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BIODIVERSITY OF THE ENDOPHYTIC FUNGI ISOLATED FROM *MORINGA OLEIFERA* OF YERCAUD HILLS

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ABSTRACT: Endophytic fungi residing inside the medicinal plants are of gaining importance for their bioactive compounds. This present study is undertaken to isolate and identify the potential endophytic fungi from *Moringa oleifera*, a traditional medicinal plant. A total of 24 segments each 12 from leaf and stem were collected, surface sterilized and was inoculated on to Sabouraud Dextrose Agar (SDA) plates. Based on the macroscopic & microscopic features the fungal isolates were identified as *Alternaria spp., Aspergillus spp. Bipolaris spp., Exosphiala spp., Nigrospora spp.,* and *Penicillium spp.* Many unidentified sterile mycelia forms were also found which were grouped under the class mycelial sterilia. The Colonization Frequency (CF) and Endophytic Infection Rate (EIR) were observed as 91.66% and 45.83% respectively. The results of this study suggest that traditional medicinal plants are a rich and reliable source of novel endophytic fungi. Further studies are required with regard to the screening of these endophytic fungi for the production of novel bioactive compounds which are medically important in the treatment of diseases.

INTRODUCTION: The endophytic fungi from the medicinal plants have been estimated as 1.38×10^6 unique fungal species isolated from the medicinal plants existing on this planet [1]. These endophytes are important components of fungal biodiversity, as many of them were important in the field of medicine. The species diversity of endophytic fungi is found to be high in tropical and temperate forest supports the high estimates of species diversity [2].

A variety of fungal relationships exists between fungal endophytes and their host plants, ranging from mutualistic or symbiotic to antagonistic or slightly pathogenic.

Endophytes are the chemical synthesizers inside plants which are symbionts living within the above ground tissue of their angiosperm hosts and are not affected by surface sterilization techniques [3].

The diversity of fungal endophytes are of gaining importance as they produce a variety of compounds which are useful to modern medicine, agriculture, industry, such as novel antibiotics, antimycotics, immunosuppressants and anti-cancer compounds [2]. The medicinal plants have been recognized as a repository of fungal endophytes with novel metabolites of pharmaceutical importance.

Moringa oleifera were traditionally known and reported to have various biological activities, including hypocholesterolemic agent, regulation of thyroid hormone status, antidiabetic agent, gastric ulcers, antitumor agent, antihyperglycemic and hypotensive agent.

The leaves as well as the flowers, roots, gums, fruits and seeds are extensively used for treating inflammation, cardiovascular action, liver disease, hematological, hepatic and renal function [4].

Thus, if a microbial source of the drug would be available, it would eliminate the need to harvest and extract the slow growing and relatively rare trees for the drug. The price for the drug would then be reduced.

Therefore, the main objective of the present investigation is to determine the diversity of endophytic mycoflora in *Moringa oleifera*.

MATERIALS AND METHODS:

Collection of samples: Healthy and mature trees were carefully chosen for sampling. Fresh mature leaves and stem of *Moringa oleifera* Lam (Moringaceae) without symptoms of ripening were collected from a healthy plant from different locations at Yercaud forest.

FIG. 1: *MORINGA OLEIFERA* LAM

The plant materials were brought to the laboratory in the sterile zip-lock bags and transported to the medical microbiology laboratory. The samples were then processed immediately to reduce the chance of contamination.

The plants were rinsed in running tap water to remove soil particles and unwanted debris. After washing, the leaves and the stem were selected for further processing under aseptic conditions. Highly sterile conditions were maintained for the isolation of endophytes and the entire process was carried out inside the laminar air flow.

Sterile glassware (Conical Flask) and mechanical things such as scissor, forceps, scalpel, and blades were used in sterile conditions for this experiment. The stem and leaves were cut into segments (0.5-1cm) by the use of sterile lancet blades.

Surface sterilization of Samples: The samples were surface sterilized by the method described [3]. In brief, the segments of stem and leaves were immersed in 70% ethanol for 5s. The branch portions were further sterilized sequentially in 4% sodium hypochlorite solution (Merck Laboratories) for 90s, and then rinsed in sterile distilled water for 10s. The excess moisture was blotted in a sterile filter paper.

Inoculation of samples on SDA: The surface sterilized segments were placed in petridishes containing Sabouraud Dextrose Agar (SDA) medium supplemented with chloramphenicol (5 mg / ml). Six segments were placed for one plate. The petri dishes were incubated at 25^{o} to 27^{o}C for 72 hrs in dark conditions and they were monitored every day to check the growth of endophytic fungal colonies from the segments.

Isolation and identification of Endophytic Fungi [5]: Most of the fungal growth was initiated within two weeks of inoculation. The incubation period for each fungus recorded was almost similar for the same species. The day of first visual growth was observed from plating date was considered as an incubation period for growth. Isolation from the master plates was done by the transfer of hyphal tips to fresh Potato Dextrose Agar (PDA) plates. The fresh PDA plates were incubated at 25^{o} to 27^{o}C for 72hrs & periodically checked for purity. The pure cultures were maintained on PDA slants. The endophytic fungi were 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 under a bright-field microscope (10X and 40X).

Statistical Analysis: The colonization frequency (CF) and Endophytic Infection Rate (EIR) were calculated as described [6]. Samples were incubated and growth was examined daily during 6 weeks and Colonization Frequency was calculated.

Colonization frequency (CF %):

$$CF = \frac{\text{No. of individual fungi recorded}}{\text{Total no. of segments screened}} \times 100$$

Endophytic Infection Rate (EIR %):

$$EIR = \frac{\text{Total no. of endophytic fungi recorded}}{\text{Total no. of segments screened}} X\ 100$$

RESULTS & DISCUSSION: Among myriad of domestic medicinal plants, which we use in our day to-day life *Moringa oleifera* Lam is one of the best known and most distributed species of Moringaceae family [4]. Moringa is an important tropical crop that is used as human food, and has several medicinal properties. This plant plays a crucial role in various biological activities such as reducing the cholesterol level, glucose level, cytotoxic activity, and also in regulating the thyroid level [10].

Therefore, the present work was initiated to find out endophytic flora associated within this widely used medicinal plant.

About 24 segments (12 segments of leaves and stem respectively) of *Moringa oleifera* were screened for the isolation of endophytic fungi. The leaf segments showed a maximum repository for endophytic fungi than the stem segments. A total of 6 endophytic fungal genera belonging to the class hypomycetes were isolated. The Colonization Frequency (CF) was 66.66% and 25% for stem and leaves respectively. The Endophytic Infection Rate (EIR%) was found to be 45.83%. The Colonization Frequency (CF%) was found to be high in leaves when compared to stem segments (**Table 1**).

TABLE 1: COLONIZATION FREQUENCY AND ENDOPHYTIC INFECTION RATE OF ENDOPHYTIC FUNGI COMMONLY ISOLATED FROM PARTS OF *MORINGA OLEIFERA*

S. No.	Plant part	No. of samples	No. of fungi isolated	Endophytes Isolated	Colonization Frequency (CF %)	Endophytic Infection Rate (EIR %)
1	Leaf	12	8	*Aspergillus nidulans* *Nigrospora spp.* *Exophiala spp.* *Alternaria spp.* *Penicillium spp.* *Mycelia sterilia* (3)	66.66%	45.83%
2	Stem	12	3	*Bipolaris* *Mycelia sterilia* (2)	25%	
	Total	**24**	**11**		**91.66%**	**45.83%**

The endophytic fungal genera isolated were *Alternaria spp., Aspergillus spp., Bipolaris spp., Exophiala spp., Nigrospora spp.,* and *Penicillium spp.,* (**Fig. 1A-D**). Many unidentified sterile mycelial forms were also found which were grouped under the class mycelia sterilia (**Table 2**).

The *Alternaria spp., Aspergillus spp.,* and *Nigrospora spp.* were also reported in the studies in which the endophytic fungi were isolated from C*alotropis gigantea* (L.) R.Br. in which they also reported about the sterile mycelial forms without any sexual and asexual conidia [3], *Calotropis procera* (Ait.) R. Br. [7].

Controversially, different endophytic fungi were reported in a Chinese medicinal plant, *Tripterygium wilfordii* [1]. The isolation of *Exophiala spp.,* was unique and was not reported in the previous studies.

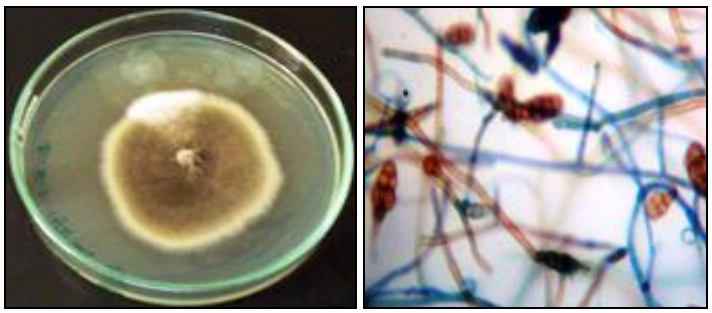

FIG. 1A

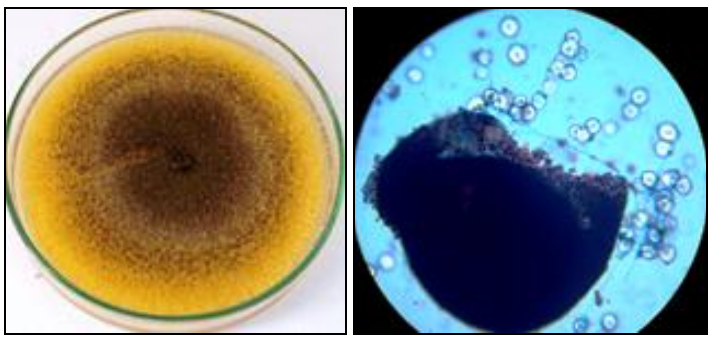

FIG. 1B

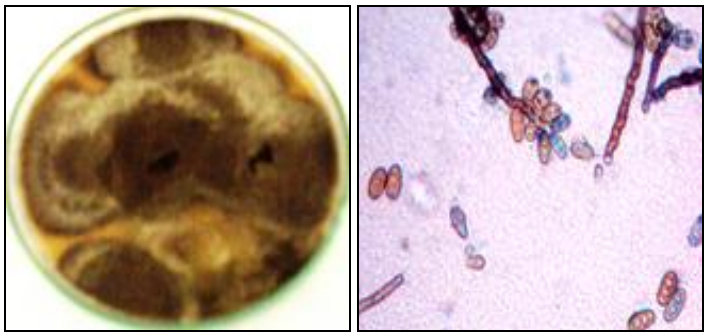

FIG. 1C

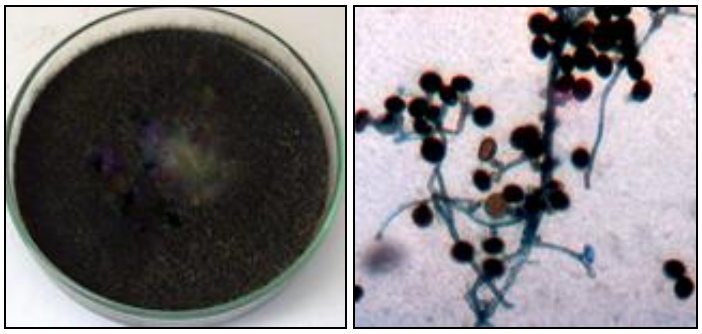

FIG. 1D

FIG. 1A: MULTICELLULAR CONIDIA (DICTYOCONIDIA) OF *ALTERNARIA SPP.*
FIG. 1B: DARK RED-BROWN CLEISTOTHECIA BEARING SPORES OF *ASPERGILLUS NIDULANS*
FIG. 1C: SYMPODIAL DEVELOPMENT OF PALE BROWN PIGMENTED, PSEUDOSEPTATE CONIDIA ON A ZIG-ZAG RACHIS OF *BIPOLARIS SPP.*
FIG. 1D: BLACK SHINING SMOOTH SPHERICAL AND DORSIVENTRALLY COMPRESSED CONIDIA OF *NIGROSPORA SPP.*

TABLE 2: ISOLATED ENDOPHYTES IN RELATION TO FUNGAL GROUP

S. No.	Isolated Endophytes	Fungal Class	Description
1.	*Alternaria spp.*	Dematiaceous Hyphomycetes (Dueteromycetes)	Colonies: black to olivaceous-black, reverse: dark brown, Conidiophores: branched, short, Conidia: multicellular conidia, obclavate, pale brown, smooth-walled
2.	*Aspergillus nidulans*	Hyaline hyphomycetes (Dueteromycetes)	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
3.	*Bipolaris spp.*	Dematiaceous Hyphomycetes (Dueteromycetes)	Colonies: grey to blackish brown, suede-like to floccose, reverse: black, Conidiophores: branched, short, geniculate or zig-zag rachis Conidia: ellipsoidal, rounded at both ends, smooth
4.	*Exophiala spp.*	Hyaline hyphomycetes (Dueteromycetes)	Colonies: smooth, greenish-grey to black, reverse: olivaceous-black, Conidia: are hyaline, smooth, thin-walled, and broadly ellipsoidal with inconspicuous basal scars.
5.	*Nigrospora spp.*	Dematiaceous Hyphomycetes (Dueteromycetes)	Colonies: white at first, later brown to black, reverse: black, Conidiophores: micronematous branched, colorless to brown, Conidia: black shining smooth, solitary, acregenous, simple and spherical.
6.	*Penicillium spp.*	Hyaline hyphomycetes (Dueteromycetes)	Colonies: green with white periphery, reverse: tan to brown, Conidiophores: were hyaline, smooth or rough-walled, Conidia: long dry chains, globose in basipetal succession from a specialized conidiogenous cell called a phialide
7.	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.

Several studies previously have reported the presence of pharmaceutical compounds like anti-cancer compounds from Thai medicinal plants [8], taxol compounds from endophytic *Phoma spp* [9].

CONCLUSION: This study had successfully isolated the endophytic fungi from *Moringa oleifera*; further studies have to be conducted to screen the novel bioactive compounds which were used in various fields like medicine, agriculture, environment etc.

Since, the renown natural sources from tropic and temperate zones of Southern regions of India has a huge consortium of medicinal plants and endophytic fungi [10] associated with, an future attempt will be made to utilize these endophytic fungi for the development of novel pharmaceutical compounds.

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