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Biodiversity of phylloplane and endophytic fungi studied on the medicinal plant; *Tinospora cordifolia*

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Abstract : Phylloplane and endophytic fungi have been proven to be a rich source of novel natural compounds with a spectrum of biological activities and a high level of structure diversity on the leaf surfaces. Bioactive natural components produced by phylloplane and endophytes have shown promising potential and use fullness in safety and human health concerns. Taking advantage of modern biotechnology and microbial fermentation process we can better understand and manipulate this important microorganism resource and make it more beneficial for the mankind. In the present study isolation and enumeration of phylloplane and endophytic fungal diversity and host relationship based on two methodologies, agar plate and moist chamber was carried out. Altogether, 16 fungal species were isolated from the plant *Tinospora cordifolia. Alternaria alternata, Aspergillus niger, Cladosporium herbarum, Penicillium chrysogenum* and white sterile were the dominant fungal taxa among the recorded species. 9 fungal species were isolated from the medicinal plant by agar plate method and 12 species were recorded by moist chamber method. *Aspergillus niger* was recorded as endophytes and phylloplane fungi like *Alternaria alternata*, *Cladosporium herbarum,* white sterile mycelia and *Penicillium chrysogenum*. *Penicillium citrinum, Fusarium* sp., *Bispora* sp., *Curvularia lunata* and *Eremascus albus* were isolated only as endophytes but not present on leaf surfaces. *Populospora* sp. and *Trichoderma harzianum* were isolated as phylloplane fungi but not recorded as endophytes. The evidence for host-preference, tissue-specification and spatial heterogeneity was found in the phylloplane and endophytes distribution based on fungal community and composition and thus the fungi isolated are dependent on our methodologies.
Key words: Biodiversity, Phylloplane, Endophytes, Medicinal plant, *Tinospora cordifolia.*

Introduction

The leaf surface is the important substrate for the growth of microorganisms as it easily provides essential nutrients required for their life and growth. There are two groups of fungi remain attached with the leaf surfaces and sub surfaces, living as residents as well as casuals i.e., Phylloplane and endophytes[1]. Residents can multiply on the surface of healthy leaves without noticeably affecting the host whereas casuals land on the leaf surface but cannot grow[2]. Advantages of a biological approach to disease control include reduced environmental damage, reduced human health risk and improved soil conditions and agricultural sustainability[3]. Phylloplane fungi have been poorly studied as compared to endophytes. Endophytes are microbes that are present in living tissue of various plants parts like, Root, fruit, stem, seed, leaf etc. Almost all vascular plants are known to harbor endophytes. These endophytes protect their hosts from infectious agents and adverse conditions by secreting bioactive secondary metabolites[4,5,6]. The present study is an attempt to isolate and

identify the phylloplane and endophytic fungi associated with the leaf surfaces of the medicinal plant, *Tinospora cordifolia*.

Materials and Methods

Sample collection

Fresh leaves of *Tinospora cordifolia* were collected from in and around of our P.G. centre, KMCPGS (Autonomous,) Lawspet. Healthy and mature leaf samples were carefully collected and brought to the Microbiology laboratory, Department of Botany, KMCPGS, Pondicherry.

Description of the Plant

Botanical name : *Tinospora cordifolia* (Willd.) Miers
Family name : Menispermaceae(moonseeds)
Common names: Tinospora (English), geloy (Hindi), Shindilakodi (Tamil), Tippa-Teega (Telugu), Amruthu, Chittamruthu (Malayalam), Amritavalli (Sanskrit), Guduchi (Marathi).

T. cordifolia is a glabrous, succulent, climbing shrub native to India and also found in Burma and Sri Lanka. It thrives easily in tropical regions, often growing to great heights and climbing the trunks of large neem plants. The bark is gray or creamy white, deeply cleft spirally and longitudinally, with large rosette-like lenticels. The wood is white, soft, porous and when freshly cut, quickly assumes a yellow tint. The branches bear smooth, heart-shaped leaves, unisexual greenish flowers in summer and red berries in winter. Long thread-like aerial roots arise from the branches. The viscous sap is light yellow, with an odor and a nauseating bitter taste.

Surface sterilization of leaves

In order to isolate the endophytic fungi, the collected healthy leaves were thoroughly washed in running tap water. Then the leaves were cut into small segments (about $1cm^2$) including midrib portion. The leaf samples were surface sterilized by 0.1 % mercuric chloride for 60 seconds and then rinsed in sterile distilled water for 10 seconds (three times). For phylloplane mycoflora study, the leaf segments were not surface sterilized since phylloplane fungi grown on the surface of the leaves. Without washing the segments, they were placed on the PDA and moist chamber plates equidistantly.

Culture of leaf samples on agar plates

Five (5) leaf segments of a centimeter square, both sterile an unsterile were placed separately on the PDA media plates equidistantly by the help of sterile forceps and pressed later on followed by incubation for 3 to 7 days.

Culture of leaf sample on moist chamber

Same like agar plates, five (5) leaf segments of centimeter square, both sterile an unsterile were placed separately on the moist chamber petriplates equidistantly by the help of sterile forceps and pressed later on followed by incubation for 7 to 21 days. The fungi on moist chamber were enumerated later on based on their growth on the leaf segments.

Isolation of fungi

After sterilization, the excess water was blotted out by sterile filter paper from the leaf segments and kept separately. Then the surface sterilized segments were placed in a petridishes containing PDA supplemented with Amoxillin as well as in moist chamber. The moist chamber plates don't need any type of medium for the growth of endophytic as well as Phylloplane fungi. In this method, the fungi grow on its own on the host, getting the moister produced from the wet condition prevailing inside the petriplates. All the plates were incubated at 25±3ºC temperature in the incubation chamber. Incubation time was maintained differently since,

7-8 days is meantt for the fungal growth of fungi in agar plate method, but in moist chamber method, 1 to 3 weeks are required for the growth of fungi. Every day watch of the petriplates and check the growth of fungi was almost necessary in our present study after 3rd day of incubation.

Identification of fungi

After three days of incubation, the fungal colonies were counted for individual species and the total number was enumerated. Microscopic slides stained with lacto phenol cotton blue were prepared from each colony of the fungus and observed microscopically under the trinacular digital photography microscope to identify up to species level. The colony which was not be identified directly from plates was sub cultured in SDA/PDA media again and identified later on. The laboratory experience and taxonomic literature were employed to identify the fungal CFUs up to species level [7,8,9,10,11]. The presence and absence based on the occurrence of individual fungus in the phylloplane and endophytic were determined and plotted in the form of tables and figures.

Results

In the present study, totally 16 fungal species were isolated and identified from the healthy leaf of the medicinal plant *Tinospora cordifolia* by Agar plate and Moist chamber methods. This plant was screened for the presence of Phylloplane and endophytic fungi. 4 Species belonging to 3 genera of Phylloplane and 7 species belonging to 7 genera of endophytic fungi were isolated and identified in Agar plate. In Moist chamber method, 7 species belonging to 6 genera of Phylloplane and 9 species belonging to 8 genera of endophytic fungi were isolated and identified. Total number of isolated phylloplane and endophytic fungi from the leaf samples of *T. cordifolia* is given in Fig 1.

In Agar plate, *Aspergillus flavus, Aspergillus niger, Penicillium chrysogenum, Trichoderma harzianum* species were identified as Phylloplane and *Alternaria Alternata, Aspergillus niger, Cladosporium herbarum, Colletotrichum* sp., *Papulaspora* sp., *Penicillium chrysogenum, Trichoderma harzianum* species were identified as endophytes.

In Moist chamber, *Alternaria Alternata, Aspergillus niger, Aspergillus* sp., *Cladosporium herbarum, Curvularia lunata, Penicillium citrinum, Stachybotrys atra* species were identified as Phylloplane fungi and *Alternaria Alternata, Aspergillus niger, Bispora* sp., *Cladosporium herbarum, Eremascus albus, Penicillium Chrysogenum, Penicillium citrinum, Fusarium* sp., *Stachybotrys atra* species were identified as endophytes. White sterile mycelia were predominantly found in both Agar plate and Moist chamber methods.

Total number of Phylloplane and endophytic fungi were recorded from the leaves of *T. cordifolia* by two different isolating methods are given in Table 1. The Phylloplane fungi like *Aspergillus niger* and white sterile were identified commonly in both Agar plate and Moist chamber methods. Endophytic fungal species like *Alternaria Alternata, Aspergillus niger, Cladosporium herbarum, Penicillium chrysogenum* were commonly identified in both Agar plate and Moist chamber methods.

Table 1: Occurrence of Phylloplane and Endophytic fungi recorded from medicinal plant; *Tinospora cordifolia* by two different methods.

Sl. No	Name of the fungi	Agar Plate		Moist chamber	
		Phylloplane fungi	Endophytic fungi	Phylloplane fungi	Endophytic fungi
1	*Alternaria Alternata*	-	+	+	+
2	*Aspergillus flavus*	+	-	-	-
3	*Aspergillus niger*	+	+	+	+
4	*Aspergillus* sp.	-	-	+	-
5	*Bispora* sp.	-	-	-	+
6	*Cladosporium herbarum*	-	+	+	+
7	*Colletotrichum* sp.	-	+	-	-
8	*Curvularia lunata*	-	-	+	-

9	*Eremascus albus*	-	-	-	+
10	*Papulaspora* sp.	-	+	-	-
11	*Penicillium chrysogenum*	+	+	-	+
12	*Penicillium citrinum*	-	-	+	+
13	*Fusarium* sp.	-	-	-	+
14	*Stachybotrys atra*	-	-	+	+
15	*Trichoderma harzianum*	+	+	-	-
16	White sterile mycelia	+	-	+	+

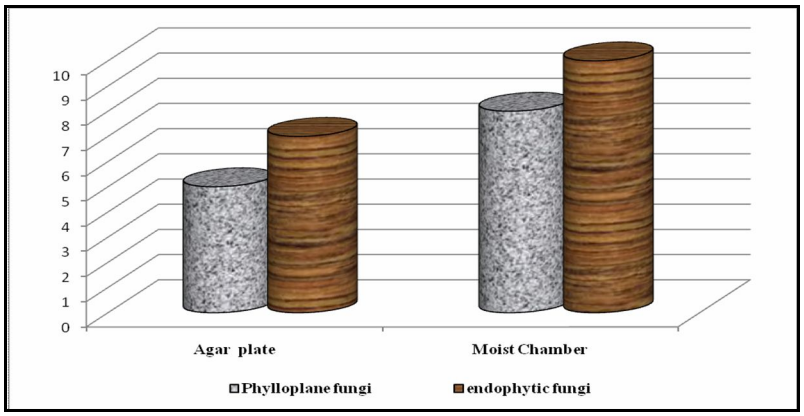

Fig 1: Total number of isolated phylloplane and endophytic fungi from the mature leaf of *Tinospora cordifolia*

Discussion

Fungi have been widely known as a source of bioactive compounds. An excellent example for this is the anticancer drug taxol, which was previously occurred only in the plant tissue[12,13,14]. Most of the isolated fungi belonged to anamorphic fungi belonging to Hyphomycetes, Ascomycetes and Zygomycetes. In our study, the isolated and identified fungi mostly belonged to the Hyphomycetes[12]. The colonization of the Phylloplane and endophytic fungi has received considerable attention as they are found to protect their host against pest, pathogens and even domestic herbivorous animals. This ubiquitous endophytes and Phylloplane fungi may lead to the production of special compound within the host plant[12]. In the present study, selection of the isolates of Endophytic and Phylloplane fungi were based on the maximum number of fungal species have been widely known as a source of bioactive components from the mature leaf sample of *T. cordifolia.* This plant has a broad spectrum of medicinal properties. The following data suggested that the smaller and the more scattered plant fragment sample was induced for probability of approaching real diversity values of Phylloplane and Endophytic fungal community. Isolation and identification of only 16 taxa of Phylloplane and Endophytic fungi showed that the medicinal properties of the plant has some roll to play in the colonization of fungi. This slow rate of colonization may be attributed to the secretion of the phyto-chemicals, since; they contain certain antifungal and antibacterial components. *Alternaria alternata*, *Aspergillus flavus*, *Aspergillus niger, Trichoderma* sp., *Cladosporium* sp., *Curvularia* sp. and white sterile mycelia are Phylloplane fungi and *Alternaria alternata, Aspergillus niger, Cladosporium* sp., *Trichoderma* sp., *Colletotrichum* sp., are Endophytic fungi were isolated from the plant, *T. cordifolia* is agreed with the previous workers who had also reported the endophytic fungi in their study [15, 16, 17]. These common endophytes and Phylloplane were isolated frequently from the leaves of medicinal plant. Compared to Phylloplane fungi, more number of endophytic fungi were isolated and identified more predominantly both agar plate and moist chamber. Moist chamber method was found suitable to isolate and record the host specific as well as systemic Phylloplane and endophytic fungi correctly compared to agar plate method[16].

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