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Isolation and Molecular Characterization of Beneficial Rhizobacteria from the Mangrove Ecosystem of Poovar, South Kerala, India

Devi R.R.[1*] and Sugunan V.S.[2]

[1]*Department of Zoology; NSS College, Pandalam*
[2]*Department of Zoology; University College Thiruvananthapuram, Kerala, India*
**Email: devirjayan@gmail.com*

Abstract

Mangroves are buffer zones between land, fresh water and the sea. Mangrove plants dominate the biomass and are subjected to a high degree of fluctuations in terms of abiotic conditions. Such a demanding environment poses the need for adopting various survival strategies by the inhabitants. In the case of mangrove flora, symbiotic relationships with bacteria are the most frequently adopted fitness strategy. A consortium of bacteria occupying the immediate soil cover of plant roots, known as rhizosphere, is called rhizobacteria. Root exudates of plants maintain selective consortia of rhizobacteria which could enhance the nutrient acquisition, disease resistance and stress tolerance of host plants. In this study, rhizobacterial colonies were isolated in pure culture from the rhizosphere of the mangrove plant *Avicennia officinalis*, from the mangrove ecosystem of Poovar, South Kerala, India. The bacterial colonies were isolated by serial dilution and spread plating. The 16 S rRNA of the selected bacteria were isolated, sequenced and analyzed for homology, with the standard sequences available in NCBI Genbank. Species-level identification was accepted with a similarity index e" 98%. The beneficial bacteria identified from the rhizosphere were *Bacillus licheniformis* (98.16%), *Bacillus subtilis* (98%) and *Lysinibacillus fusiformis* (98.37%). The isolates are beneficial for the host plant as they aid in phosphate solubilization, nitrogen fixation, nematicidal and antifungal efficiency and horizontal transfer of desirable genes. In general, rhizobacteria contribute to the bioremedial potential of the selected mangrove ecosystem.

Keywords: Rhizosphere, Phosphate, Gene transfer, Vegetation

1. Introduction

Mangroves are heterogeneous ecosystems rich in plants, animals and microorganisms. Mangroves render several ecological services in the biogeochemical cycling of nutrients and minerals and maintenance of local climate (Prakash *et al.*, 2015). Complex plant-animal-microbe interactions exist in mangrove ecosystems in which the participants are mutually benefitted. The most common among them is the plant-bacterial interactions, which assist the plant partner in better nutrient acquisition, disease resistance and growth amidst the fluctuating environment (Kloepper & Schroth, 1981). These beneficial bacteria can inhabit many of the available microhabitats, including the host plant's rhizosphere, phyllosphere, or endosphere (Kloepper & Beauchamp, (1992).

Hiltner coined the term rhizosphere in 1904 to describe the part of the soil that is influenced by plant roots. Rhizosphere can extend 5mm or more as a series of gradients of organic substrates, pH, O_2, CO_2 and water (Dodd, 2009). Essentially two regions are recognized in the rhizosphere – 1) the rhizosphere soil and 2) the rhizoplane soil, the soil in direct contact with the plant root. The rhizosphere harbours diverse consortia of microorganisms that interact with each other and with the plant root. These interactions affect the growth and physiology of the plant and the physical and chemical properties of the soil. In recent years, rhizobacteria have been used to promote plant growth and crop yields and biotechnological applications (Osorio, 2007).

Mangrove plant roots produce various compounds like exudates, secretions, lysates and plant mucilage which favour the growth of their bacterial partners (El Zahar *et al.*, 2008) and, in return, the rhizobacteria play significant roles in mediating nutrient decomposition for plant growth. Rhizobacterial consortia render a beneficial influence on host plant growth and metabolism. Hence, this study has been undertaken to isolate and identification of the bacterial colonies residing in the rhizosphere of the mangrove plant *Avicennia officinalis* from the mangrove ecosystem of Poovar, South Kerala India. Molecular characterization of the isolates based on 16S rRNA sequencing is envisaged for the species-level identification and functional appreciation of the isolates.

2. Materials and Methods

A. Study site and sampling: Poovar mangrove (Fig.1) lies very close to Vizhinjam, a natural harbour in Thiruvananthapuram, Kerala, India. The geographical location is 81^0 09' 054''N 77^0 03'44.6'' E. The dominant vegetation is the mangrove plant *Avicennia officinalis* (Fig.2a). For the isolation of viable rhizobacteria, rhizospheric soil samples were aseptically collected in triplicates from the rhizosphere zone of *Avicennia officinalis* (Fig. 2b&c) during the low tide of summer of 2019 and transferred to the laboratory in an icebox, within 3hours for isolation.

B. Isolation of rhizobacteria: Using a sterile blade, intact roots were cut out from a depth of 10-30 cm, without losing

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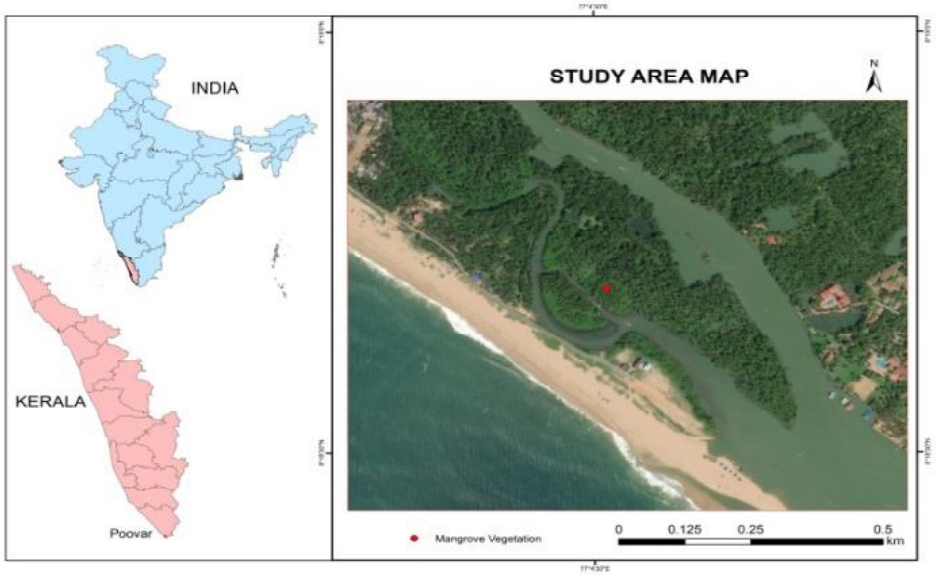

Fig. 1. Study site

the soil and transferred to the laboratory in sterile sample collection bottles. 1 g of rhizosphere soil was mixed with 10 mL sterile distilled water and vortexed vigorously for 10 min. The resulting suspension was serially diluted to 10^{-2}, and 100 µl aliquot was spread plated in triplicate onto nutrient agar plates and incubated at room temperature for 24 hrs (Dweipayan *et al*., 2014). Colonies with distinct morphology were streak plated on agar slants for the propagation of pure culture.

C. 16S rRNA Isolation: The bacterial suspension was centrifuged at 5000 rpm, mixed with 10 microliter Tris-EDTA buffer (pH 8.0) and centrifuged again until a pellet was obtained. The Tris-EDTA buffer and lysozyme were added to the bacterial pellet and then incubated at 37°C for 30 minutes. The cells were lysed by adding 3 microliters of 10% Sodium dodecyl sulphate (SDS) and three microliters of acetyl trimethyl ammonium bromide. The aqueous phase was extracted using chloroform: isoamyl alcohol (24:1) and centrifuged at 10,000 rpm. Isopropanol (0.6ml) was added to the supernatant, and the mixture was again centrifuged at 10,000 rpm for 5min, after which the supernatant was removed. The ethanol washed, air-dried pellets were suspended in Tris - EDTA buffer and stored at 4°C until used (Cheneby et *al*., 2000; Nishiguchi *et al*., 2002).

D. Molecular characterization and identification of rhizobacterial isolates: The 16 S rRNA isolated from the selected bacteria was sequenced using Universal primer 27 F (5'-AGAGTTTGATCATGGCTCAG-3') as the forward primer, following standard protocols (Rajapandi *et al*., 2016). The raw data of codon sequences were compared with the DNA database deposited in the NCBI Library using Megablast programmer. Species-level identification of the clone was accepted when the 16S rRNA sequence had a similarity of ≥ 98 % with that of the prototype sequences.

Fig. 2(a, b, c). Rhizosphere of *A. officinalis*

3. Results and Discussion

The bacterial isolates obtained in culture varied significantly in terms of colony morphology, Gram staining responses and cellular morphology (Fig.3, Table 1), indicating the presence of various species of bacteria in the rhizosphere consortia. Based on 16S rRNA sequence homology the isolates were identified as *Bacillus licheniformis* (B 1), *Bacillus subtilis* (B 2) and *Lysinibacillus fusiformis* (B 3). The BLAST analysis summary and the GenBank accession numbers of the isolates are presented in Table 2.

In this investigation, the rhizobacteria identified from the mangrove plant *A. officinalis* belonged to the genus *Bacillus*. *Bacillus* occupies a dominant position in mangrove rhizobacterial consortia, since their metabolic versatility and morphological plasticity and adaptability allow them to better survive under the harsh, demanding, and ever fluctuating ecosystem of mangrove rhizosphere, with beneficial contributions towards the growth and survival of the host plant (Newton *et al.*, 2010). The benefits offered by *Bacillus sp.* include their ability to synthesize a vast array of beneficial substances, phosphate solubilization, nitrogen fixation, biocontrol attributes like production of HCN, siderophore, hydrolytic enzymes and antibiotics, resistance against insects, nematodes and fungus attack (Tian *et al.*, 2007; Kaki *et al.*, 2013) and increase in nutrient availability (Samuel *et al.*, 2011). *B. subtilis* and *B. licheniformis* are phosphate solubilizers and indispensable for mangrove plants since mangroves are limited phosphate ecosystems and available phosphorus for plant absorption solely depends on bacterial metabolism (Guedon *et al.*, 2005). *B. licheniformis* is reported to render nematicidal and antifungal efficiency to the host plant and produce plant growth promotion factors (Vazquez *et al.*, 2000; Insunza *et al.*, 2002). The presence of *L. fusiformis* in the mangrove rhizosphere is attributable to its ability to enhance the salt tolerance of host plants (Damodaran *et al.*, 2019). *B. subtilis* plays a significant role in plant disease suppression and acts as a vector for the horizontal transfer of genes for nodule formation and disease resistance (Kinsella *et al.*, 2009).

B. subtilis, *B. licheniformis* and *L. fusiformis* produce resistant endospores, and the spore-forming ability may be a reason for the dominance of *Bacillus* in the rhizosphere (Shahanawaz *et al.*, 2016). *B. subtilis* release resuscitation promotion factors (Rpf). In mangroves, the probiotic bacterial consortia occupying the host-associated niches adopt spore formation to overcome the environmental adversities and the Rpf released by *B. subtilis* are required for the revival of the cells in dormancy (Commichau & Halbedel, 2013). *B. subtilis is* also identified as a potential bioinoculant agent for *P. vulgaris and M. phaseolina*, associated with root rot disease (Kumar *et al.*, 2012). Polythene degrading ability of *L. fusiformis* is detected in the studies of Shahanawaz *et al.* (2016), and their presence at the study site accounts for the highly investigated biomedical potentials of mangrove ecosystems. The study by Rojas *et al.* (2001) report *B. licheniformis* as a tool to enhance reforestation and restoration of dwindling mangrove forests, since prior-plantation treatment of mangrove seedlings with *B. licheniformis* result in better and faster growth.

Since *Bacillus* is a polyphyletic taxon, molecular taxonomy is considerably useful in their systematic positioning (Wang, 2009). This genomic heterogeneity accounts for the metabolic versatility of *Bacillus*, which is of environmental relevance (Yadav *et al.*, 2010). The identified bacteria can aid plants in better nutrient acquisition, disease resistance and growth amidst fluctuations and also offer tremendous biotechnological applications. Mangroves thus act as repositories of highly beneficial bacteria, especially those which can be used in our agricultural sector. Mangrove rhizosphere bacteria can also be used for mangrove restoration. Further studies are

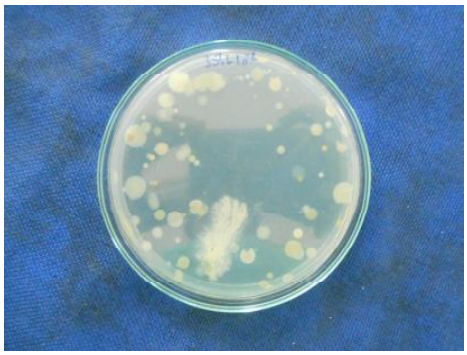

Fig. 3. Isolated rhizobacterial colonies on Petri plate

Table 1. Colony and cell morphology of rhizobacterial isolates

Sl No:	Sample code	Colony morphology	Gram staining morphology	Spore formation
1	B1	Punctiform, convex, off-white colonies with entire margin	+ve, rod	+
2	B2	Circular, mucoid, pulvinate White colonies with entire margin	+ve, rod	+
3	B3	Irregular, Light brown undulate colony	+ve, rod	+

Table 2. BLAST analysis summary, identification and 16S rRNA sequence accession numbers of selected rhizobacteria

Sl No:	Sample code	% Identity of 16S rRNA sequence	Identification	GenBank Accession No:
1	B 1	98.16	*Bacilus licheniformis*	NR 074923.1
2	B 2	98	*Bacillus subtilis*	NR112686.1
3	B 3	98.37	*Lysinibacillus fusiformis*	NR112569.1

required for the isolation and identification of such useful bacteria and their characterization to exploit their full potential for bioremediation, bio farming, and derivation of probiotic molecules.

4. Conclusion

Rhizosphere of plants maintain a stable consortium of bacteria for better exploiting their environment in terms of nutrient uptake, inhibition of pathogenic microbes and exchange of desired genetic sequences. Here the rhizosphere of the plant *A. officinalis* from the mangrove ecosystem of Poovar, South Kerala, is benefitted by the presence of *B. licheniformis*, *B. subtilis* and *L. fusiformis*, among their rhizobacterial consortia. The identified bacterial species are highly beneficial for the host plant, and they also help in ecosystem maintenance and restoration.

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Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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