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Isolation and identification of endophytic fungi from *Avicennia marina* in Ramanathapuram District, Karankadu, Tamilnadu, India

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ABSTRACT

Avicennia marina (Tamil : Alai : Alai atti) : Avicennia marina is the dominant mangrose species in Karankadu and accounts for about 95% of the vegetative cover plant in Indian Sub-continent was investigated for endophytic mycoflora as a possible source of bioactive secondary metabolites. Being salinity resistant, this species dominate most mangrove area of the country including karankadu. Four hundred seventy three segments from 7 plants of Avicennia marina collected from different locations of karankadu mangrose forest in 2010 to 2011, were processed for me presence of endophytic fungi. A total 10 fungal species viz. Aspergillus flavus, A.niger, Aspergillus sp., penicillium sublateritium, phomo chrysanthemicola, P.hedericola, phoma sp. and candida albicans were isolated. Among the endophytic flora, phoma was the most prominent genus. Interestingly no endophytes was isolated from 110 leaves samples and overall colonization frequency from surface. Sterilized stem was 8.85%.

INTRODUCTION

Endophytic fungi that live inside the tissues of living plants are under - explored group of microorganisms. 1. Estimated that there may be at least one million species of endophytic fungi alone. Recently they have received considerable attention after they were found to protect their host against insect pests, pathogens and even domestic herbivores, 2. 3, almost all the plant species (400,000) harbor one or more endophytic organisms, 4. To date only a few plants have been extensively investigated for their endophytic biodiversity and their potential to produce bio-active secondary metabolites. Endophytic fungi generally live peacefully with their host, while these fungi under different conditions may act as facultative pathogen. One of the important roles of endophytic fungi is to initiate the biological degradation of dead or dying host-plant which is necessary for nutrient recycling 5.

The primary productivity from mangroves is enormous and various organisms such as wood borers, fungi and bacteria are involved in recycling the detritus. Such nutrient recycling helps in maintaining ecological balance in the estuarine environment of all fungi have been found to play a major role in the nutrient regeneration users. The fungi colonizing mangrove substrate can be divided in to terrestrial mycota, colonizing the plant parts above the water column and marine fungi colonizing the parts inundated either completely or intermittently by the tidal waters. At the interface a mixture of both terrestrial matine fungi occur, 7. Marine mycology, as a specialized branch of science, has evolved relatively recently. Neatly 1500 species excluding those that form lichens, have been estimated although 444 formely described higher marine fungi have been reported recently.

MATERIALS AND METHODS

Isolation of *endophytic fungi* from *Avicennia marina* plant

Stems and leaves of *Avicennia marina* (Tamil : Alas atti) were sampled for the investigation of endophytic fungal communities. Healthy and mature plants were carefully chosen for sampling. Samples from different sites of each plant were randomly collected and brought to the laboratory in sterile bags and processed within a few hours after sampling. Samples from different sites of each plant were randomly collected and brought to the laboratory in sterile bags and processed with in a few hours after the sampling to reduce the chances of contamination.

Isolation of endophytic fungi was carried out according to the method described by the samples were rinsed gently in running water to remove dust and debris. After proper washing, stem sample were cut into long 0.5-1 cm pieces. Whereas leaves were cut into 3-4 mm x 0.5-1 cm pieces with any without midrib under aseptic conditions. Surface sterilization was done by 1-13% sodium hypochloride (NaOCl) according to the type of tissues (for example higher concentration was used for leaf samples). Each set of plant material was treated with 75% ethanol for 1 min followed by immersion in sodium hypochlorite and again in 75% ethanol for 30 sec. Later the segments were rinsed three times with sterile distilled water. The plant pieces were blotted on sterile blotting paper. The efficiency of surface sterilization procedure was ascertained for every segment of tissue following the imprint method (11) In each petridish, 5-6 segments were placed on potato Dextrose Ager (PDA) supplemented with penicillin – G @ 100 units / ml and streptomycin @ 100 µg / ml concentrations. The dishes were sealed with parafilm and incubated at $27^{o}C \pm 2^{o}C$ for 4 – 6 weeks in dark room.

Fungi growing out of the plant segments were purified and identified after reference to 2, 13, 14 & 15 species of *Aspergillus* and *Penicillium* were grown on PDA and (2 Starch Dextrose agar) for identification.

Colonization frequency (CF) was calculated was described by (16) samples were incubated and growth was examined daily during 6 weeks and colonizing frequency was calculated.

$$\text{Colonization frequency (\%)} \quad \frac{\text{Number of segments colonized by an endophyte}}{\text{Total number of segments analyzed}} \times 100$$

Table 1: Endophytic fungi isolated from different parts of *Avicennia marina*

Site of isolation	*Number of Sample*	*Number of Fungi Isolated*
Leaves	110	11
Stems	110	9
Total Number of Isolates	-	20

Table 2: Name and Colonizing frequency of Endophytic fungi isolated from *Avicennia marina*

S. No.	*Fungi*	*Number*	*% Frequency of Colonization*		
			Leaf	*Stem*	*Number of Isolates*
1.	*Ascomycota*	209	3	3	1
	Candida albicans	379	4	4	1
	Deuteromycota	346	1	1	1
1.	*Aspergillus flavus*	286	6	6	1
2.	*Aspergillus niger*	211	7	3	1
3.	*Aspergillus terreus*	197	5	1	1
4.	*Aspergillus* sp.	177	1	1	1
5.	*Penicillium sublateritium*				1
6.	*Phoma chrysanthemicola*	156			1
7.	*Phoma hedoricola*				
8.	*Phoma* sp	138			

RESULTS

The plant materials were collected from karankadu mangroes forest and sample specimen was deposited the A.V.V.M. Sri Pushpam College, Thanjavur and identified by Prof. Dr. A. Panneerselvam, Department Botany and micro biology, Thanjavur, Tamilnadu.

Two - Hundred - Twenty (110 leaf samples and 110 stem samples) segments from 10 plants of *Avicennia marina* were processed for the isolation of endophytic fungi. A total of 10 fungi belonging to species were isolated (Table 1) Except *Avicennia marina.* Which belongs to the class. Ascomycetes, all the other isolates belong to the class Deuteromycetes. Most prominent endophyte in *Avicennia marina* was found to be genus *Avicennia.* Different Species of *Avicennia* including *Avicennia marina* were isolated and identified. Interestingly all the fungi were isolated only from leaves of plant while no leaf segments were also examined. All the isolated and identified fungi were submitted to the A.V.V.M. Sri Pushpam College culture collection (A.V.V.M.) (Table 2). Many fungi did not produce any reproductive structure, as they produced staile mycelia and in some cases sterile pycnidium.

i. *Aspergillus flavus* Link: *Aspergillus flavus* grew rapidly with floccosity when cultured on Czapek's Dox agar. It produced light greenish-yellow colour colony. Reverse side of the colonies were yellowish at primary stage of growth and brownish in mature age.

Conidiophores arose from submerged hyphae, were 400-1000 x 5-15 µ in size. Walls of conidiophores were pitted, rough and uncoloured. Conidial heads were hemispherical to subglobose. Vesicles were dome-like and 10-30 µ in diameter. Sterigmata were mostly in two series. Single series sterigmata were also produced. Primary sterigmata were 7-10 x 3-4 µ and secondary sterigmata were 10-15 x 3-5 µ in size. Conidia were pyriform to almost globose, nearly colourless and varied in size between 3-4 µ.

ii. *Aspergillus niger* van Tieghem: *Aspergillus niger* grew rapidly on Czapek's Dox agar. Colonies were carbon black in colour. It produced abundant submerged mycelia in the medium. Conidiophores were smooth with thick walls, unseptate, 200-1000 µ long and 7-10 µ thick. They were uncoloured near the vesicle. Conidial heads were fuscous black, globose, up to 300-500 µ in diameter. Vesicles were colourless and globose, thickwalled up to 35 µ in diameter. Conidial chains were present over the entire surface of vesicles. Conidia were rough, globose and 3-4 µ in diameter.

iii. *Penicillium sublateritium* Biourge: Colonies of *P. sublateritium* were restricted when grown on Czapek's Dox agar. It reached 1-2 cm in diameter in 12 days at 27 °C. Colonies were velvety, orange-green in colour with thin white margin. Reverse side of the colony were pale orange. Mature colonies were deeply radiantly wrinkled. Spores were abundant with grey-green shades. Colonies did not produce odour and exudates. Conidiophores were mostly 70-80 x 2 µ in size and smooth walled. Phyloides were strictly monoverticillate, consisting of small verticels. Five to eight or ten parallel sterigmata were present on verticels. Sterigmata were mostly 10-12 x 0.2-2.5 µ, occasionally 15 µ in length. Spores arranged in chain. Conidial chains were up to 100µ long. The mature conidia were elliptical, smooth and 4.0-5.0 x 3.0µ in size.

iv. *Phoma chrysanthemicola* Hollos: *Phoma chrysanthemicola* produced irregular, olivaceous grey colonies with darker patches on PDA medium. Reverse side of the colonies were blackish brown. Mycelia were immersed, branched, septate and brown in colour. Conidiomata were pycnidial, dark brown in colour, semi-immersed and thin-walled. Pycnidia of *P. chrysanthemicola* developed separately or aggregately on medium. Pycnidia sometimes became erumpent, unilocular and globose. Conidia were straight cylindrical with 1-4 guttules, 5-6.5 x 1-1.5µ in size. Clamydospores were multicellular. Pseudosclerotia were formed by aggregated Clamydospores which were pale to dark brown in colour.

v. *Phoma hedericola* (Dur. & Mont.) Boerema: *Phoma hedericola* produced brown coloured colonies with regular margin on PDA medium. Reverse side of the colonies were brown. Mycelia were immersed, branched, septate and dark brown in colour. The fungus produced semi-immersed pycnidia in medium which were globose, thinwalled and brown in colour. Sometimes the pycnidia were erumpent and unilocular. Pycnidia were separate or aggregated and occasionally confluent on medium. Conidia 4-6.5 x 2.5-3.5µ in size.

vi. *Candida albicans* (C.P. Robin) Berkhout: Colonies of *C. albicans* were smooth and off-white in colour when it grew on PDA medium. Reverse side of the colonies were also off-white. The diameter of colonies was 3-4 cm at 10 days at 27 °C. Older colonies developed radial furrows. Pseudomycelia developed by elongation of cell. Cells were small, oval, budding, 2.5 x 4 x 6 µ in size. *C. albicans* produced clamydospores in medium.

DISCUSSION

The Endophytic fungi are one of the most unexplored and diverse group of organisms that make symbiotic associations with higher life forms and may produce beneficial substances for host (Weber, 1981 : Shiomi *et al*., 2006) Fungi have been widely investigated as a source of bio alive compounds. An excellent example of this is the anticancer drug, taxol, which had been previously supposed to occur only in the plants (Strobel & Daisy, 2003).

Endophytic organisms have received considerable attention after they were found to protect their host against insect pests, pathogens and even domestic herbivorous (Weber, 1981). However

only a few plants have been studied for their endophyte biodiversity and their potential to produce bioactive compounds. Recently studies have been carried out about the endophytic bio diversity, taxonomy, reproduction, host ecology and their effort on host (Petrini, 1986 : Arnoldetal 2001 ; Clay & Schardl, 2002; Selosse & Schardl, 2007). Endophytes, are now considered as an out standing source of bioactive natural products, because they occupy unique biological niches as they grow in so many unusual environments (Strobel & Daisy, 2003 Strobel *et al*., 2009).

A study of endophyte biodiversity of the two dry and moisture of mangrove forest in Ramanathapuram District, Karankadu in Tamilnadu, India was conducted by the suryanarayana *et al*., (2003). They have reported diversity of fungal species vanging from 10 to 26 in the host. Among the one plant species the lowest number of fungal diversity was 10 in Gmelina arborea Roxb. In the present study 8 different species with 8.86% colonization frequency were isolated from. *Avicennia marina* which is slightly less than the above cited study Suryanarayanan *et al*., 2003.

During the present study mainly *Aspergillus, penicillium* and *phomase* were isolated as endophytic fungi. Deuteromycota fungi were largely prevalent. Majority of endophytic fungi belongs to Ascomycota and Deuteromycota (Frohlich & Hyde, 1999).

In this study profuse pycnidia were found on the fresh plant of *Avicennia marina* which is the Characteristic of the fungus *phoma* the most common endophyte of this plant. The *phoma* species are reported to produce cellulytic encymes, necessary to degrade plant material (Urbanek *et al*., 1998).

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