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Isolation of Cellulolytic *Bacillus* sp. from the Viviparous Seedling Hypocotyls of Red Mangrove – *Rhizophora mucronata* Lam.

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Abstract

Mangroves are one of the world's most unique tropical coastal ecosystems. They are a rich repository of biological wealth, including specially adapted flora and fauna. The microbiome component of this ecosystem is a fascinating world that is yet to be fully explored for its functional and ecological inter-relationships with its hosts. The mangrove ecosystem is a hidden treasure of microbial diversity, without which mangrove biology is incomplete. In the present study, the isolation of a cellulase-producing, endophytic *Bacillus* sp. from the hypocotyl region of viviparous seedlings is described. This study urges us to look into the microbial diversity of mangrove propagules, by presenting a glimpse of a member of the endospheric microbiome of viviparous hypocotyls.

Keywords: *Mangrove, Rhizophora, Endophyte, Vivipary, Bacillus*

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INTRODUCTION

Mangroves are a unique, tropical coastal ecosystem, mostly comprising salt-tolerant tree species.[1-3] These plant species have unique morphological and physiological adaptations, which enable them to encounter harsh conditions of high salinity and pH range of 4-10.[4-6] Mangrove biology has always remained an intriguing subject and currently, there is a resurgence in its studies.[7-14] Microbiome component, especially endophytes of 'mangrove holobiont',[15,16] is one such area that needs further investigations to unveil the functional relationship between endophytes and their host.[17-22] The term 'holobiont' is comprehended as a 'single dynamic entity', formed by the functional interaction between the macroorganism host and its associated microbes.[23-26] In the case of mangroves, the interactions between the microbes and its mangrove host, forming a single dynamic entity can be apprehended as 'mangrove holobiont'. Endophytes like *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Gordonea terrae*, *Trichoderma harzianum, Piriformospora indica* etc are non-pathogenic intra or intercellular, microbial residents of host plants.[27-31] They are found to be ubiquitous, sharing an ecological niche similar to that of plant pathogens, and often, they enter into a mutualistic relationship with host plants, contributing to the overall host fitness.[32-37]

Rhizophora mucronata Lam. is a mangrove tree species, belonging to the family *Rhizophoraceae*. Members of this family, commonly called 'red mangroves', are one of the prominent mangrove taxa possessing several adaptive features common to mangroves including the viviparous type of seed germination.[38-41] Vivipary is an important developmental phenomenon seen associated with the mangrove tree species.[39,40] Here, the seeds germinate while still attached to the mother plant and later, seedlings detach and drop into the muddy environment where it grows further into an adult plant, overcoming extreme pH and salinity.[40, 42] Exploration of the microbiome of these seedlings will help us to identify and understand the role of microorganisms in the establishment and growth of mangroves, and further, it will shed light on mangrove biology and its survival. In the present study, attached viviparous seedling hypocotyls of *Rhizophora mucronata* are explored for the presence of culturable endophytes.

MATERIALS AND METHODS

Study site and collection of plant samples

Viviparous seedling hypocotyls of *R. mucronata*, in the attached condition, were cut from the plant and collected in polyethylene bags. They were collected from multiple mangrove sites at Koduvally (11°767′N, 75°482′W) and Ozhayilbhagam, Dharmadam (11°46'30.14" N 75°28'25.5" E) located near Thalassery, Kannur, Kerala, India.

Isolation of pure culture

The samples were cleaned thoroughly using 5% teepol solution, followed by surface sterilisation using 70% ethanol for 1 min and treatment with 5% sodium hypochlorite for 3 min.[43,44] After surface sterilisation, the samples were washed three times using sterile distilled water and air-dried. Afterwards, the seedlings were cut into small pieces of 1-2 cm in length. The samples were split open longitudinally and the outer green-coloured epidermal portion was removed. Inner 'core' parts were placed on Luria-Bertani agar medium (Titan Biotech, Kerala, India) and incubated for 1 to 2 days at room temperature along with a control plate with sterile water.

Biochemical characterisation of endophytes

The following tests were carried out to characterise the biochemical properties of the bacterial isolates.

a) Test for Indole acetic acid (IAA) production

The bacterial isolates were cultured in Luria-Bertani broth containing L-tryptophan (1 µg/ml) for 24 h.[45,46] Culture supernatant was collected by centrifugation (8000 rpm; 10 min) and 1 ml of it was taken and mixed with freshly prepared Salkowsky reagent (2 ml). The reaction mixture was incubated at 28°C for 25-30 min., and colour change noticed.

b) Cellulase activity

Fresh cultures of the bacterial isolates were spot inoculated on nutrient agar containing carboxy methyl cellulose (0.2%) and incubated at 30°C for 3-4 days.[47] After the incubation, the culture plates were flooded with Congo-red

stain solution (1 µg/ml) for 10-15 min., and washed with NaCl (1 M).

c) Starch hydrolysis

Nutrient agar medium containing soluble starch (0.3%) was inoculated with freshly grown bacterial cultures and incubated for 2-3 days at 30°C. The culture plates were stained with Gram's iodine by flooding.[44]

d) Production of ammonia

For the detection of ammonia production, the bacterial cultures were grown in nutrient broth (30°C for 24-48 h) in a rotary shaker (Remi Instruments, India). After the incubation, Nessler's reagent (0.5 ml) was added to each tube and colour development is noticed.[46]

e) Catalase activity

Fresh bacterial cultures were used to inoculate Yeast extract tryptone broth tubes and incubated for 3 days at 30°C. For testing catalase activity a few drops of 3% H_2O_2 were added to both cultures.[48]

f) Gelatin hydrolysis

Freshly grown bacteria was inoculated on nutrient gelatin medium and incubated for 3 days at 30°C along with control tubes. These tubes were then placed in ice to test the gelatinase activity.[49,50]

g) Phosphate solubilisation

To detect phosphate solubilisation activity, freshly grown bacterial cultures were spot inoculated on Pikovskaya agar medium, and incubated at 30°C for 1-8 days.[51]

Molecular analysis of 16S rRNA region of bacterial isolates

a) Amplification of 16S rRNA

Forward primer 5'-CCGAATTCG TCGACAACAGAGTTTGACCCTGGTTCAG-3'; and Reverse primer 5'-CCCGGGATCCAAGCTTAC GGCTACCTTGTTACGACTT-3' was used to amplify the 16S rRNA gene from the microbe under the following PCR conditions: 98°C - 2 min; 30 cycles of 98°C - 30 sec; 55°C - 30 sec; 72°C - 1 min and Final extension, 72°C - 10 min.

The amplified products were analysed on 0.8% EtBr-Agarose gel.

b) Sequencing of PCR product

The PCR product were purified and subjected to direct sequencing in an automated sequencing machine (ABI prism, Applied biosystems, CA,USA). Forward primer given above was used for the sequencing purpose.

c) BLAST analysis of sequences

Good quality sequences were subjected to BLAST analysis (https://blast.ncbi.nlm.nih.gov). The sequences were searched against nucleotide database of NCBI to identify the bacterial strain.

d) Phylogenetic analysis

Phylogenetic analysis of the current bacterial isolate were carried out based on the 16SrRNA sequences using the software MEGA version 11.[52] Neighbour- Joining Method was used to generate the phylogenetic tree (cladogram).

RESULTS

Isolation and characterization of endophytes from plant tissues

Out of the several bacterial colonies obtained, the bacterial colony which is found fast growing and emerging directly out of the tissues was selected and named as VpR. It was found to have rod-shaped morphology when observed under a microscope. The culture was positive for Gram's stain reaction and also for biochemical tests like cellulase, catalase and ammonia production. The culture did not show any prominent hydrolytic activity for starch or gelatin. Tests for IAA production and Phosphate solubilisation were also negative. A summary of morphological and biochemical characterization is shown in Table. Figure 1 shows the results of the biochemical test for cellulase activity.

16S rRNA amplicon sequence analysis of bacterial isolates

Molecular analysis of bacterial isolates was carried out based on the 16S rRNA region. 16S rRNA region of the bacterial culture samples was amplified and separated on 0.8% EtBr-Agarose gel. Sequencing of the purified PCR products was carried out in ABI prism automated sequencer. The 16S rRNA sequence showed maximum homology to that of *Bacillus subtilis*. The sequence obtained was deposited in GenBank**:** MT968034 (VpR)**.** Result of the phylogenetic analysis is shown as a bootstrap consensus tree in Figure 2. The evolutionary relatedness of the present bacterial

isolate with other bacterial groups can be inferred from this cladogram.

DISCUSSION

The microbiome of mangroves is an underexplored ecological as well as functional component of 'mangrove holobiont'.[15] Several studies have highlighted the importance of microbial associates of mangrove plants which includes both plant protection and plant growth promotion.[53,54] Isolation of salt-tolerant microbes from the mangroves is an example to apprehend the role microbes in mangrove ecosystem which is a unique ecosystem characterized by salinity.[8] They can facilitate the uptake of nutrients like Phosphorus, Nitrogen, etc. which is very much crucial for the plant growth. Many of the resident microbes are capable of producing growth promoting phytohormones like IAA (indole acetic acid) besides the production of hydrolytic enzymes like cellulases. They can also 'prime' the plant defense mechanism.[55] Many bioactive compounds are isolated from mangrove endophytes which could also find applications in agriculture as well as in medical fields besides their role in mangrove ecosystem.[8,54] In general, they are partners in mangrove biology rendering many ecosystem services including nutrient cycling.[13,56-60]

In the present study, isolation and identification of *Bacillus subtilis* from the core tissues of viviparous seedling hypocotyls of *Rhizophora mucronata* is described. The occurrence of *Bacillus subtilis* in the endosphere of the undetached viviparous seedling hypocotyl of *R. mucronata* points to the various possibilities that this endophyte could have in the lifecycle of its host. This particular bacterial culture showed cellulase, catalase and ammonia-producing activities. Ammonia production by endophytic bacteria is a feature that is generally considered to be plant growth promotion in nature,[61] but, contrary views and opinions are also presented by researchers.[62,63] From an ecological point of view, the feature of ammonia production by bacteria and its relevance to mangrove biology will be an interesting study to follow. Microbial cellulase activity is considered to contribute significantly to the carbon cycle at the ecosystem level.[64,65] Catalase activity is generally considered as a cellular mechanism to mitigate abiotic stress, but the endophytic catalase activity observed here could be an attribute of the microbe to adapt itself to the endophytic lifestyle.[66]

Table. Results of morphological and biochemical characterization of bacterial endophytes isolated from *Rizhophora mucronata*. VpR- Viviparous seedling hypocotyl inner core tissue

No.	Reaction	VpR
1.	Gram staining	+
2.	Cell morphology	Rod
3.	Cellulase activity	+
4.	Starch hydrolysis	-
5.	Catalase activity	+
6.	Gelatine hydrolysis	-
7.	Test for IAA production	-
8.	Production of ammonia	+
9.	Phosphate solubilisation	-

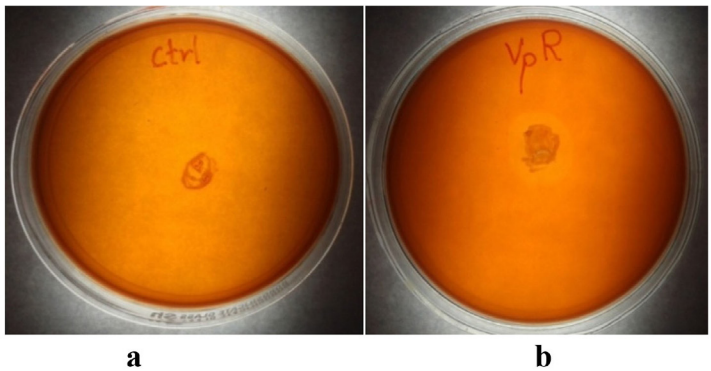

Figure 1. Cellulase activity by endophytic isolates from *Rhizophora mucronata* Lam.
a) Control plate (*E. coli*), b) VpR. The 'clear zone' seen around the colony is due to the cellulolytic activity of bacteria

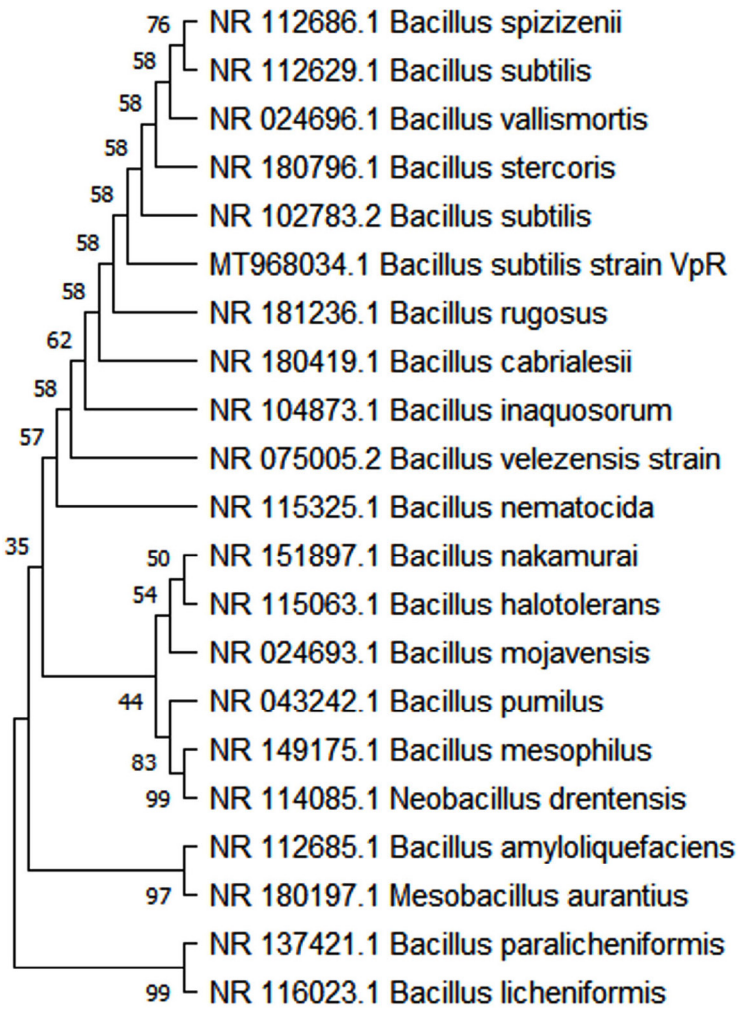

Figure 2. Phylogenetic analysis of Bacterial isolate VpR – *Bacillus subtilis* (MT968034.1).
Bootstrap consensus phylogenetic tree is shown here. The sequences were aligned and analysed using MEGA Software (version 5.0.5). The branching pattern of the tree is derived by Neighbour-Joining method

In most cases, endophytes are known to impart plant-beneficial attributes. They are found to reduce the severity of stress encountered by the host plant.[30-32, 61,67,68] *Bacillus subtilis* and other members of the group *Bacillus*, occur in diverse habitats including endophytic conditions where they contribute to the benefit of host plants, especially in adaptive features like salinity and drought resistance.[69-71] Endophyte-mediated plant resistance has emerged as a successful alternative agriculture strategy in many instances. Various species of *Bacillus*, (*Bacillus cereus*, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus tequilensis*, etc.), in several cases, have been shown to be successful as biocontrol agents in crops.[29,36,37,54,68,70, 72-74]

The term 'holobiont' is conceptualized as 'a single dynamic entity' formed of the functionally related partners – the macroorganism host and its associated or interacting microbes.[23-26] Endophytes of these salt-tolerant trees are important partners of the 'holobiont' without which the mangrove biology will be incomplete. Besides, the microbiome component of the mangrove ecosystem is a promising resource for novel microbes with hitherto unknown attributes or features with biotechnological and agricultural application potential.[7, 75-79]

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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