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Devi R R

Vinod Prabhakar

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Abstract

Microhabitats are smaller pockets of ecosystems with unique conditions and they serve as major contributors of species richness. Mangrove plants offer a diverse array of such microhabitats (rhizosphere, phyllosphere, endosphereetc), and the inhabitant bacteria benefit the host by providing better salinity tolerance, faster and better nutrient mobilization, protection from phytopathogens and assistance in seed germination. Identification of such beneficial bacteria,their growth requirementsandmetabolism, willhave direct application in the field of agriculture and ecosystem restoration. Hence in this study, effort has been made to elucidate the seasonal numerical and compositional profile of pneumatophore bacterial consortia of *Avicennia officinalis*, from the mangrove ecosystemof Poovar, South Kerala, India. The physico-chemical parameters of mangrove water, micronutrient availability within the pneumatophore and the count,morphologyand biochemical characteristics of the endosymbiont bacterial consortia were analyzed simultaneously. Significant variation is observed across the rainy and the non-rainyseasons, in the water parameters and in the endobacterial CFU count. Antagonistically,the micronutrient contentand the endobacterial composition of the pneumatophore remained stable across the seasons. The biochemicalprofile of the endobacterial consortia illustrated the metabolic co-dependence and complementarity among the host and the symbiotic consortia,which substantiate the need for such a solid core bacterial consortium within the plant host. Further studies are warranted in this lane to trace the beneficial metabolic pathways of symbiotic bacterial strains, which could find wide application in the field of agriculture and human health.

Keywords

Pneumatophore, bacteria, biochemical, endosphere, symbiotic.

RESEARCH PAPER

Study on the Seasonal Endobacterial Profile of *Avicennia officinalis*, Focusing on Core Bacterial Consortia Within Mangrove Pneumatophores

Devi R. Radhakrishnan [a,*], Vinod Prabhakar [b]

[a] Department of Zoology, NSS College Nilamel, Kerala, India
[b] Department of Zoology, NSS College, Pandalam, Kerala, India

Abstract

Microhabitats are smaller pockets of ecosystems with unique conditions and they serve as major contributors of species richness. Mangrove plants offer a diverse array of such microhabitats (rhizosphere, phyllosphere, endosphere etc), and the inhabitant bacteria benefit the host by providing better salinity tolerance, faster and better nutrient mobilization, protection from phytopathogens and assistance in seed germination. Identification of such beneficial bacteria, their growth requirements and metabolism, will have direct application in the field of agriculture and ecosystem restoration. Hence in this study, effort has been made to elucidate the seasonal numerical and compositional profile of pneumatophore bacterial consortia of *Avicennia officinalis*, from the mangrove ecosystem of Poovar, South Kerala, India. The physico-chemical parameters of mangrove water, micronutrient availability within the pneumatophore and the count, morphology and biochemical characteristics of the endosymbiont bacterial consortia were analyzed simultaneously. Significant variation is observed across the rainy and the non-rainy seasons, in the water parameters and in the endobacterial CFU count. Antagonistically, the micronutrient content and the endobacterial composition of the pneumatophore remained stable across the seasons. The biochemical profile of the endobacterial consortia illustrated the metabolic co-dependence and complementarity among the host and the symbiotic consortia, which substantiate the need for such a solid core bacterial consortium within the plant host. Further studies are warranted in this lane to trace the beneficial metabolic pathways of symbiotic bacterial strains, which could find wide application in the field of agriculture and human health.

Keywords: Pneumatophore, Bacteria, Biochemical, Endosphere, Symbiotic

1. Introduction

Symbiotic bacteria live in plant-provided microhabitats and form positive, negative, or neutral associations with their hosts. Mangrove plants support an extremely diverse array of such bacteria, since they grow in a harsh, ever fluctuating environment, where such inter-communal relationships remain crucial for survival. The pneumatophore, or negatively geotropic roots, of mangrove plants, is a particularly interesting microhabitat because it exposes bacteria to both the stable microhabitat provided by the plant and the seasonal physico-chemical fluctuations in the mangrove water caused by porosity and tidal submergence. Though a multitude of studies have addressed the plant-bacteria relationships in the mangroves [1−4], majority of them had discussed individual beneficial/harmful species [5,6]. Lacunae are felt at addressing the interesting questions like 1) how the symbiotic bacteria of the mangrove plants respond to the fluctuations in the environment of the host? And 2) are they responding as individual cells or as a single core consortium? Understanding such response patterns will find application in sectors like agriculture, health and animal husbandry, where the use of probiotic bacteria, which occupy specific microhabitats, is gaining considerable momentum.

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* Corresponding author.
E-mail address: devirjayan@gmail.com (D.R. Radhakrishnan).

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Therefore, the goal of this study is to examine how seasonal variations in the physico-chemical and nutritional conditions of the mangrove ecosystem affect the numerical and compositional profile of the pneumatophore bacterial consortia.

2. Materials and methods

The samples were collected and analyzed during the summer, pre-monsoon, monsoon and post monsoon seasons of 2019, from the mangrove ecosystem of Poovar (81°09′054″N77°03′44.6″E), South Kerala, India. The pneumatophore bacterial consortia were isolated from the mangrove plant *Avicennia officinalis* (Fig. 1).

2.1. Physico-chemical parameters analyzed

Secondary data on precipitation and relative humidity were received from the Meteorological Department, Thiruvananthapuram substation Kerala. Water turbidity, temperature, pH, dissolved oxygen (DO), biochemical oxygen demand (BOD), total hardness (TH) and total dissolved solids (TDS) were measured following the protocol IS 3025 (part 10, 38 & 16 respectively), across the seasons.

2.2. Micronutrient analysis of pneumatophores

The elemental composition of the pneumatophore samples was analyzed using SEM-EDAX (AMETEK,USA), at a resolution of 126.7eV (set using octane plus detector) and an applied tension of 20 KV.

2.3. Isolation and colony forming unit (CFU) count of pneumatophore consortia

Intact pneumatophore was cut at a length of 8–10 cm from the top using a sterile blade and transported to the laboratory in sealed sterile containers. The pneumatophore was surface sterilized following the 3 steps protocol discussed by Ref. [6]. The surface sterilized samples were extracted with sterile distilled water and serially diluted and pour plated on standard agar plates and incubated at room temperature. CFU was taken after 24 h of incubation, at 10^{-5} dilution.

2.4. Biochemical profiling and identification of isolated bacteria

Isolated bacterial colonies were metabolically analyzed using the Vitek 2 system, an automated microbiology system working on growth-based technology. The instrument uses advanced photometric technology to determine individual biochemical reactions contained in colorimetric microbial identification cards, named reagent cards. Vitek system has four reagent cards, each specified for a particular group of bacteria – 1. GN card (Gram negative fermenting and non-fermenting bacilli) 2. GP card (Gram positive cocci and non-spore forming bacilli) 3. BCL card (Gram positive spore forming bacilli) and 4. YST card (Yeast & yeast like organisms). Reagent cards were designed based on the reactions standardized in *Bergey's manual of determinative bacteriology*, to measure the unique identifying metabolic features of specific groups of bacteria. Each reagent card has 64 wells. Each well contains an individual test substrate conjugated with a pH indicator and each measures a specific metabolic activity like carbon source utilization, enzyme production, alkalinization, acidification, growth in the presence of inhibitory substances and antibiotic resistance. Metabolic reactions within the wells result in pH change, which is identified by the colour change of the indicator. An optically clear

Fig. 1. Pneumatophores of A. officnalis.

film is present on both sides of the card and the colour intensity is readily measured by the machine's internal optical system. Positive and negative responses towards the substrates were recorded using the Vitek 2 database and the elucidated biochemical profiles of the unknown bacterial isolates were compared with the standardized profiles in the database for identification. The biochemical fingerprinting of bacterial isolates followed 5 steps:

a) Reagent card selection: Reagent cards were selected on the basis of the Gram staining response of colony members. In this study three types of Reagent cards were used, namely GP card, GN card and BCL card. For the Gram-positive bacilli isolates 45 biochemical reactions were carried out using the BCL card, for the Gram-negative rods 47 reactions were carried out using the GN Card and for the Gram-positive cocci 43 reactions were carried out using the GP cards.

b) Sample preparation: The isolated pure colonies were suspended in 3 ml of 0.5 % sterile saline solution (pH 4.5 to 7) in 12 × 75 mm clear polystyrene test tubes and the turbidity of the suspensions was adjusted to the McFarland turbidity range, as required by the selected reagent cards (Table 1). Turbidity was checked using Densicheck™.

c) Reagent card inoculation: The reagent cards were inoculated with the bacterial suspension automatically, within the vacuum chamber of the Vitek analyzer. For card inoculation the aseptically prepared suspension tubes of the unknown bacteria were placed on to the cassette in the Vitek analyzer. Each cassetteaccommodates 15 test tubes. The cassettes were automatically shifted into the vacuum chamber of Vitek analyzer and the microbial suspension from each test tube was pumped through the transfer tubes into the micro-channels of respective reagent cards that fill all the wells in it.

d) Card sealing and incubation: The inoculated reagent cards were sealed by cutting off the transfer tube and were transferred into the incubation chamber of the analyzer. Cards were incubated at 35 °C for 7–14 h.

e) Reaction reading and identification: Each card was automatically removed from the carousel incubator every 15 min of incubation and was transferred to the optical system of the analyzer for the colorimetric reading of test results. Each card was returned to the chamber after reading, and the procedure was repeated until the completion of incubation time. The positive and negative responses towards the substrates were recorded and were compared with the standard database available in the Vitek system software. Similarity above 99 % with the standard database biochemical profile was accepted for identification of the unknown bacterial isolate at the species level.

Table 1. McFarland turbidity range for reagent card inoculation.

Reagent Cards Used	MC Farland turbidity range for Inoculation (NTU)
BCL	1.80–2.20
GN	0.50–0.63
GP	0.50–0.63

2.5. Statistical analysis

The seasonal fluctuations in the variables were assessed with Two-way ANOVA and Tukey's test (with 95 % family-wise confidence level), using Vegan package in R and the corresponding numerical response pattern of the pneumatophore bacteria was visualized using line diagram in MS Excel 2010 version.

3. Results and discussion

The physico-chemical parameters of the mangrove water (Table 2) and the endobacterial CFU count (Fig. 2) varied significantly across the rainy (Monsoon and post-monsoon) and the non-rainy

Table 2. The seasonal measures of physico-chemical parameters of mangrove water.

Measured parameters	Summer	Pre-monsoon	Monsoon	Post monsoon
Precipitation (mm)	15.9	118.80	188.10	282.00
Relative humidity (%)	78	75	81	78
Water salinity (0/00)	28.74 ± 1	18 ± 0.9	17.96 ± 1.5	15.52 ± 1.36
Water pH	8 ± 0.09	7.35 ± 0.1	7.29 ± 0.15	6.78 ± 0.09
Water temperature	26 ± 1.8	23 ± 0.09	21 ± 0.08	18.6 ± 1
Turbidity (ntu)	0.3 ± 0.01	0.9 ± 0.02	1.7 ± 0.05	1.5 ± 0.05
BOD (mg/l)	8.39 ± 0.23	13.47 ± 2.05	16.56 ± 1.2	20.23 ± 2.09
DO (mg/l)	8.8 ± 1.05	7.01 ± 0.96	5.1 ± 0.91	4 ± 0.05
Total hardness (mg/l)	336.2 ± 2	89 ± 1.76	73 ± 2.0	66 ± 2.16
TDS (g/l)	1.5 ± 0.97	1.84 ± 0.77	2.27 ± 0.98	2.83 ± 1.03
Cfu of pneumatophore bacteria (10^{-5}dilu)	95 ± 4.2	89 ± 2.26	62 ± 2.08	60 ± 1.5

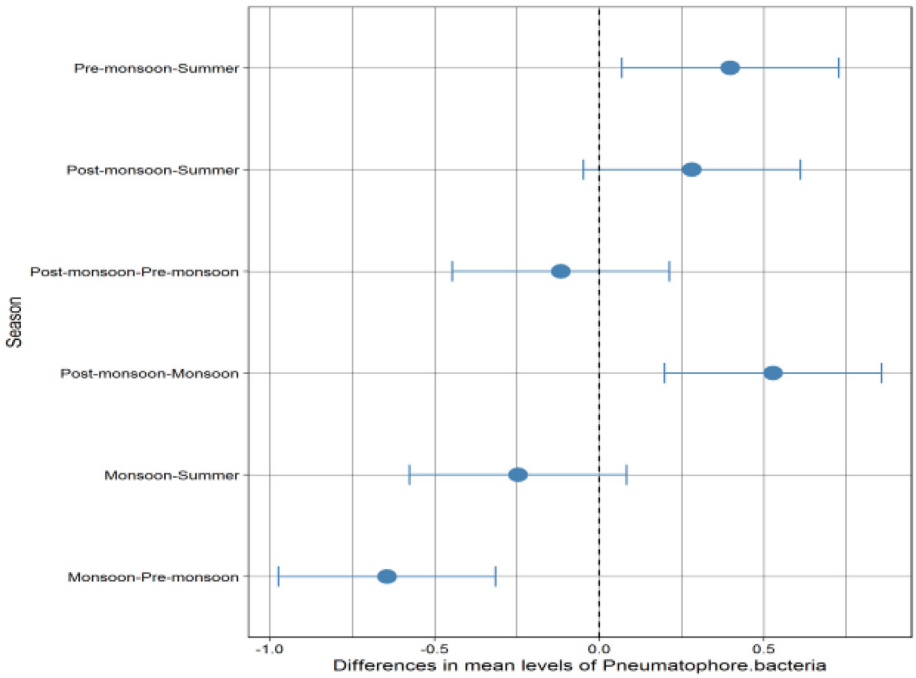

Fig. 2. Tukey's plot of seasonal pneumatophore bacterial CFU.

seasons (summer and pre-monsoon), but the element overlay (Fig. 3(a-d)) of the pneumatophore samples during the meantime showed no statistically significant variation. This observation clearly indicates that the bacterial consortia within the pneumatophore show growth response tothe changes in the water parameters although their microhabitat ensured a steady nutrient supply. During the summer and pre-monsoon of the study period, the rainfall was below 120 mm whereas during the monsoon and the post monsoon it ranged above 180 mm.

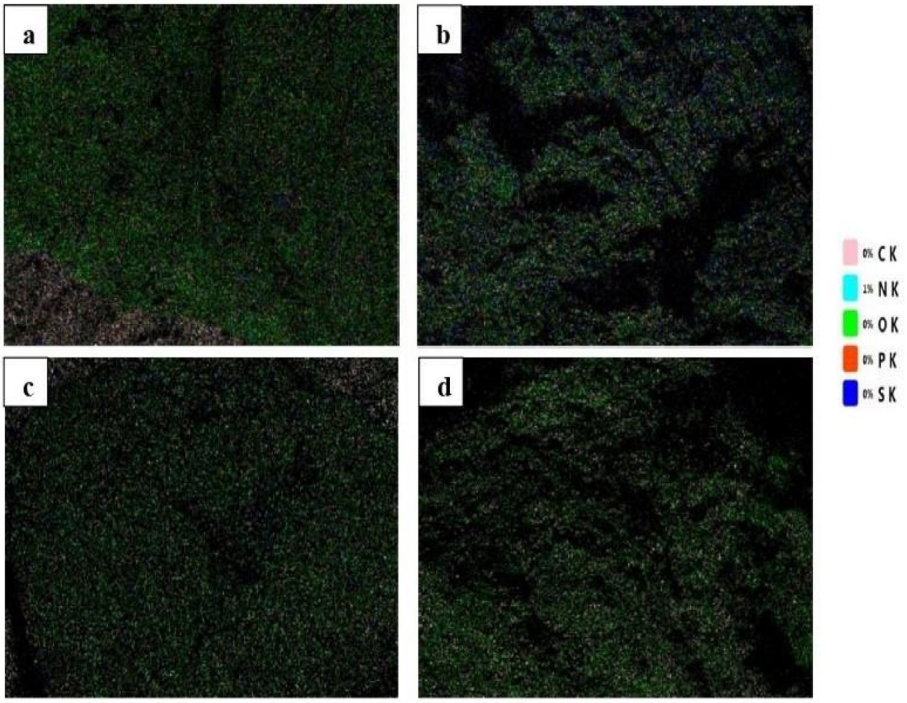

Fig. 3. Element overlay of pneumatophore samples during a) summer b) Pre-monsoon c) Monsoon & d) post-monsoon.

Since precipitation underlies the chances of freshwater influx and sea water inundation in mangrove ecosystems [7], whilst it accounts for the significant variation recorded in the water parameters. The rate of photosynthesis and oxygen diffusion in the pneumatophore declines with submergence [8] and hence, accounts for the fall in the CFU count of aerophilic bacteria during the rainy seasons. Here the observed normal pencil morphology and the steady mineral profile of the pneumatophore confirm the pristine nature of the study site and rules out chances of population fluctuations due to pollution [9].

The seasonal numerical response pattern of the endobacterial consortia was analogous with the physico-chemical fluctuations of water (Fig. 4), but compositionally it remained stable throughout the year, with 14 identified species (Table 3). Though the lack of comparable studies limits the discussion on this observation, it does support the earlier studies on plant-microbe relationships, where the host is recorded to maintain a stable internal environment to outlast a consortium of desired endosymbionts. The ability of mangrove pneumatophore to resist the extreme environmental fluctuations through salt exclusion, salt excretion [10,11] and gas diffusion [12], further strengthen the existence of a stable pneumatophore endosphere.

Since pneumatophore contains enzyme inhibitors and cytotoxic substances [13], the persistence of a core endobacterial consortium, amidst the seasonal fluctuations, demands metabolic plasticity and complementarity among the host and the bacterial consortia. The elucidated metabolic profile of the pneumatophore consortia included the enzymes arylamidases (leucine, proline, tyrosine, alanine, aspartate, L-pyrolydonyl), α-glucosidase, galactosidase, lipases and β N acetylglucosaminidase. The consortia were found positive for the utilization of sucrose, D-maltose, D-cellobiose, citrate, sorbitol, adonitol, L-arabitol, palatinose, esculin and

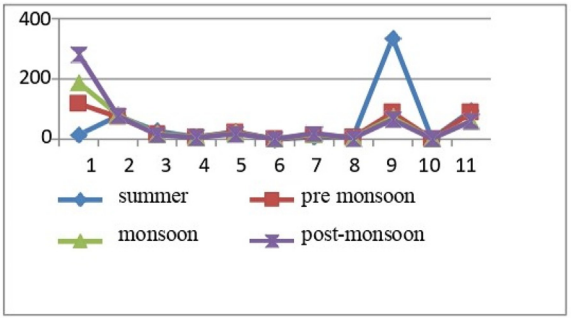

Fig. 4. Seasonal numerical response pattern of pneumatophore endobacterial consortia.

Table 3. Species composition of the pneumatophore bacterial consortia.

Slno:	Bacterial species
1	*Pseudomonas medocina*
2	*Aeromonas salmonicida*
3	*Aeromonas sobria*
4	*Kocuria rhizophila*
5	*Brevundimonas diminuta*
6	*Sphingomonas spiritivorum*
7	*Sphingomonas paucimoilis*
8	*Bacillus pumilus*
9	*Pasteurella canis*
10	*Aeromonas caviae*
11	*Pseudomonas stutzeri*
12	*Comamonas testosteroni*
13	*Aeromonas taiwanensis*
14	*Pseudomonas alcaligenesis*

succinate, as their carbon sources. This broad metabolic profile of the bacterial consortia complements the host since, pathways involving glucosidases and arylamidases are involved in the synthesis of secondary metabolites of host preference [14]. Glutamylarylamidase and lipase activity was found in the pneumatophore consortia and is attributable to the regulatory role of plant-microbe interaction on bacterial enzyme production. Surprisingly Beta galactopyranosidase was absent in the pneumatophore even when the consortium use xylose. The reason for such an observation could be that the bacteria were either depending on the pyranosidase of host plant [15] or was selectively up taking the monosaccharides in the furanose form or had pathways to bypass the steps catalyzed by pyranosidase. The detection of D-xylose in the pneumatophore consortium confirms the observation by Ref. [16] that unlike L-xylose, D-xylose exists in furanose form in nature. Our study confirms the earlier works of, suggesting metabolic co-evolution among the host and the endosymbionts. Co-evolution of partners, thus establish stable, functionally efficient and dynamically regulated communities under fluctuating ecosystems. Natural selection favoring such efficient functional associations, ultimately contribute towards the functional superiority of mangrove ecosystems. Understanding such fundamental biological interactions among the mangrove microhabitats and the resident bacterial consortia will definitely help in managing mangrove ecology and conservation practices.

4. Conclusion

This study addresses the numerical and compositional fluctuation patterns of the symbiotic bacterial consortia that inhabit the pneumatophore of the mangrove plant *A. officinalis*, across the seasons. The

numerical composition of the pneumatophore consortia varied in accordance with the changes inthe physical and chemical parameters of mangrove water but the composition of the bacterial consortium remained stable. The micronutrient availability during the mean time was also maintained stable by the host plant. Though the pneumatophore bacterial consortium is directly exposed to the fluctuations in the mangrove water, the results suggest that the bacterial consortium is responding to these fluctuations as a single unit, thus making numerical adjustments to resist compositional alterations. The metabolic profile of the identified bacteria indicates co-evolution of the host and the symbiotic bacterial consortium, which explains the need for maintaining such a core bacterial consortium. Similar studies can generate knowledge to tackle the problem of maintaining beneficial bacteria within symbiotic microhabitats that can have wide range of application in the field of agriculture, health and animal husbandry.

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Ethical information

This work don't present data on plant or animal experiments, hence ethical clearance is not applicable.

Disclosure statement

The authors declare that no competing interest exists.

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