in different plant tissues in different seasons and geographical locations. *T. cordifolia* is highly genetically diverse plant and the supporting plant (Neem) enhances the therapeutic efficacy of the main plant by causing variations in their metabolic profile.

Our finding indicated that different tissues of plant in different environmental conditions and geographical locations harbor distinct endophytic fungal communities which could be promising source for unique metabolites of biological activities. Although less work has been done for the isolation of fungal endophytes from valuable medicinal plants, therefore, more research is required for identification of the significance of these endophytes in agriculture and medicinal areas.

Conclusion

Endophytic fungi are a group of microorganisms which have the potential to synthesize same or different metabolites of the plants in which they reside. In the present study, 36 different endophytic fungi in *Tinospora cordifolia* have been screened from its leaves and stems explants. These fungal species can be explored further for their bio-constituents production capabilities being economical, more convenient and easy to scale up microbes based processes.

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