Endophytic fungal community associated with the mangrove plant, *Aegiceras corniculatum* with the predominance of *Penicillium citrinum* and *Colletotrichum siamense*: A reservoir of biocatalysts and antibacterial molecules

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Abstract
This study explored the endophytic fungal community associated with the leaves of a mangrove plant, Aegiceras corniculatum. An isolation frequency of 9.53% was observed and 102 endophytic fungi could be isolated and maintained. The isolates displayed diverse colony morphologies, predominantly belonging to Ascomycota (95%) and a minor portion to Basidiomycota (5%). Within Ascomycota, the most represented classes were Sordariomycetes, Eurotiomycetes and Dothideomycetes. The most abundant genera were Penicillium (27%), Colletotrichum (24%) and Alternaria (13%). Totally seventeen endophytic fungal species were identified from Aegiceras corniculatum.

Penicillium citrinum (22%), Colletotrichum siamense (21%) and Alternaria alternata (13%) were the predominant species. The colonization frequency indicated Penicillium citrinum as the most prevalent species at 2.3%, followed by Colletotrichum siamense at 2% and Alternaria alternata at 1.2%. The isolates demonstrated significant hydrolytic enzyme production and antimicrobial activity, indicating their potential utility in aquaculture and medicine.

Keywords: *Aegiceras corniculatum*, Endophytic fungi, Diversity, Mangroves, Antibacterial, Hydrolytic enzymes.

Introduction

Endophytic fungi are a group of microorganisms that inhabit plant tissues without causing any visible symptoms of infection. Endophytic fungi have adapted to live inside plant tissues where they establish a mutually beneficial relationship with the host plants. They contribute to the host plant's health by enhancing nutrient uptake, improving stress tolerance and providing protection against pathogens. This unique symbiotic interaction between the fungi and their host plants transforms them into a potent source of diverse natural products. These natural compounds encompass a broad range of chemical classes including alkaloids, polysaccharides, polyketones, terpenes, sterols, anthraquinones, flavonoids, xanthones, phenols, anthrene derivatives, furandione, cyclic peptides etc.[24]

Numerous investigations have demonstrated the biological activities associated with these compounds, such as antibacterial, antidiabetic, antifungal, anti-inflammatory, antiprotozoal, antituberculosis, insecticidal, immunomodulatory, antiviral, anticancer and anthelmintic properties[31]. These bioactive compounds hold potential applications across various industries including pharmaceuticals, agriculture and food production. Furthermore, these microorganisms are widely acknowledged as abundant reservoirs of enzymes. These enzymes play crucial roles in plant defense against pathogens, nutrient uptake from the host and the decomposition of leaf litter. Additionally, their potential applications span across diverse industries including detergents, pharmaceuticals, food processing and the leather industry.

Mangroves are woody plants that uniquely grow at the interface between land and sea in tropical and subtropical regions. These environments are characterized by high salinity, extreme tides, strong winds, elevated temperatures and muddy, anaerobic soils. No other plant types exhibit such advanced morphological and physiological adaptations to thrive under these conditions. Consequently, mangroves provide a unique habitat for a diverse array of organisms including epibenthic, infaunal and meiofaunal invertebrates, as well as phytoplankton, zooplankton and fish. Furthermore, this unique habitat induces novel metabolic pathways in the plants, resulting in the synthesis of unique bioactive compounds. These compounds pave the way for the development of new therapeutic precursors and industrial raw materials.

Mitra et al[27] documented a list of phytochemicals with diverse biological activities isolated from various mangrove species. Numerous clinical trials on extracts from *Excoecaria agallocha, Bruguiera sexangula* and *Avicennia africana* have been conducted, revealing their potential as antiviral, anticancer, anti-HIV and antitumor agents[16,34,40]. *Aegiceras corniculatum*, commonly known as black mangrove, belongs to the Myrsinaceae family and is distributed in coastal and estuarine regions from India

through Southeast Asia to Southern China, New Guinea and Australia. Traditionally, this plant has been used in medicine to treat asthma, microbial infections, diabetes, pain, inflammation, cancer and arthritis.

A diverse number of bioactive secondary metabolites such as flavonoids, benzoquinones, triterpenes, polyphenolic acids, stilbenes, tannins and macrolides, has been identified in different parts of this plant[38]. The endophytic fungi associated with the plants also exhibited biological activities. For example, Penicillinols A_1 and B_1, purified from the endophytic fungus *Penicillium* sp. associated with *A. corniculatum*, demonstrated cytotoxic effects against the HL-60 cell line with IC_{50} values of 0.76 µM and 3.20 µM respectively[23].

Research on endophytic fungi and their natural products has emerged as a promising field for discovering novel bioactive compounds and elucidating their ecological functions within plant ecosystems. The present study is focused on isolating endophytic fungi from the black mangrove plant, *Aegiceras corniculatum* and evaluating their bioactive properties.

Material and Methods

Sample collection: Leaves were randomly collected from ten healthy *Aegiceras corniculatum* (Fig. 1a) plants in Kollam, India (9°07'13.8"N 76°28'40.5"E). The samples were carefully placed in polyethylene bags, transported to the laboratory and kept at a temperature of 4 °C in a refrigerator. All samples were processed within 24 hours of collection (Fig.1).

Isolation of Endophytic fungi: Fungal endophytes were isolated from the leaf samples using the method detailed by Kumaresan and Suryanarayanan[18]. The leaves were thoroughly rinsed under running tap water to remove dirt and dust. Next, surface sterilization was done by dipping the leaves in 70% ethanol for 5 seconds, followed by a 90 second treatment with sodium hypochlorite (4% v/v). Post-sterilization, the samples were rinsed three times for 10 seconds each in sterile water and any excess water on the leaf surface was dried with blotting paper. The leaves were cut into small pieces, about 0.5 cm^2 each, under sterile conditions in a laminar flow chamber. To confirm the effectiveness of the disinfection process, imprints of the leaf samples were made on potato dextrose agar (PDA) medium, the absence of fungal growth on the medium after incubation confirmed successful surface sterilization.

A total of 1080 leaf segments were placed in Petri dishes containing PDA supplemented with 150 mg/L chloramphenicol. These Petri dishes were incubated for four weeks at 28 ± 2 °C, with regular observations for fungal growth. Fungal colonies that emerged from the leaf segments (Fig. 1b) were transferred to PDA slants and three day old pure cultures in potato dextrose broth (PDB) were stored at -80 °C in a deep freezer (Thermo Fisher Scientific, USA).

Isolation frequency, Colonization frequency and Percentage occurrence of each taxon:
Isolation Frequency (IF) % = number of leaf segments with fungal growth/total number of leaf segments plated × 100

Colonization Frequency (CF) % = number of leaf segments colonized by an endophytic fungal species/total number of leaf segments plated × 100

Percentage occurrence of a taxon (class/ order / genus/ species) = Number of isolates of a particular taxon (class/ order / genus/ species) from a plant species/ Total number of isolates from a plant species (or all plant species) × 100

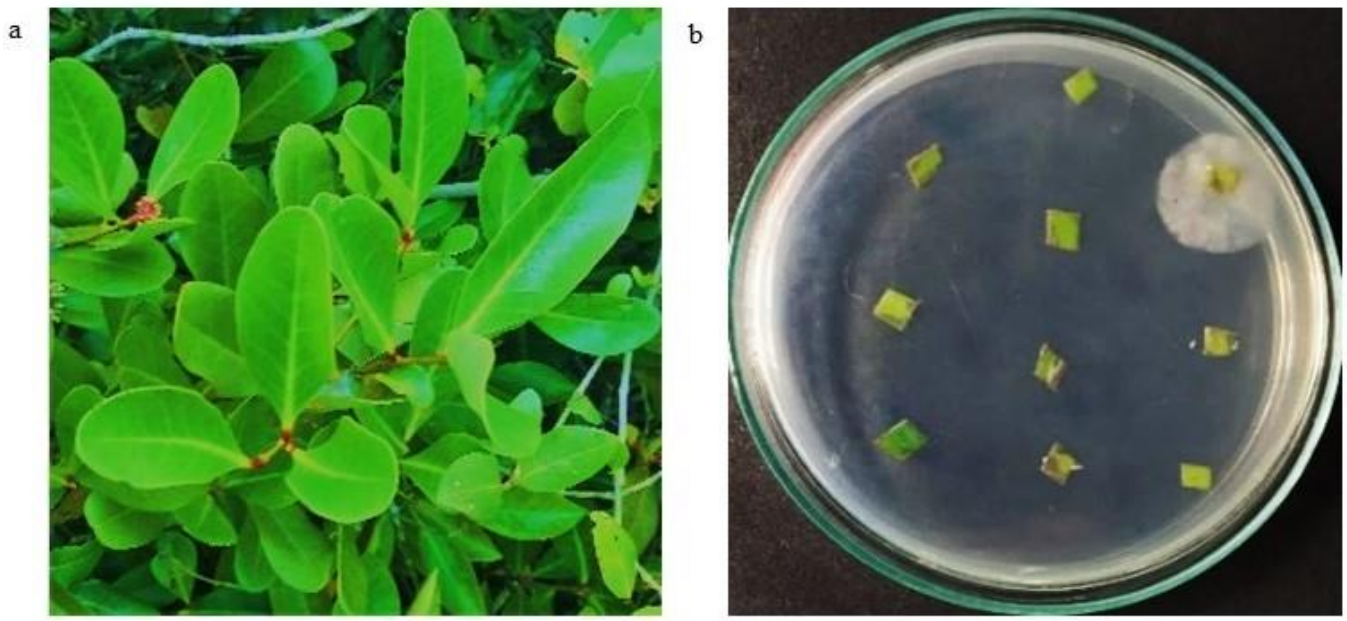

Fig. 1: a) Host plant *Aegiceras corniculatum* selected for sampling b) endophytic fungal growth from the leaf segments

Identification of endophytic fungi

Morphological identification: The isolates were spot inoculated onto PDA plates and kept in an incubator at 28 ± 2 °C for one week. Afterwards, a thorough examination of their morphological traits such as colony characteristics, growth and color (both front and reverse) was done. The Scotch tape method[20] was utilized to examine microstructural features which involved placing the adhesive side of clear cellophane tape onto colony surface and transferring it onto a slide with lactophenol cotton blue. Spores and hyphae were examined using an Inverted Research Microscope (Zeiss, Axio Observer 3, Germany). Identification of the samples at the genus level was based on spore morphology, following procedures outlined by Rogerson et al[37].

Molecular Characterization: Fungal genomic DNA was extracted using the salting out method described by Miller et al[26]. Pure fungal cultures were grown in potato dextrose broth (PDB) under static conditions for 24–48 hours. The mycelia were homogenized in 500 µL of solution 1 (50 mM Tris HCl pH 8.0, 20 mM EDTA pH 8.0, 2% SDS), with the addition of 0.5 µL of proteinase K (20 mg/mL). This mixture was vortexed and then incubated for 2 hours at 55 °C in a water bath. After incubation, the lysed cell suspension was chilled on ice for 10 minutes. Next, 250 µL of 6M NaCl was added and the lysate was mixed by inversion. After an additional 5-minute ice chill, the lysate was centrifuged for 15 minutes at 5700g.

The resulting clear supernatant was carefully transferred to a new vial and two volumes of absolute isopropanol were added. The mixture was then incubated at 4 °C for 12-18 hours to precipitate the DNA. The DNA pellet was collected by centrifugation at 10900g for 15 minutes, washed three times with 500 µL of 70% ethanol, followed by two washes with 95% ice-cold ethanol and air-dried. The pellet was dissolved in 30 µL of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) and stored at -20 °C. The internal transcribed spacer (ITS) regions of the rDNA was amplified using the primer pairs ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-3')[45].

Polymerase chain reaction (PCR) was conducted in a final volume of 25 µL, containing 1X standard Taq buffer (10 mM Tris–HCl, 50 mM KCl, pH 8.3), 3.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each primer, 1U Taq DNA polymerase (Fermentas, Inc.) and 1-2 µL of DNA template (10-100 ng). The PCR protocol consisted of an initial denaturation at 94 °C for 5 minutes followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 56 °C for 45 seconds, extension at 72 °C for 1 minute and a final extension at 72 °C for 10 minutes.

The PCR products were analyzed by electrophoresis on a 1% agarose gel and visualized under UV light after staining (Bio-Rad, USA). The resulting PCR products were used for amplified ribosomal DNA restriction analysis (ARDRA).

Amplified ribosomal DNA restriction analysis (ARDRA): The ITS amplicons were digested using three different restriction enzymes: *Hinf* I, *Sdu* I (Fermentas, France) and *Cfo* I (Sigma, USA) in separate reactions. Each reaction mixture (20 µL) contained 8 µL of PCR product, 2 µL of buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl2, 1 mM dithiothreitol), 8 µL of Milli-Q water and 2 µL (5 units) of the respective restriction enzyme. The mixtures were incubated at 37 °C for 16 hours followed by enzyme inactivation at 65 °C for 15 minutes. The resulting digestion products were separated by 2% agarose gel electrophoresis and visualized using a gel documentation system (Bio-Rad, USA).

A dendrogram was constructed based on the ARDRA profiles of different fungal isolates using PRIMER 6.0 and one representative strain from each cluster was chosen for further analysis. The ITS amplicons from these representative strains were sent for sequencing at Scigenome, Kochi. The obtained sequences were subjected to nucleotide BLAST search against the GenBank database at NCBI to identify the fungi based on sequence similarity.

In cases, where the sequences showed similar percentages of homology with multiple fungal species in the BLASTn analysis, amplification of the ß-tubulin (Ben A) gene was performed. The Ben A gene was amplified using the same thermocycler program as for ITS, with an annealing temperature of 55 °C using primers Bt2a (5' GGTAACCAAATCGGTGCTGCTTTC 3') and Bt2b (5' ACCCTCAGTGTAGTGACCCTTGGC 3')[10]. The PCR amplicons of Ben A were sequenced at Scigenome, Kochi. The nucleotide sequences of Ben A were subjected to a nucleotide BLAST search against the GenBank database at NCBI, confirming the identity of the isolates. All sequences were deposited in GenBank (NCBI). The phylogenetic tree was constructed using MEGA 11.0.11.

Screening of Antibacterial activity: The antibacterial activity of crude extracts from endophytic fungi was assessed using the Kirby-Bauer disk diffusion method[2]. The study involved testing against 12 bacterial pathogens including *Escherichia coli* (MTCC 1610), *Edwardsiella tarda* (MTCC 2400), *Pseudomonas aeruginosa* (MTCC 741), *Bacillus cereus* (MTCC 1272), *Staphylococcus aureus* (MTCC 3061), *Aeromonas hydrophila* (MTCC 1739), *Vibrio cholerae* (MTCC 3906), *Vibrio parahaemolyticus* (MTCC 451), *Vibrio alginolyticus* (MTCC 4439), *Vibrio harveyi* (BCCM 4044), *Vibrio proteolyticus* (BCCM 3772) and *Vibrio vulnificus* (MCCB 130).

The endophytic fungal isolates were cultured in potato dextrose broth (PDB) under static conditions for 14 days at room temperature (28 ± 2 °C). After incubation, the crude broth (devoid of mycelia) was applied to sterile filter paper discs (5 mm), which were then placed onto Muller Hinton agar (MHA, HiMedia) plates inoculated with the respective

bacterial strains. The plates were supplemented with NaCl (15 g/L) for vibrios.

Incubation was conducted under specific conditions: 37 ± 2 °C for human pathogens including *Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus, Staphylococcus aureus* and *Vibrio cholerae* and 28 ± 2 °C for aquaculture pathogens such as *Aeromonas hydrophila, Edwardsiella tarda, Vibrio parahaemolyticus, Vibrio alginolyticus, Vibrio harveyi, Vibrio proteolyticus* and *Vibrio vulnificus*. Following overnight incubation, the diameter of the inhibition zone surrounding each disc was measured to assess the antibacterial activity of the fungal extracts against the tested pathogens.

Screening of hydrolytic enzyme production: The fungal isolates were tested for various enzymatic activities including amylase, lipase, protease, cellulase, chitinase, tyrosinase, laccase, asparaginase, glutaminase, ligninase, pectinase and DNase. This was done by spot-inoculating the isolates on assay plates containing specific substrates. The plates were then incubated at 28±2 °C for 5-7 days. After the incubation period, the agar medium was flooded with appropriate reagents and the development of a clear zone or coloration was observed and recorded. Detailed information about the media, reagents and the criteria for identifying positive reactions are provided in Table S1 (Supplementary file).

Results

Endophytic Fungal Community: A total of 102 endophytic fungal isolates were obtained from the leaves of *Aegiceras corniculatum*, with an isolation frequency of 9.53%. On potato dextrose agar (PDA), the colony morphologies of the isolates varied, appearing as powdery, cottony, velvety, leathery, or glabrous, with surface topologies including flat, rugose, umbonate and verrucose (Fig. 2). Among these isolates, 95% belonged to the phylum Ascomycota and 5% to Basidiomycota. Within Ascomycota, the classes identified were Sordariomycetes (39%), Eurotiomycetes (34%), Dothideomycetes (20%) and Saccharomycetes (2%) and they were distributed among eight orders: Eurotiales (34%), Hypocreales (30%), Pleosporales (13%), Capnodiales (7%), Diaporthales (6%), Saccharomycetales (5%), Sordariales (2%) and Glomerellales (1%). In Basidiomycota, only the class Agaricomycetes (5%) and the order Agaricales (5%) were recorded.

Table S1
Media and reagents used for testing the enzyme production by endophytic fungi

Enzymes Tested	Medium (Per litre of seawater of salinity 25 psu)	Reagents used	Positive reaction
Amylase	Glucose yeast extract peptone (GYP) agar (glucose 1.0 g; yeast extract 0.1 g; peptone 0.5 g; agar 20 g) + 2% starch	Lugol's iodine (1% iodine and 2% potassium iodide in distilled water)	Clear zone
Lipase	GYP agar + 1% tributyrin	Nil	Clear zone
Protease	GYP agar + 2% gelatin	15% Mercuric chloride solution (HCl, 20 ml; distilled water, 80 ml)	Clear zone
Cellulase	GYP agar + 1% carboxy methyl cellulose	0.2% aqueous Congo red followed by destaining with 1M NaCl	Clear zone
Chitinase	GYP agar + 5% colloidal chitin	Nil	Clear zone
Tyrosinase	GYP agar + 0.5% tyrosine	Nil	Clear zone with brownish periphery
Laccase	GYP agar + 0.05% α-naphthol	Nil	Reddish brown
Asparaginase	Glucose 2 g; KH_2PO_4 1.52 g; KCl 0.52 g; $MgCl_2$ 0.52 g; $FeSO_4.7H_2O$ 0.01 g; phenol red 0.009%; agar 20 g + 1% L-asparagine	Nil	Pink colouration around colony
Glutaminase	Glucose 2 g; KH_2PO_4 1.52 g; KCl 0.52 g; $MgCl_2$ 0.52 g; $FeSO_4.7H_2O$ 0.01 g; phenol red 0.009%; agar 20 g + 1% L-glutamine	Nil	Pink colouration around colony
Ligninase	Crawford's agar (glucose 1 g; yeast extract 1.5 g; Na_2HPO_4 0.45 g; K_2HPO_4 0.1 g; $MgSO_4$ 0.02 g; $CaCl_2$ 0.5 g; agar 20 g) + 0.02% methylene blue	Nil	Clear zone
Pectinase	Yeast extract agar (yeast extract 10 g; agar 20 g) + 0.5% pectin	Lugol's iodine solution (1% iodine and 2% potassium iodide in distilled water)	Clear zone
DNase	DNase agar (peptone 5 g; beef extract 3 g; DNA sodium salt 2 g; agar 20 g)	1N HCl	Clear zone

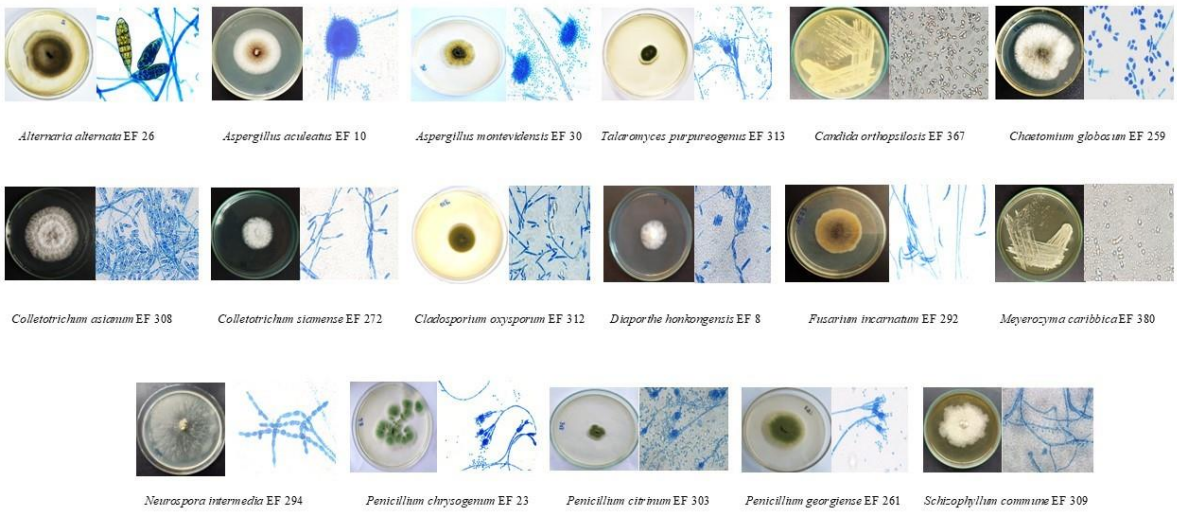

Fig. 2: Macro and Microscopic images of different endophytic fungal isolates of *A. corniculatum*

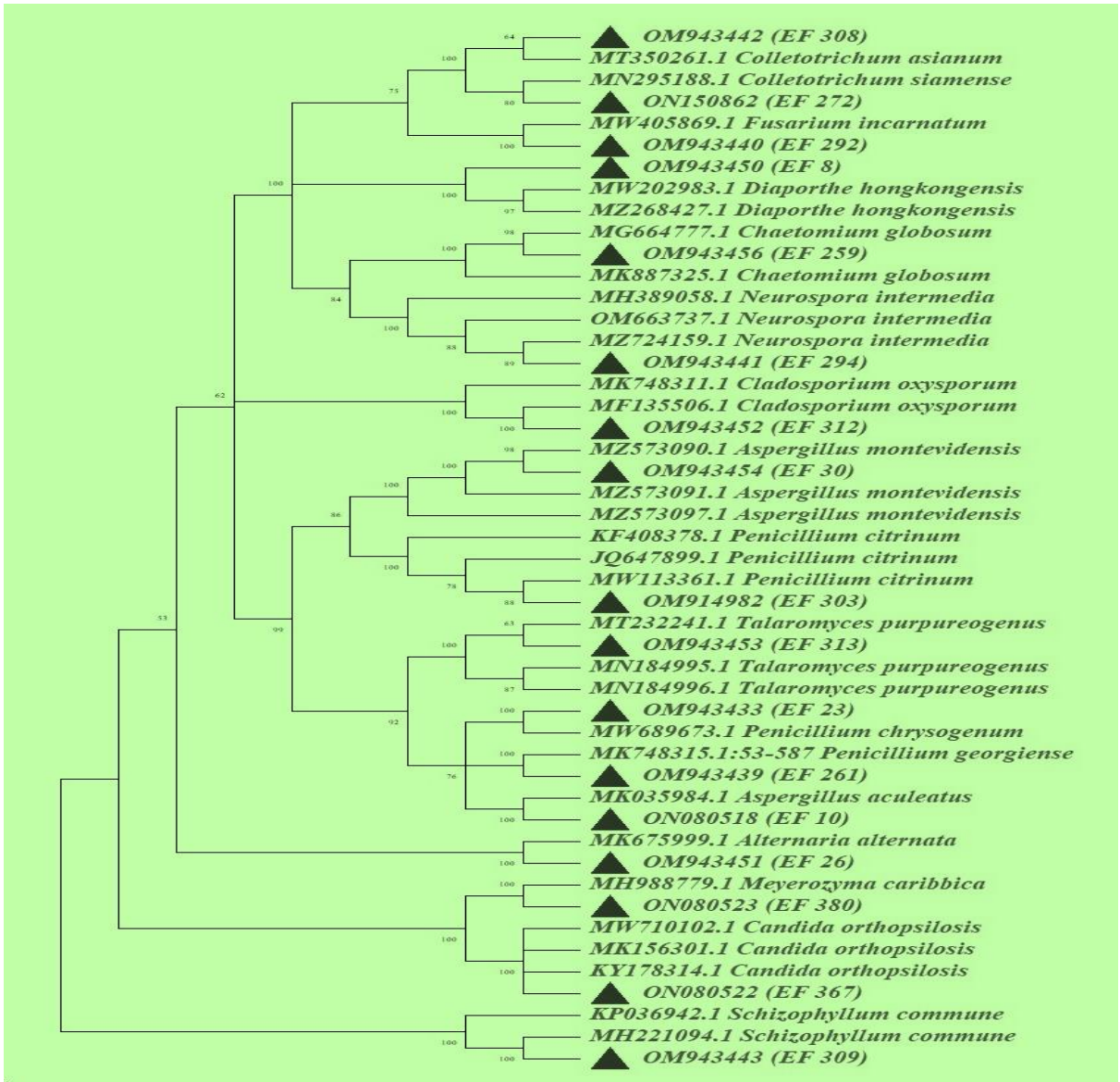

Fig. 3: Neighbor-joining phylogenetic tree based on ITS sequence of fungal endophytes from *A. corniculatum* and related sequences from GenBank, NCBI

Thirteen genera were identified with *Penicillium* (27%) being the most abundant followed by *Colletotrichum* (24%), *Alternaria* (13%), *Aspergillus* (7%), *Cladosporium* (7%), *Diaporthe* (6%), *Fusarium* (5%), *Schizophyllum* (5%) and others. Phylogenetic analysis provided insights into the evolutionary relationships of the isolates (Fig. 3). Seventeen species were isolated and their sequences were submitted to GenBank (Table 1). The most prevalent species were *Penicillium citrinum* (22%), *Colletotrichum siamense* (21%) and *Alternaria alternata* (13%). *Cladosporium oxysporum* accounted for 7%, *Diaporthe hongkongensis* for 6% and *Fusarium incarnatum* for 5% (Fig. 4). The colonization frequency revealed that *Penicillium citrinum* had the highest prevalence at 2.3% followed by *Colletotrichum siamense* (2%) and *Alternaria alternata* (1.2%) (Table 2).

Table 1
Details of BLAST analysis and GenBank accession numbers for the nucleotide sequences of the endophytic fungal isolates

Isolate	Sequence length (bp)	Identity (%)	Query coverage (%)	Species identified	Accession number	
					ITS	Ben A
EF 26	518	100	*100*	*Alternaria alternata*	OM943451	
EF 10	517	100	*100*	*Aspergillus aculetaus*	ON080518	
EF 30	500	100	*100*	*Aspergillus montevidensis*	OM943454	
EF 367	469	100	*100*	*Candida orthopsilosis*	ON080522	
EF 259	484	100	*100*	*Chaetomium globosum*	OM943456	
EF 312	493	100	*100*	*Cladosporium oxysporum*	OM943452	
EF 308	515	100	*100*	*Colletotrichum asianum*	OM943442	
EF 202	514	100	*100*	*Colletotrichum siamense*	OM943438	
EF 8	533	100	100	*Diaporthe honkongensis*	OM943450	
EF 292	486	100	100	*Fusarium incarnatum*	OM943440	
EF 292	284	100	100	*Fusarium incarnatum*		ON803447
EF 380	540	100	100	*Meyerozyma caribbica*	ON080523	
EF 294	532	100	100	*Neurospora intermedia*	OM943441	
EF 294	437	100	100	*Neurospora intermedia*		ON803448
EF 23	540	100	100	*Penicillium chrysogenum*	OM943433	
EF 303	516	100	100	*Penicillium citrinum*	OM914982	
EF 261	533	100	100	*Penicillium georgiense*	OM943439	
EF 309	574	100	100	*Schizophyllum commune*	OM943443	
EF 313	547	100	100	*Talaromyces purpureogenus*	OM943453	

Table 2
Colonization frequency of endophytic fungal species isolated from *A. corniculatum*

Species	Colonization frequency (%)
Alternaria alternata	1.2
Aspergillus aculeatus	0.3
Aspergillus montevidensis	0.1
Candida orthopsilosis	0.1
Chaetomium globosum	0.1
Cladosporium oxysporum	0.5
Colletotrichum asianum	0.4
Colletotrichum siamense	2.0
Diaporthe hongkongensis	0.5
Fusarium incarnatum	0.4
Meyerozyme caribbica	0.1
Neurospora intermedia	0.2
Penicillium chrysogenum	0.1
Penicillium citrinum	2.3
Penicillium georgiense	0.1
Schizophyllum commune	0.4
Talaromyces purpureogenus	0.1

Antibacterial activity: All isolates were screened for antibacterial activity against 12 selected bacterial pathogens. Out of these, 16 endophytic fungal isolates exhibited growth inhibition against at least one of the pathogens. Specifically, 7.8% of the isolates inhibited the growth of *S. aureus*, 4.9% inhibited *B. cereus* and 2.9% inhibited *E. tarda*, *V. alginolyticus* and *V. cholerae*. Additionally, 1.9% of the isolates inhibited *V. parahaemolyticus* and *V. harveyi* (Fig. 5a). The potent isolates chosen for further study were *Aspergillus aculeatus* EF 10 and *Penicillium citrinum* EF 19 (Table S2)

Hydrolytic enzyme production: Production of hydrolytic enzymes by the isolates was also evaluated. More than 90% of the isolates tested positive for amylase, over 80% showed activity for pectinase and lipase, while more than 65% were positive for protease and cellulase. Around 57% were positive for glutaminase and 42% for asparaginase. However, only 7% of isolates exhibited ligninolytic activity and 14% showed laccase activity (Fig. 5b). Isolates that demonstrated significant hydrolytic enzyme production included *Aspergillus montevidensis* EF 30, *Cladosporium oxysporum* EF 316, *Colletotrichum fructicola* EF 22, *Colletotrichum siamense* EF 272, *Meyerozyma caribbica* EF 347, *Schizophyllum commune* EF 309 and *Talaromyces purpureogenus* EF 313 (Table S3).

Discussion

Endophytic fungal community: In this study, phylogenetic analysis using ITS and Ben A sequencing revealed the presence of 17 distinct species distributed across 13 genera. Among these, 12 genera were classified under the phylum Ascomycota and only a single genus under phylum Basidiomycota. This observation aligns with the existing information that mangrove endophytic fungi predominantly belong to Ascomycota[12,36]. Among the isolated fungal strains, the genus *Penicillium* accounted for 27% of the total isolates, making it the predominant genus. This was followed by *Colletotrichum*, comprising 24% of the isolates and *Alternaria*, which constituted 13%. In a study investigating the diversity of endophytic fungi in 20 different mangrove species on Andaman Island, *Diaporthe pseudomangiferae* was identified as the predominant species in *Aegiceras corniculatum*[36].

Fig. 4: Taxonomic classification of endophytic fungi isolated from *A. corniculatum*

Table S2
Antibacterial activity of the endophytic fungi isolated from *Aegiceras corniculatum* by disk diffusion assay

Endophytic fungi	Zone of inhibition (mm)												
	Ah	Bc	Ec	Et	Pa	Sa	Va	Vc	Vh	Vp	VPro	Vf	Vv
Alternaria alternata EF 7		7											
Aspergillus aculeatus EF 10		12		10			9	10	20	10			
Aspergillus aculeatus EF 12							13	10					
Penicillium citrinum EF 15				9						10			
Colletotrichum siamense EF 18			12										
Penicillium citrinum EF 19		14		8		15	5	21	5				10
Alternaria alternata EF 26						12							
Colletotrichum siamense EF 33		16											
Penicillium citrinum EF 277		10											
Penicillium citrinum EF 282						10							
Penicillium citrinum EF 283						10							
Penicillium citrinum EF 296						10							
Cladosporium oxysporum EF 311						20							
Talaromyces purpurogenus EF 313	5									16			
Penicillium citrinum EF 315						16							
Cladosporium oxysporum EF 316						27							

Table S3
Hydrolytic enzyme production by selected endophytic fungi isolated from *Aegiceras corniculatum*

Endophytic fungi	Amylase	Protease	Lipase	Cellulase	Asparaginase	Glutaminase	Dnase	Tyrosinase	Pectinase	Ligninase	Laccase
Aspergillus montevidensis EF 30	+++	-	++	++	+++	++	++	-	-	-	-
Cladosporium oxysporum EF 316	+	+++	+++	++	+	+	+	-	-	-	-
Colletotrichum fruticola EF 22	+	+	+	-	++++	+	-	-	+	-	-
Colletotrichum siamense EF 272	++	-	+	+	+	-	-	-	+++	-	-
Meyerozyma caribbica EF 347	+++	+++	+++	-	++	++	++	-	-	-	-
Schizophyllum commune EF 309	+++	+	+	-	+	+	++	-	-	++	++

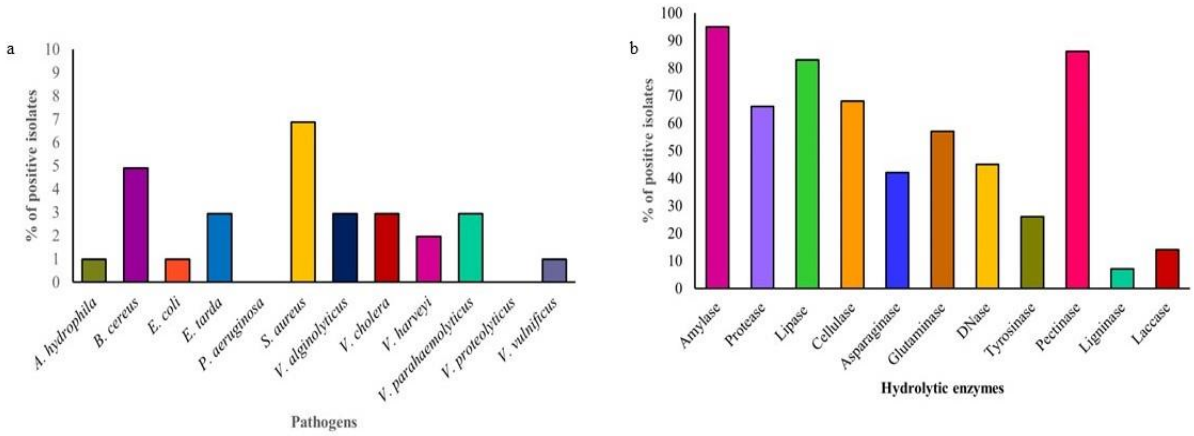

Fig. 5: a) Antibacterial activity profile of the endophytic fungi
b) Percentage of positive isolates for enzyme production from *A. corniculatum*

Penicillium species are commonly found in the rhizosphere of various plants where they play a fundamental role in the soil phosphorus cycle. *Penicillium* species are among the most crucial groups of fungi for promoting plant growth, capable of suppressing numerous plant pathogens, inducing plant systemic resistance and exhibiting wide adaptability to diverse environments as well as tolerance to various abiotic stresses [14,17]. Furthermore, these genera were identified as potent candidates for the remediation of heavy metals and other industrial pollutants. This widespread occurrence suggests that *Penicillium* species has adapted to thrive within the unique ecological niches provided by mangrove ecosystems. Their presence suggests potential ecological and functional roles in these environments such as contributing to plant health, nutrient cycling and adaptation to environmental stresses commonly found in mangrove habitats.

The *Penicillium* species in the current study primarily consisted of *Penicillium citrinum, Penicillium chrysogenum* and *Penicillium georgiense*. A diverse array of species belonging to the genus *Penicillium* has been consistently identified as endophytes in numerous mangrove plant species. *Penicillium citrinum*, known for being a potent source of the mycotoxin citrinin, is widely distributed worldwide and isolated from various sources such as soil, plants and indoor environments. *Penicillium georgiense*, a soil fungus is commonly known as infectious agent in *Allium cepa*[48] whereas *Penicillium chrysogenum* utilized in the production of the antibiotic β-lactam, has been reported as an endophyte in various mangrove plants.

The second most abundant genus, *Colletotrichum*, included species such as *Colletotrichum siamense* and *Colletotrichum asianum*. Its presence is associated with promoting plant growth through mechanisms such as enhanced nutrient uptake, production of growth-promoting compounds and stimulation of the plant's defense mechanisms. Moreover, certain strains of *C. siamense* have demonstrated biocontrol activity against other pathogens, offering an additional layer of protection to the host plant[4]. *Alternaria alternata* is commonly isolated from a diverse range of plants, functioning both as an endophyte and a pathogen. In the current study, the occurrence of this species in *A. corniculatum* was 13% of the total isolates. *Alternaria alternata* was already reported as an endophyte in *A. corniculatum* and *Avicennia marina*[21].

Cladosporium species comprising 7% of the isolates exhibit a cosmopolitan distribution in plants as endophytes and in various organic materials such as foods and textiles as saprobes. *Cladosporium* can also act as a pathogenic agent for plants, animals and humans. *Cladosporium oxysporum* was previously isolated from the roots of *Avicennia officinalis* through direct plating and damp chamber incubation [1].

Aspergillus species comprised of 6% of the total endophytic isolates identified in the study. The species within this genus included *A. aculeatus* and *A. montevidensis*. *Aspergillus aculeatus*, identified in this study was reported as an endophyte of *Rhizophora stylosa* collected from Hainan Province, China[47]. It has been shown to confer salt stress tolerance to plants by producing indole-3-acetic acid and siderophores[22]. *Diaporthe*, comprising 6% of the total isolates in the present study and commonly found as an endophyte or plant pathogen with a wide host range, has been recorded as one of the most predominant endophytic fungi in mangroves [6]. A study on a Brazilian mangrove forest indicated the presence of *Fusarium* in all sampling sites across all plant parts and selected plant species[7].

Fusarium is drawing significant attention due to its plant pathogenicity, causing infections in nearly all economically important plants affecting the agriculture industry. Additionally, certain *Fusarium* species can act as opportunistic human pathogens, causing infections in the cornea, nails and skin[11]. Fusarium species constituted 5% of the total isolates in our study. A study on a Brazilian mangrove forest indicated the presence of *Fusarium* in all sampling sites across all plant parts and selected plant species[7].

Schizophyllum commune, the only Basidiomycota isolated in this study, is widely distributed and has been identified from all continents except Antarctica[30]. Previously, it has been reported as an endophyte in *Avicennia officinalis*[15]. Research indicates that *Schizophyllum commune* can cause infections in both humans and plants, although it predominantly functions as a saprobic fungus responsible for white rot[19].

In summary, the diversity and composition of endophytic communities within plants are influenced by a combination of host-related factors such as plant species and physiology and environmental factors, including climatic conditions and geographical locations[8,35]. Understanding these influences is crucial for studying the ecological roles and potential applications of endophytic fungi in plant health and ecosystem functioning.

Antibacterial activity: Numerous researchers have documented the bioactive potential of diverse fungal endophytes isolated from various mangrove species across the globe. Among the total isolates obtained in our study, 16 endophytic fungal isolates exhibited growth inhibition against at least one of the pathogens. The crude extracts from *Glomerella*, *Guignard*ia and *Cladosporiu*m, isolated from the leaves of *A. corniculatum*, were reported to have inhibitory activity against the five tested pathogens, namely *B. cereus, P. aeruginosa, E. coli* and two MDR strains, *Klebsiella pneumoniae* and *Acinetobacter baumannii*[3].

Several bioactive secondary metabolites have been well-characterized from endophytes associated with the mangrove *A. corniculatum*. For example, an antibacterial compound, pestalol B, purified from the endophytic *Pestalotiopsis* sp. AcBC2 associated with the plant collected from the Nansha mangrove wetland in Guangdong province, China, showed inhibitory potential against *M. tuberculosis*, comparable to the control drugs, isoniazid and rifampin[41]. Additionally, isoindolone derivatives isolated from the endophytic fungus *Emericella* sp. were identified as potent antiviral agents against the influenza A virus[49].

Enzyme production: Fungal endophytes are known to be prolific producers of various extracellular enzymes which are crucial for their ability to penetrate and colonize host plants. In recent years, numerous studies have documented the enzyme production capabilities of these endophytes and their practical applications. Using agar plate assays for qualitative screening, we found that the majority of the fungal isolates from *A. corniculatum* were potent producers of the various enzymes tested. Amylases are one of the essential enzymes in industries such as food, chemicals, detergents and textiles, where they convert starch into simple sugars. Glucoamylases, commonly found in fungi, are often sourced from *Aspergillus* sp. and *Rhizopus* sp. for industrial applications[32].

In our study, over 90% of the isolates tested positive for amylase production which agreed with earlier findings on mangrove-derived fungi[25]. More than 80% of the isolates tested positive for lipase and pectinase production. Fungal lipases have various industrial applications including baking, beverage manufacturing, cheese production, butter emulsification, fat removal from meat and the development of digestive aids. In a study on endophytic fungi from Thai medicinal plants, 10 out of 65 isolates were found to be positive for lipase activity[33]. Pectinases produced by endophytic fungi play a dual role, they serve as a defense molecule against pathogenic organisms and facilitate nutrient uptake from the host.

Pectinases are among the leading products in the commercial enzyme industry, with crucial applications in the wine industry for juice clarification, the food industry for processing and extraction and the paper industry for pulp bleaching and waste paper recycling. In a study on endophytic fungi from Thai orchids, 57% of the isolates showed pectinase activity[39]. Proteases, which hydrolyze peptide bonds in proteins, are extensively used in detergents, leather industry, food industry, pharmaceutical industry and bioremediation processes.

Recent research has focused on fibrinolytic enzymes, a type of protease used in thrombosis therapy. A novel fibrinolytic enzyme was purified and characterized from the endophyte *Fusarium* sp. associated with chrysanthemum stems[46]. In our study, 65% of the isolates tested positive for protease activity. Cellulases, which have diverse applications in the textile, paper, food, feed, biofuel and detergent industries, as well as in agro-waste management, were detected in 66% of the endophytic isolates. Previously, 27.67% of endophytic fungal isolates from medicinal plants in the Western Ghats were found to be cellulase positive[43].

Glutaminase and asparaginase are important enzymes in modern healthcare, especially as anticancer agents. Previous studies have reported that two endophytic fungi, *Cladosporium* sp. and *Trichoderma* sp., produce L-glutaminase[9]. Microbially-derived L-asparaginase is preferred over chemical drugs due to its biodegradability and non-toxicity[29]. Endophytic fungi such as *Lectosphaerella, Fusarium, Stemphylium, Septoria, Alternaria, Didymella, Phoma, Chaetosphaeronema, Sarocladium, Nemania, Epicoccum, Ulocladium* and *Cladosporium*, isolated from Asteraceae family of plant species, are known to produce asparaginase[13]. In our study, 57% of the total isolates tested positive for glutaminase and 42% for asparaginase.

DNase, a DNA-degrading enzyme, is commonly utilized in healthcare for the treatment of cystic fibrosis and as an anticancer agent. In our study, 45% of the fungal endophytes tested positive for this enzyme. Previous research has reported that fungal isolates recovered from mycotic keratitis possess the ability to produce DNase [28]. Tyrosinase, a copper-containing enzyme, catalyzes the synthesis of melanin in plants and animals. In our study, 26% of the isolates tested positive for tyrosinase activity. An

unidentified endophytic fungal isolate screened for tyrosinase activity from four plants viz. *Calotropis gigantea, Azadirachta indica, Ocimum tenuiflorum* and *Lantana camara* revealed that isolates from *Azadirachta indica* and *Ocimum tenuiflorum* exhibited the highest enzyme activity[42].

In the current study, only 14% of the isolates tested positive for laccase production while 7% tested positive for ligninase. Ligninolytic enzymes have found applications in various fields including the food industry, textile industry, synthetic chemistry, cosmetics, soil bioremediation and biodegradation of environmental phenolic pollutants. Endophytic fungus *Monotospora* sp. isolated from *Cyanodon dactylon* have demonstrated the ability to produce laccases[44]. Ligninolytic enzymes, including laccase and lignin peroxidase, are produced by the endophytic fungus *Phomopsis liquidambari* when cultured in submerged fermentation using phenolic 4-hydroxybenzoic acid as the sole carbon and energy source, revealing its potential for bioremediation[5]. In the present study, we identified *Aspergillus, Cladosporium, Colletotrichum, Schizophyllum* and *Talaromyces* as potent producers of the tested enzymes. Previous research has documented that these genera are capable of producing a variety of hydrolytic enzymes with extensive applications across various industries.

Conclusion

In conclusion, our study provides an account of the endophytic fungi associated with the leaves of the mangrove plant, *Aegiceras corniculatum* and their bioactive potential. A total of 102 isolates were identified, with Ascomycota constituting 95% and Basidiomycota 5%. Molecular analysis identified 17 species with *Penicillium citrinum*, *Colletotrichum siamense* and *Alternaria alternata* being the dominant species. Our results also highlight the potential of these endophytic fungi as sources of bioactive compounds, as several isolates exhibited antibacterial activity against various pathogens.

Additionally, majority of the isolates demonstrated significant hydrolytic enzyme production, indicating their metabolic versatility. This research advances our understanding of the diversity and biotechnological potential of endophytic fungi associated with *Aegiceras corniculatum* and emphasizes their significance in ecological and industrial applications.

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