



Indian Journal of Applied Microbiology

ISSN (Online): 2454-289X, ISSN (Print): 2249-8400

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Volume 21 Number 1
April - June 2018, pp. 58-68

Isolation and Identification of Endophytic Fungi from *Melia azedarach L.* of Kalvarayan Hills, Eastern Ghats, Tamil Nadu, India

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Abstract: To investigate the Endophytic fungi that are occupied asymptotically in domestic tissues of all higher plants are of growing interest as promising sources of biologically active agents. The exploitation of novel and eco-friendly secondary metabolites used in medicine, the pharmaceutical industries and agriculture. In this research we select the *Melia azedarach L.* medicinal plant (exotic tree from Kalvarayan hills) to isolate and identify the endophytic fungi from living apparently symptomless flower, stems, leaves and fruits of medicinal plant will grow very well and is apparently resistant to many pathogenic microorganisms like bacteria, fungi and parasitic infection and it has insecticidal compound. Each segment from the plant of leaves, flowers, fruits and stem each 15 segment of plant parts were be sampled and, total of 47 fungi were isolated. The isolates of endophytic fungi from this plant parts including *Cylindrocarpon lichenicola* followed by, *Chaetomium spp.*, *Curvularia fallax*, *Fusarium solani*, *Alternaria alternata*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus nidulans*. The study the shows the hyphomycetes were more than dematiaceous and mycelia sterilia. The genera of *Chaetomium lichenicola* and *Aspergillus* were the most common in that plant.

Key words: Medicinal plant, *Melia azedarach L.*, Endophytic fungi, Medicinal properties, *Aspergillus sp.*

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1. Introduction

Endophytes are commonly refer to a set of fungi that survive asymptotically within the living plant tissues for entire or part of their whole life cycle with no causing any visible symptoms of their presence (1). They are present in host plant bury and intra cellular and produce certain bioactive natural products or drugs and derivatives; without causing any damage or disease to their hosts. These plants serve as a reservoir for a large numbers of microorganisms known as endophytes (2). Endophytic fungi have been reported from various plant species, which contribute to diversity of microorganisms in natural environments and produce various bioactive compounds that play a major role in inherent surroundings (3). Within the hosts, these fungi inhabit all the tissue including leaves, stems, twigs, bark, root, fruit, flower and seeds. Endophytic fungi have been recognized as sources for new secondary metabolites with useful biological activity. These represent a virtually untapped source of chemical reservoir that finds use in agriculture and therapeutics (4). The various natural products produced by these endophytic fungi have a unique structures and great bioactivities, representing a huge reservoir which offers an enormous potential for exploitation for agricultural, industrial uses and medicinal properties (5). The endophytic fungi were screened for a new compound have high amount of antioxidant, anticancer, antimicrobial properties. To discover the valuable compound like taxol producing fungi improved the importance of endophytes and shifted natural products research to endophytic fungi (6, 7).

Melia azedarach L. (Meliaceae) is the best known species known as chinaberry or pride of India. This is an exotic tree from Kalvarayan hills possessing high insecticidal compounds. This plant is a good producer of limuloids which are terpenoidic natural substances with enormous insecticidal activities. *Melia azedarach* L, commonly known as Indian lilac has been used to fighting many diseases like rheumatism, leprosy, rashes, etc. The leaves of the *Melia azedarach* L used in treatment of diuretic, deobstruent, leprosy, scrofula, anthelmintic and resolvent (8). 26.37% of endophytic fungi were active against one or more pathogens in antimicrobial activity (9). In this present study an attempt has been made to isolate and identify the endophytic fungi from *Melia azedarach* L. from the tropical evergreen forest ecosystem of Kalvarayan hills located in the Eastern Ghats.

2. Materials and methods

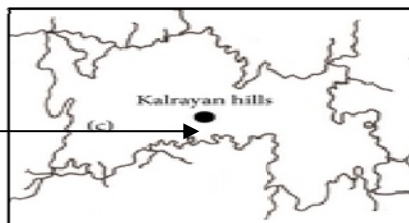
2.1 Collection of Plant Materials

The plant *Melia azedarach* L. were collected from the tropical evergreen forest ecosystem of Kalvarayan hills (Latitude: 11°46'41.21"and Longitude: 78°49'49.88), Tamil Nadu, India (Fig: 2.1.1). The plant materials were taxonomically recognized and voucher specimen was deposited there with register number AUT/PUS/082 at the ABS Botanical Conservation, Research & Training centre, Kaaripatti, Salem, Tamilnadu. Mature healthy plants of *Melia azedarach* L. devoid of any signs and symptoms of disease were collected by random sampling from different regions of the tropical evergreen forest (Fig: 2.1.2). The collected plant materials were stored in sterile polythene bags in ice box and transported to the laboratory for isolation.

Fig: 2.1.1 Sampling site of Eastern Ghats,
Tamil Nadu



Fig: 2.1.2 Plant located in the tropical
evergreen Forest of Kalvarayan hill



The plant material was rinsed gently in running water to remove the dust and debris. Leaves were cut into 3-4 mm in diameter and 0.5-1.0 cm in length with and without midrib. The surface sterilization was done by sodium hypochlorite (NaOCl) and 75% ethanol. The time of treatment and concentration of sodium hypo chloride was changed according to the type of plant tissue. The concentration for NaOCl used was 1-13% and time of sterilization was 3-10 minutes. Each set of plant material was treated with 75% ethanol for 30 seconds. Later the segments were rinsed three times in sterile distilled water. The plant pieces were blotted onto sterile blotting paper. The efficiency of surface sterilization procedure was ascertained for every segment of tissue following the imprint methods.

In each Petri plates 5-6 segments of each plant tissue (leaf, stem, flowers and fruit) was placed on to Potato Dextrose Agar (PDA) supplemented with Chloramphenicol 100 units/ml. The plates were incubated at 28- 30°C monitored every day for the growth of endophytic fungal colonies. The endophytic fungi was identified according to their macroscopic (front and reverse side of fungal colonies) and microscopic characteristics such as the morphology of fruiting structures and spore morphology by employing standard isolation methods. The identified fungi were maintained on Potato Dextrose Agar (PDA) slants for further study. The fungal isolates that failed to produce spores even after 3-4 weeks of incubation were referred to as mycelia sterilia.

2.2 Statistical Analysis

The colonization frequency for the endophytic species in the leaf, stem, flowers and fruit tissue was calculated by using the following formula (10).

2.3 Colonization Frequency (CF %)

$$CF\% = \frac{\text{Number of segments colonized by an endophytic fungal species}}{\text{Total number of segment}} \times 100$$

3. Results

About 72 segments (12 segments of each part respectively) of the medicinal plant were screened for the isolation of the endophytic fungi. A total of 49 endophytic fungi was isolated and

identified from medicinal plant of *Melia azedarach* L. from different parts of stem, leaves, flowers and fruit of the medicinal plant. The leaf segments showed a maximum repository for endophytic fungi than the other segments. Tables 3.1 and 3.2 showed the CF value and taxonomic position of endophytic fungi.

The predominant endophytic fungi isolated belonged to the genera of *Cylindrocarpon lichenicola*, *Curvularia* sp, *Alternaria alternata*, *Fusarium solani*, *Chaetomium* sp, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus nidulans* and *Aspergillus fumigatus*. Some fungi which did not produce any reproductive structure, as they produced sterile mycelia and in some cases sterile pycnidium were also grouped under mycelia sterilia. These fungi did not sporulate in spite of repeated subculturing on to sporulating media (Potato dextrose agar and, Tap water agar) and hence are grouped on *Mycelia sterilia*.

Table: 3.1. Endophytic fungal genera and their colonization frequency (CF %)

S. No	Plant parts	No of Segments	No of Fungi Isolated	Colonizing Frequency (CF %)
1	Leaf	18	16	32.5
2	Flower	18	15	30.4
3	Fruit	18	10	20.2
4	Stem	18	08	16.1

Among the 49 endophytic fungi, the predominant endophytic fungi isolated in this study belonged to the genera of *Cylindrocarpon lichenicola*, *Chaetomium* sp, *Curvularia fallax*, *Fusarium solani*, *Alternaria alternata*, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus* and *Aspergillus nidulans* were mentioned in the Fig 3.3 and Table 3.2.

Fig: 3.3 Endophytes Isolated in relation to Fungal Groups

Cylindrocarpon lichenicola

(a) Colony appearance



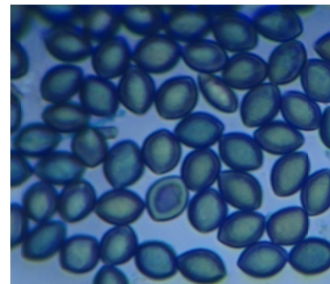
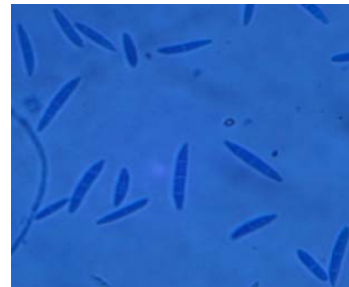
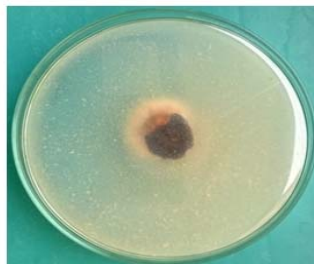
Front



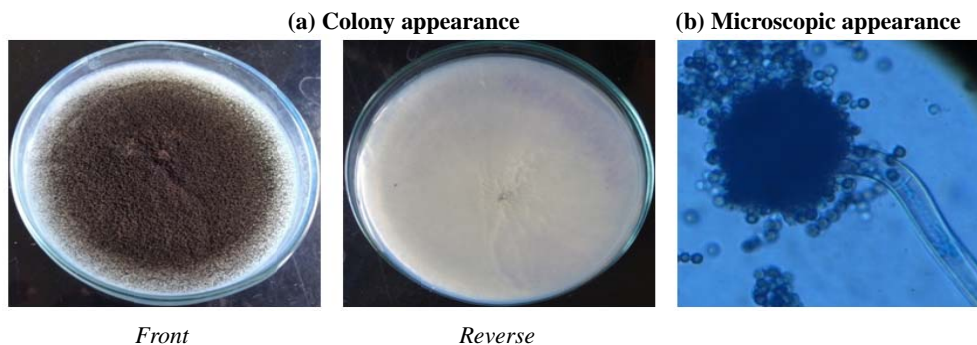
Reverse

(b) Microscopic appearance



2. *Chaetomium sp.***(a) Colony appearance***Front**Reverse***(b) Microscopic appearance****3. *Fusarium solani*****(a) Colony appearance***Front**Reverse***(b) Microscopic appearance****4. *Curvularia fallax*****(a) Colony appearance***Front**Reverse***(b) Microscopic appearance**

5. *Aspergillus niger*



6. *Mycelia sterilia*

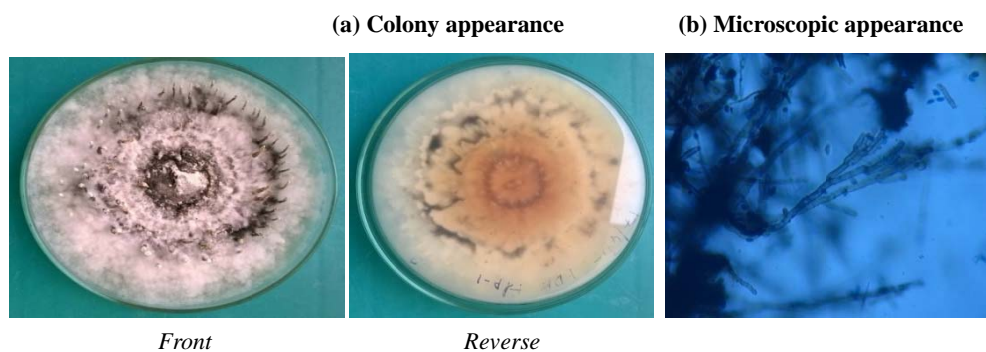


Table: 3.2. Endophytes Isolated in relation to Fungal Groups

Sl. No	Isolated Endophytes	Fungal Class	Description
1	<i>Cylindrocarpon lichenicola</i>	Hyaline Hyphomycetes	Colonies are fast growing, bright-coloured, suede-like or woolly. Arranged in brush-like structures. Phialides are cylindrical to subulate, producing hyaline, smooth-walled conidia, arranged in slimy masses.
2	<i>Chaetomium</i> sp.	Hyaline Hyphomycetes	Colonies: darkly-pigmented terminal hairs, globose, ovoid, barrel to flask-shaped, ostiolate ascocarps (perithecia). Ellipsoidal or lemon-shaped.

3	<i>Curvularia fallax</i>	Dematiaceous Hyphomycetes	Colonies are suede-like to downy, brown to blackish brown with a black reverse. Conidia are pale brown, cylindrical or slightly curved, the central cells being larger and darker. Germination is bipolar and some species may have a prominent hilum.
4	<i>Fusarium solani</i>	Hyaline Hyphomycetes	Colonies are pale or brightly coloured (depending on the species) and aerial mycelium may present. The colour of the thallus varies from whitish to yellow, brownish, pink, reddish or lilac shades. Chlamydoconidia may be present or absent.
5	<i>Alternaria alternata</i>	Dematiaceous Hyphomycetes	Colonies: black to olivaceous-black, reverse: dark brown, Conidiophores: branched, short, Conidia: multicellular conidia, obclavate, pale brown, smooth-walled.
6	<i>Aspergillus niger</i>	Hyaline Hyphomycetes	Colonies consist of a compact white or yellow basal of dark-brown to black conidial heads. Conidial heads are large, Conidia are globose to subglobose, dark brown to black and rough-walled.
7	<i>Aspergillus flavus</i>	Hyaline Hyphomycetes	Colonies are granular, flat, often with radial grooves, yellow at first but quickly becoming bright to dark yellow-green with age. Conidial heads are typically radiate. Some strains produce brownish sclerotia.
8	<i>Aspergillus fumigatus</i>	Hyaline Hyphomycetes	Colonies show typical blue-green surface, dense felt of conidiophores. Conidial heads are typically columnar and uniseriate. Conidia are produced in basipetal succession forming long chains and are globose to subglobose, green and rough-walled.
9	<i>Aspergillus nidulans</i>	Hyaline Hyphomycetes	Colonies: plain green in color with dark red-brown cleistothecia, reverse: olive to purple-brown, Conidiophores: short, brownish and smooth walled, Conidia: globose and rough-walled.
10	Sterile Forms	Mycelia sterilia	Many fungi do not produce any recognizable sexual/asexual conidia state in culture. Such forms are frequently classified for convenience in the Mycelia sterilia.

The fungi which did not produce any reproductive structure were grouped under *Mycelia sterilia*. In this study majority of the fungi belonged to hyaline Hyphomycetes. The colonization frequency was few with fruit, leaves, flower and stem respectively 32.5%, 30.4%, 20.2% and 16.1% as mentioned in the pie diagram.

Further study of endophytic ecology in natural system promises to elucidate both potential applications of endophytic fungi for human use and ecological roles of these ubiquitous associates of healthy plant tissues. Since, medicinal plants were known to have pharmaceutical properties; it was assumed that associated endophytes could also be able to produce similar substances. The rationale of the proposed work was that after successful completion, identification of novel bioactive compounds could lead to the development of novel pharmaceutical agents against human diseases

4.1. Discussion

Endophytes have proven to be a rich source of novel natural compounds with a wide-spectrum of biological activities and a high level of structural diversity. Bioactive natural compounds produced by endophytes have shown promising potential and usefulness in safety and human health concerns. Taking advantage of modern biotechnology such as genetic engineering, metabolic technology and microbial fermentation process, we can better understand and manipulate this important microorganism resource, and make it more beneficial for the mankind. Discovering new chemical compounds from natural products is very important for formulating new drugs (11).

There are 2,50,000 higher plant species on earth, around 5000 species have specific therapeutic value. The medicinal plants used in Ayurveda can provide biologically active molecules and lead structures for the development of modified derivatives with enhanced activity and reduced toxicity (12). Medicinal plants are regarded as potentially safe drugs, therefore the use of Ayurveda medicines has increased now days (13). The most important of these bioactive compounds, the principles are alkaloids, phenolic, flavanoids and tannins compounds that may be evolved in plants as self defense against pests and pathogens (14). The bioactive metabolites produced by endophytic fungi originate from different biosynthetic pathways and belong to diverse structural groups. Endophytes therefore, represent a chemical reservoir for new compounds such as, anti-cancer, immune-modulatory, anti-oxidant, anti-parasitic, anti-viral, anti-tubercular, insecticidal, anti-microbial etc., for use in the pharmaceutical and agrochemical industries (11).

The study also showed such a trend of diversity of endophytic fungi which was apparent with the leaves, stem, flowers and fruit part of medicinal plant *Melia azedarach* collected from Kalvarayan hills. Out of 72 segments plated each 18 from stem, leaves, flowers and fruit of plant, 49 different endophytic fungal isolates were obtained.

The endophytic fungi species isolated were belong to the genera of *Cylindrocarpon lichenicola*, *Chaetomium sp.*, *Curvularia fallax*, *Fusarium solani*, *Alternaria alternata*, *Aspergillus niger*,

Aspergillus flavus, *Aspergillus fumigatus* and *Aspergillus nidulans* Many fungi did not produce any reproductive structure, as they produced sterile mycelia and in some case sterile pycnidium were groups under Mycelia sterilia. Out of 72 segments screened, the predominant fungal genus isolated was *Cylindrocarpon lichenicola* and *Aspergillus* spp. (15) These results were similar to the studies, who isolated *Balansia* spp. *Aspergillus* spp. *Fusarium* spp. *Gilmaniella* spp. *Nigrospora* spp. *Penicillium* spp. and *Trichoderma* spp. in the part leaf, fruit, stem and root of *Melia azedarach* L. In another study reported *Penicillium janthinellum* were reported in fruit (16) and, reported *Penicillium brasilianum* found in the root bark of *Melia azedarach* L (17). Three fungal species of *Aspergillus* viz., *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus* sp, and one species of *Nigrospora* spp. were isolated from leaves (18). The *Aspergillus flavus* and *Trichoderma koningii* isolated from leaves and root of *Melia azedarach* L.

Except *Curvularia fallax* and *Alternaria alternata* (Dematiaceous Hyphomycetes), all the other *Aspergillus* sp, *Cylindrocarpon lichenicola*, *Fusarium solani* and *Chaetomium* sp, genera belong to Hyaline Hyphomycetes of group fungi Imperfect or Duteromycetes (Hyphomycetes). The colonization frequency (CF%) were observed in total segment 72 of 68.05%. The colonization frequency were observed in leaf segment to be 22.2% and 10.8% respectively which was found to be greater when compared to study (18). This might be due to that fact that these endophytic fungi vary with the geographical distribution of the plant species, as this plant was collected from Kalvarayan Hills, Villupuram (DT). Variation in the endophytic fungi isolation was seen in this study.

4.1. Conclusion:

The medicinal plant *Melia azedarach* L. have a rich source of medicinal properties. In this study the leaf, stem, flowers and fruits materials of *Melia azedarach* L. were collected from the Kalvarayan hills, Villupuram (DT). About 72 segments (18 segments of each part respectively) of the medicinal plant were screened for the isolation of the endophytic fungi. The leaf segment showed a maximum repository of endophytic fungi than the stem, flower and fruit. A total of 49 endophytic fungi were isolated and identified from 72 segments of the medicinal plant *Melia azedarach*. Among that the predominant endophytic fungi was *Cylindrocarpon lichenicola*, *Chaetomium* sp, *Curvularia fallax*, *Fusarium solani*, *Alternaria alternata*, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus* and *Aspergillus nidulans*. Majority of the fungi isolated in this study belonged to hyaline Hyphomycetes. In this study the Hyphomycetes were more prevalent than Dematiaceous and Mycelia sterilia. The genera of *Chaetomium* sp, and *Aspergillus* were the most common in that plant. The colony frequency of the leaf was high and showed maximum repository for endophytic fungi 68.05% respectively. *Melia azedarach* L, a well-known medicinal plant contains various chemical compounds. Isolation of endophytic fungi from this plant produces novel bioactive compounds.

Acknowledgement

I wish to express my deep sense and sincere thanks to DST-FIST and Department of Microbiology, Periyar University, Salem- 11, for providing me the fund and all facilities to carry out this project work successfully in the department Microbiology.

Conflict of Interest

There is no conflict of interest

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