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Diversity of *Bruguiera cylindrica* and *Rhizophora candelaria* from Ayiramthengu mangrove ecosystem, Kerala

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ABSTRACT

Mangrove is a complex ecosystem, possessing a diverse group of plants, animals and microorganisms. Interestingly a fascinating group of endophytes is residing in mangrove plants and these have a major role to enhance the growth of plants in the unique environmental condition. So the diversity and the activity of endophytic fungi need to be screened for high biotechnological potential and a valuable source of useful metabolites. In this study 31 endophytic fungi were isolated from mangrove plants, belonging to various genera such as Phoma sp., Mucor sp, Pencillium sp, Aspergillus sp., Phomopsis sp., Acremonium sp., Cheatonium sp., Arnium sp., Eutypella sp., Fusarium sp., Botrytis sp., Geotrichum sp., Aphyllophorals sp. and Nigrospora sp. Screening of these fungal isolates for their antimicrobial potential was done and two prospective isolates MEF 14 and MEF21 were selected. MEF14 and MEF21 were identified as Eutypella sp and Geotrichum candidum respectively, based on 18S rDNA analysis. G. candidum showed antimicrobial activity against human pathogens, thereby suggesting a new source of antimicrobial compounds. Eutypella was found to be a potent source of many exoenzymes such as cellulases, proteases, laccases, lipases and amylases. The findings of this work should pave a way for identification of potential natural product for its application in industrial and pharmaceutical level.

Keywords: Endophyte, Mangrove, Geotrichum, Eutypella, Bruguiera, Rhizophora.

INTRODUCTION

Endophytic fungi are microbes, which spend the whole or part of its life cycle colonizing inter- and/or intracellular spaces inside the healthy tissues of the host plants, typically causing no apparent symptoms of disease [1]. Mangrove forests occurring at the interface of terrestrial and marine ecosystems made of harsh environment of salinity, high temperature and moisture and anaerobic condition. So they developed different mechanisms to put up with their environment, in this view a fascinating group of endophytic fungi possess. It has a major role to sustain the environment and adapts to the harsh environment. Endophytes by occupying the localized point of entry or by spreading within the plant, they produce an array of bioactive metabolites and hydrolytic enzymes to survive in the unique chemical environment of the host plant [2].

Rhizophoraceae (Bruguiera and Rhizophora) is a well known group in mangrove community and also for its medicinal potentials. The leaves of *Bruguiera* species are used for reducing blood pressures and the barks of *Rhizophora* species have astringent, antidiarrhoea and antiemetic activities [3]. Root, leaf and stem extracts of

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Rhizophora trees have inhibitory properties, affecting the growth of various human pathogenic organisms. So exploitation of the medicinal value of these endangered plants is essential, but to a greater extent it is limited, due to scarce availability. Moreover, the loss of mangrove habitat throughout its range, primarily due to extraction and coastal development is a threat to these plants, and there has been an estimated 24% decline in mangrove area within this species range since 1980 [4].

The ability to produce pharmacologically important natural products previously only known from plant sources is also inherent to endophytic microorganisms [2]. Endophytes thus could serve as a potential source of many plant borne bioactive compounds. This would not only reduce the need to harvest slow growing and possibly rare plants, but also preserve the world's ever-diminishing biodiversity. Furthermore, it is recognized that a microbial source of a valued product may be easier and more economical to produce effectively reducing its market price [5]. Thus, methods to tap the bioactive potential of these plants, along with its simultaneous preservation gains relevance. The current study made an effort to find out the biodiversity of endophytic fungi present in two mangrove plants *Bruguiera cylindrica* and *Rhizophora candelaria*. The antimicrobial potential and extracellular enzyme production of selected endophytes were studied. While much of the interest in endophyte bioactive compound is for medicinal use, compounds that may have industrial or agricultural applications are also gaining attention. Previous works on the endophyte diversity of *Bruguiera* and *Rhizophora* are merged and thus the work gains much relevance. Attempts to explore the bioactive compounds from their resident endophytes than host plants could help to protect these endangered plants to a large extent.

MATERIALS AND METHODS

Collection of samples and visualization of endophytic fungi

Plant materials of two healthy mangrove plant *Bruguiera cylindrica* and *Rhizophora candelaria* were collected from Ayiramthengu, Kollam district, Kerala, India for isolating endophytic fungi. Plant tissues were brought to the laboratory in closed polythene bags and were processed within 3 hrs of collection. 2, 3, 5- Tri phenyl Tetrazolium Chloride (TTC) staining was carried out to visualize the presence of endophytic fungi in the collected leaf samples. Surface sterilized plant material was incubated overnight in TTC stain and cross sections were examined under the light microscope for the presence of stained fungal hyphae [6].

Isolation of endophytic fungi

Plant samples were cleaned by washing under running tap water, tween 80 and surface-sterilized by dipping successively into 70% ethanol for one minute, 4% sodium hypochlorite for 45 sec, and finally rinsed three times in sterile distilled water [7]. The surface sterilized plant samples were air dried under aseptic condition, then it was cut into 1cm long segments with a sterile blade and inoculated on isolation medium (Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA)) supplemented with streptomycin (30 μ g/mL). The inoculated plates were incubated for 7 days until the fungal mycelia grew from the plant samples. Pure cultures of the endophytic fungi were subcultured on PDA agar slants and stored at 4°C for the further studies.

The effectiveness of surface sterilization was assessed by plating sterile distilled water aliquots used for the final rinse onto the surface of PDA plates supplemented with streptomycin. The plates were examined for fungal growth after incubation at 25 ± 2 ^oC for three days along with suitable controls [8]. The colonization frequency was calculated by recording the number of isolates from each sample [7] using the formula,

Colonisation frequency of endophytes =	Number of segments colonised by fungi	x 100
	Total number of segments observed	

Taxonomic identification of the isolated endophytic filamentous fungi was done through observation of macroscopic and microscopic characters [9].

Screening of the isolates based on antimicrobial activity

The isolates were screened for their antibacterial activity using disc diffusion method [10]. Potential human pathogenic bacteria such as *Staphylococcus aureus, Escherichia coli, Salmonella typhi, Klebsiella pneumonia, Proteus* sp. were selected for this study. A suspension of 12 hrs old culture of pathogenic bacteria was spread on a sterile Mueller Hinton agar plate onto which a five day old disc (5 mm diameter) of endophytic fungi was kept and

incubated at 25 ± 2 °C for 18-24 hrs. Antimicrobial activity was calculated by measuring zone of inhibition produced by endophytic fungi against pathogenic bacteria.

The antibacterial activity of positive endophyte fungal cultures was further verified using cell free extracts [11]. The positive endophyte cultures were grown in potato dextrose broth, filtered to separate the mycelia and were extracted 2-3 times with ethyl acetate (v/v) in a separating funnel. Ethyl acetate extracts of filtrate were mixed, evaporated till dryness under vacuum at 50°C and dissolved in Dimethyl Sulfoxide. Antimicrobial activity of culture extract was assessed with a disk diffusion method.

Qualitative screening of fungal enzyme activity

The potential of the isolates to produce various extracellular enzymes such as amylases, Laccases, Lipases, Protease [12] and Cellulase [13] production was assessed. Production of these enzymes by the fungal endophytes was studied by digestion of suspended or dissolved substrate in agar plates after inoculation with 3 mm mycelial plugs and incubated for 3-5 days at 25±2°C. Amylase activity was assessed by growing the fungi on glucose yeast extract peptone (GYP) agar medium (glucose, 1 g; yeast extract, 0.1g; peptone, 0.5g; agar, 16 g; distilled water, 1000 mL; pH 6) with 2% soluble starch. After incubation, the plates were flooded with 1% iodine in 2% potassium iodide. For cellulase, the fungi were cultured on yeast extract peptone agar medium (yeast extract, 0.1g; peptone 0.5 g; agar, 16 g and distilled water, 1000 mL) supplemented with 0.5% Na-carboxymethyl cellulose (CMC). After incubation, the plates were flooded with 0.2 aqueous Congo red and destained with 1M NaCl for 15 minutes. Laccase activity was assessed by growing the fungi on GYP agar medium amended with 1- naphthol, 0.005% (pH, 6) and incubated. On oxidation of 1-naphthol by laccase, the medium changes from clear to blue. For lipase activity, the fungi were grown on peptone agar medium (peptone, 10 g; NaCl, 5 g; CaCl2 2H2O, 0.1 g, agar, 16 g, distilled water, 1000 mL; pH 6) supplemented with Tween 20 (separately sterilized and added 1 mL to 100 mL medium). Protease assay was performed by growing the fungi on GYP agar medium amended with 0.4% gelatin (gelatin, 8 g/100 mL distilled water, sterilized separately and mixed with sterile GYP agar medium) adjusted the pH to 6. After incubation, plates were flooded with saturated aqueous ammonium sulphate.

The zone of enzyme activity surrounding the fungal colony was measured after incubation. A clear zone formed surrounding the colony in agar plates was considered positive for amylase, cellulase, lipase and protease activity, whereas medium change from clear to blue indicated positive laccase activity.

Molecular identification of the selected endophytic fungal isolates

Molecular identification of selected fungal cultures showing positive antibacterial activity or enzyme activity was done using using ITS-PCR universal primers specific for fungal genomic DNA (ITS 1:5'-TCCGTAGGTGAACCTGCGG-3' ITS4: 5'-TCCTCCGCTTATTGATATGC-3'). The PCR product of 1.5 kb was purified using illustra GFX PCR DNA and gel band purification kit (GE Healthcare). The purified amplicon were sequenced using big dye terminator v3.2 cycle sequencing chemistry for ABI Bioprism (Applied Biosystems). The sequences were analyzed using the BLAST (www.ncbi.nlm.nih.gov) search algorithm and aligned to their nearest neighbors. The sequence was deposited in the NCBI GenBank database.

SEM analysis of dried culture was performed by mounting culture on specimen stubs with double adhesive tape and coated with platinum in a sputter coater and examined under JEOL 6390 SEM JSM microscope at 10KV.

Auxanographic tests were done for identification of yeast-based on their ability to assimilate carbohydrates and nitrate [14]. The assimilation of carbohydrate was assessed using Yeast nitrogen base agar seeded with a heavy suspension of the yeast - top agar combination (5.0 mL/100 mL of media) in a petri plate. Filter paper discs impregnated with saturated solutions of the carbohydrates (dextrose, maltose, sucrose, lactose, galactose) were placed on the surface of solidified media and incubated for 48 h at 25 ± 2 ⁰C. Growth of the yeast around individual discs was detected visually and it indicated assimilation of that particular compound. When the compound was not utilized no enhanced growth was seen. YCB (yeast carbohydrate base) media were prepared and seeded with yeast as stated above to check the nitrate assimilation. In this case, two discs, one impregnated with KNO₃ and one with peptone were placed on the agar and incubated.

RESULTS AND DISCUSSION

Isolation and identification of endophytic fungi

Fungi have proven themselves as valuable sources of natural products for agriculture as well as biomedical development. The mangrove plants *Bruguiera cylindrica* and *Rhizophora candelaria* from Ayiramthengu region of the Quilon district of Kerala state were collected as the experimental plants and surface sterilized healthy leaves were used for the visualization and isolation of endophytic fungi. The residence and invasion of endophytic fungi inside the plant tissues were confirmed by Triphenyl Tetrazolium Chloride staining (fig 1), which is based on the activity of respiratory enzymes [6]. Tri phenyl Tetrazolium chloride is a water soluble heterocyclic organic salt that can be easily reduced to a highly colored (dark red) insoluble product called Formazann. As the fungal hyphae grew, the dye got incorporated into the fungal cell and it was reduced to an insoluble dark red pigment formazann. The dark red color is found only in the area where the fungus grows.

A total of 31 different endophytic fungal isolates were obtained from healthy *Rhizophora candelaria* and *Bruguiera cylindrica*. A high colonization frequency of endophytes was found in *Rhizophora candelaria* (42%) compared to *Bruguiera cylindrica* (35%). Bacterial and fungal colonies were not observed on sterility control plates after 3 days of incubation. Based on their morphological and microscopic properties the fungal isolates were identified as *Phoma* sp., *Mucor* sp., *Pencillium* sp., *Aspergillus niger*, *Phomopsis* sp., *Acremonium* sp., *Cheatonium* sp., *Arnium* sp., *Dematacieae*, *Eutypella* sp., *Fusarium* sp., *Aphanoascus* sp., *Botrytis* sp., *Geotrichum* sp., *Aspergillus flavus*, *Aphyllophorals* sp. and *Nigrospora* sp. Most of the endophytic fungal isolates were identified by morphological observations (Fig 2a and 2b). But the remaining isolates couldn't be completely identified because of lack of spore formation in the media. It was also correlated to the viewpoint that identification of microorganisms isolated from plants, mainly filamentous fungi, is very complex [15].

In this study, some of the endophytic fungi, such as Phoma sp, Fusarium sp., Penicillium sp. were not host specific and were isolated from both the host plants. This observation substantiates former reports suggesting endophyte assemblages were common to more than one host mangrove variety [16]. Mucor, Cheatomium sp., Eutypella sp., Botrytis sp., Acremonium sp were also isolated from mangrove plants under study. The results of this study support previous findings [7] who reported that the variation in distribution of fungal endophytes, were not restricted to a single species, genera and family. Endophytes do not show host specificity, however, certain fungal lineages appear with greater frequency in plants representing particular families and thus denote host preference [17].

Antimicrobial activity of the isolates

The antimicrobial activity of isolated endopytic fungi were tested against human pathogens (*Staphylococcus aureus*, *Escherichia coli, Klebsiella pneumonia* and *Proteus*) by disk diffusion method. Two endophytic fungal isolates (MEF 21 and MEF 17) inhibited all the four test microorganisms and were selected. The isolate MEF 21 exhibited highest antimicrobial activity against pathogens compared to second selected isolate MEF 17. The crude extract of ethyl acetate was dried in oven, dissolved in DMSO and again tested for the antibacterial activity (table 1). The need for new antimicrobial agents, in general, comes from the increasing rates of resistance to existing antibiotics. Medicinal plants have been recognized as the repository of fungal endophytes with novel metabolites of pharmaceutical importance [18].

Enzyme activity of the fungal isolates

Wood-inhabiting marine fungi serve as a potential source of exoenzymes [19], [20]. All the endophytic fungi were tested for their enzyme activity. Fig 3 represents the percentage of enzyme activity of endophytic fungal isolates. Among the isolates MEF14 gave a positive reaction for all the tested enzymes (fig 4), while 11 of the isolates were able to degrade starch by amylase enzyme. Among the isolates some fungi showed significant zone of clearance in starch agar plate indicating their ability to produce a good amount of amylase enzyme. Proteases are important enzymes used in clinical applications, especially in treatments of diseases like diabetes [21]. In this study five isolates showed significant protease production.

Endophytes are able to decompose polymer carbohydrates such as cellulose [22]. The present study also shows that six of the mangrove endophytes could produce cellulases. Most of the soil fungi produce lignocellulose-modifying exoenzymes like laccase. In this study ten of the isolates showed laccase activity, representing their involvement in the litter degradation. Laccase activity already reported in many marine fungi [23], [7] but only a few researchers has only studied the physiology and biochemistry of these fungi. Only 2 fungal isolate showed lipase activity. These

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fungi have the ability to utilize fat as their energy source. If the endophytes are capable of producing extracellular enzymes [24], it is highly likely that the endophytes can also act as wood degrading fungi. Wood degrading fungi are well-known in mangrove habitats.

Identification of the selected isolate

The isolate MEF 21 was selected based on its antimicrobial property and MEF 14 was selected for the enzyme activity test. Colony characteristics of the isolate MEF 21 suggested that it was yeast, so it was tested for the biochemical test of assimilation of carbohydrate and nitrate test. The selected isolate was able to utilize only glucose and galactose and unable to assimilate nitrate. From these findings MEF 21 was identified as *Geotrichum* sp. (fig 5). MEF 14 formed white and cottony, cream-colored in reverse, with no fruiting structures. After about 1 week, MEF 14 was developed black spotters, and the reverse side became almost black in agar plate, while in 2-3 weeks it was produced yellow pigmentation in the potato dextrose broth (fig 6). Based on molecular analysis of MEF 14 and MEF 21 was identified as *Eutypella* sp. and *Geotrichum candidum* respectively. The ITS- specific regions of MEF 14 and MEF21 were sequenced and deposited in NCBI database under accession numbers JX477425 and JX477426 respectively. *Eutypella* is a filamentous fungus and *Geotrichum candidum* is yeast, both of the isolate belongs to the phylum *Ascomycota*.

Table 1: Measurement of zone of inhibition of fungal culture extract against pathogenic bacteria

Extract of the fungal isolates	Klebsiella	Proteus	Staphylococcus aureus	E.coli
MEF 17	7mm	9mm	10mm	7mm
MEF 21	10mm	12mm	12mm	16mm





Fungal hyphae

Fig 2a Morphological observations of endophytic fungal isolates (a) *Mucor* sp. (b) *Fusarium* sp. (c) *Phoma* sp. (d) *Penicillium* sp.



Fig 2b Microscopic structures of reproductive stages of endophytic fungi by lactophenol cotton blue staining (a)Botrytis (b) Mucor sp (c) Fusarium sp. (d) Aspergillus sp.(e)Cheatomium





Fig 3 Graphical representation of percentage enzyme activity of fungal isolates

Fig 4 Enzymatic activity of endophytic fungal isolates



(a) Blue coloration around the colony indicated the oxidation of GYP (Glucose, Yeast extract Peptone) medium containing naphthol by laccase enzyme of MEF 14 and MEF 23



(c) Clear zone showing degradation of gelatin in GYP medium by the protease enzyme of MEF 12, MEF 13 and MEF 14



(b) Clear zone showing degradation of sodium carboxymethyl cellulose in YPA medium by cellulase enzyme of MEF 14



(d) Clear zone indicating degradation of starch in GYP medium by amylase enzyme of MEF 14 and MEF 21



(e) Clear zone around the colony indicated lipase positive fungi shown by MEF 14 only.

Fig 5. Identification of MEF 21 (A) colony morphology (B) lactophenol cotton blue staining (C) SEM Analysis



Fig.6 Identification of MEF 14 (a) colony morphology (b) Pigmentation observed in culture filtrate



CONCLUSION

Studies on mangrove endophytic fungi were showed that it has an extremely diverse fungal flora that together contribute to the stress adaptiveness and productivity of mangrove plants. These endophytes contribute significantly to the growth of its host plants by secreting various antimicrobial compounds, thereby inhibiting its invasion by pathogens. Secretion of a wide range of extracellular enzymes by endophytes also complements to the enhanced absorption availability of nutrients by plants. Screening of these fungal resources could help us to discover many valuable sources for novel metabolites and enzymes. The use of easily growing yeast *Geotrichum candidum* as a source of antimicrobial compounds as suggested by this study is an example. The isolate *Eutypella* proved to be a valuable candidate for the production of multiple enzymes and it could be exploited in many industries to make economically important innovations. To the best of our knowledge, the current work is unique being the first report on the endophytic potential mycotic diversity of *Bruguiera* and *Rhizophora*.

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