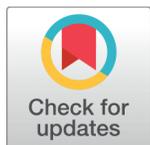


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Diversity of Endophytic Bacteria from Marine Associated Plant Leaves

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Abstract

Objectives: To study endophytic bacterial population from 3 marine plants such as *Avicennia marina*, *Salicornia brachiata* and *Suaeda maritima*. **Methods:** The fresh leaves from the above plants were collected at Kattumavadi, Kottaipattinam and Sethubhavachathiram mangrove ecosystems during the period of December month. The leaves were surface sterilized and the inner tissues of the leaf bits were macerated and serially diluted for the isolation of bacterial endophytes and grown on Nutrient Agar (NA) Petri plates. The endophytic bacteria grown in the nutrient agar was identified with the help of gram staining and biochemical assessments. The statistical method of endophytic bacterial diversity was measured in phrases of Shannon's and Simpson's indices. **Findings:** Absolutely 219 colonies are recorded in three marine plant leaves consisting of *Avicennia marina* (109), *Salicornia brachiata* (52) and *Suaeda maritima* (58). The nine microorganisms were recognized and denoted by letter code such as BMBT 1 to BMBT 9 consisting of *Bacillus amyloliquefaciens*, *B. Megaterium*, *B. Subtilis*, *Pseudomonas aeruginosa*, *Corynebacterium sp*, *B. licheniformis*, *Citrobacter sp*, *Halomonas sp* and *Mycobacterium sp*. The results confirmed that most of the bacterial diversity belonged to *Bacillaceae* family. Endophytic bacterial range indices have been measured using a statistical method, which revealed variations in plant variety and species richness. The Simpson's diversity indices of 3 marine leaves inclusive of *Avicennia marina* (0.345), *Salicornia brachiata* (0.368) and *Suaeda maritima* (0.341) have been cited with the maximum for *Salicornia brachiata* in comparison with different plant leaves. **Novelty:** These novel natural merchandise from marine *Bacillus sp* have been employed for the use of drugs, pesticides, carotenoids, and tools for bioremediation of heavy metallic toxicity.

Keywords: Endophytic bacteria; *Avicennia marina*; *Salicornia brachiata*; *Suaeda maritima*; Shannon and Simpson diversity; *Bacillus subtilis*

1 Introduction

Endophytic bacteria are endosymbionts that reside in the tissues of the host plant that does not show any harmful effects to the plant and are found to be highly beneficial to the host. These creatures are typically symbiotic and sometimes their association could be obligate or facultative with the host plant. It is generally agreed upon that the endophytes, which are ubiquitous organism colonizers, can play a crucial part in controlling host growth and development, including improved yield and phytoremediation. Recent research has revealed that endophytes have the potential to be used in biomedicine since they can create a wide range of bioactive secondary metabolites, including alkaloids, peptides and steroids. Numerous studies have demonstrated how microbial communities facilitate plant defense and their significant positive effects on host plants, including enhanced nutrient uptake, accelerated growth, resilience against pathogens, and improved resistance to abiotic stress like heat, drought, and salinity^(1,2). Considering their extensive plant growth-promoting activities, endophytes synthesize phytohormones, antimicrobial substances, and many agro-based bioactive metabolites that can be utilized as a safe and affordable alternative to chemical pesticides and fertilizers⁽³⁾. Most plant species possess one or more endophytic bacteria or fungi, significantly contributing to microbial diversity. It is best and most productive to isolate critical bacterial strains from crop wild relatives to use the endophytic bacterial isolates. Only a few plants have been thoroughly studied for their endophytic biodiversity till now. The endophytic microorganisms in terrestrial plants have been well examined, and it has been discovered that they have antimicrobial, anticancer, antiviral, antidiabetic, and antioxidant properties^(4,5). However, there has been little progress in isolating endophytic microbes from marine plants. In some instances, bacterial endophytes can also hasten the emergence of seedlings and aid in the establishment of plants under adverse environmental conditions⁽⁶⁾. Studies on endophytic bacteria from mangrove halophytic plants are also understudied. The main goal of earlier research on endophytes was to identify culturable microorganisms and understand how they function in the environment. Therefore, the current study is the first report on the endophyte diversity of *Avicennia marina*, *Salicornia brachiata* and *Suaeda maritima* plants in Kattumavadi, Manakkadu, and Sethubhavachathiram mangrove forests.

2 Methodology

2.1 Sample collection

The young leaves of selected halophytic plants were collected from Kattumavadi (10.1283° N, 79.2240° E), Kottaiappattinam (9.9800° N, 79.1972° E), and Sethubhavachathiram (10.2490° N, 79.2780° E), Tamil Nadu, India. The sample is stored in unopened plastic bags to avoid exposure to moisture. The authentication of plant species was given by Dr. M. Ayyanar, Assistant Professor, Department of Botany and Microbiology, A.V.V.M. Sri Pushpam College, Poondi-Thanjavur, Tamil Nadu, India.

2.2 Isolation of endophytic bacteria

In order to get rid of surface-contaminating bacteria, collected leaf samples were extensively surface-sterilized with 70% ethanol and air-dried inside a laminar-flow chamber. The outer tissue of the sample was cut away using a sterile knife blade, and the interior tissues of the sample were removed, excised, and macerated with sterile distilled water using a mortar and pestle. The macerated materials were serially diluted, plated in triplicates in nutrient agar medium, and cultured for 24 hours at 37 °C in an incubator. The colonies were counted using a colony counter after achieving discernible growth. Different heterotrophic bacterial strains were chosen based on their morphology and streaked onto a nutrient agar slant for subsequent use.

2.3 Identification of bacteria

The isolated endophytic bacteria were identified by standard manuals such as Bergey's Manual of Determinative Bacteriology for identifying bacteria⁽⁷⁾.

2.4 Statistical analysis

The diversity index is a mathematical measure of species diversity in a given community which is computed based on the species richness (number of species present) and species abundance (number of individuals)⁽⁸⁾. There are two indices: dominance and information statistic, which provide information about species in a specific community.

The Shannon index is an information statistic index, which assumes all species are represented in a sample and randomly sampled⁽⁹⁾. In contrast, the Simpson index refers to a dominance index because it gives more weight to common or dominant species. The following equations (1) to (6) are used to determine the species richness, species evenness, and diversity indices:

Margalef’s richness Index (D_M) = $\{(S-1)/\log N\}$ —————→ Eqn . (1)

Menhinick’s richness Index (I) = S/\sqrt{N} —————→ Eqn . (2)

Pielou’s Evenness (J) = $[H'/\ln(S)]$ —————→ Eqn . (3)

McIntosh’s Evenness (E) = $\{(\sum x_i^2)/(\sqrt{[(N-S+1)^2+(S-1)])}\}$ —————→ Eqn . (4)

Shannon’s Diversity Index (H') = $-\sum p_i \cdot \ln p_i$ —————→ Eqn . (5)

Simpson Diversity Index (D) = $\sum p_i^2$ —————→ Eqn . (6)

Where **S** is the number of species, **N** is the total number of individuals found, **x** is the numeric vector of species counts, **p** is the proportion of individuals in one particular species (**n**) divided by the total number of individuals found (**N**), and the range of **i** value lies between 1 and **S**⁽¹⁰⁾.

Where, **p** is the proportion (**n/N**) of individuals of one particular species found (**n**) divided by the total number of individuals found (**N**), Σ is still the sum of the calculations, and **S** (number of species) **N** (total number of individuals) Σ (sum) of p_i^2 (**n/N**) 2Σ (sum) of $p_i \ln p_i$.

3 Results and Discussion

The halophytic plants were collected from the following sites, as marked in Figure 1 and which include Kattumavadi, Kottaiappattinam, and Sethubavachatram of the eastern coastal regions of Tamil Nadu.

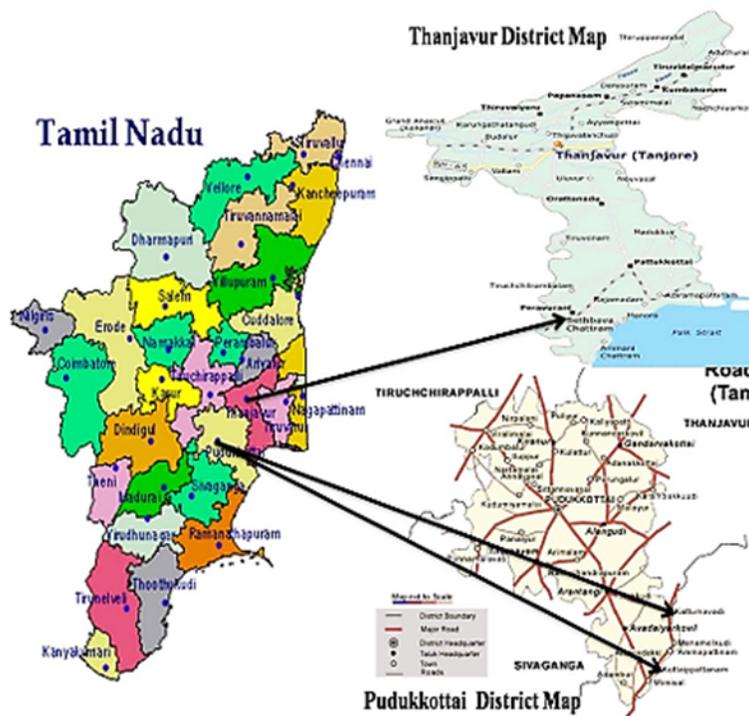


Figure 1: Sites chosen to study the Endophytic Microbial Diversity of Mangrove Ecosystem

Fig 1. Sites chosen to study the Endophytic microbial diversity of Mangroove ecosystem

The pure cultures were then isolated by serially diluting the leaf extracts and plating them in nutrient agar plates. The colonies obtained from three different plants were harvested from three remote locations, and the pure cultures obtained from different bacterial species are stated in Tables 1 and 2. The maximum number of colonies (109 CFUs/mL) was obtained from the *Avicennia marina* compared to the other two species of plants.

Table 1. Isolation of endophytic bacteria from marine-associated plants leaves on the east coast of Tamilnadu, India

Places	Total no. of colonies (CFU/ml)		
	<i>Avicennia marina</i>	<i>Salicornia brachiata</i>	<i>Suaeda maritima</i>
Kattumavadi	29	10	15
Kottaipattinam	33	18	23
Sethubavachatram	47	24	20
TOTAL	109	52	58

Table 2. Identification of bacterial strains from Endophytic bacteria from different places

S.No	Places	<i>Avicennia marina</i>	<i>Salicornia brachiata</i>	<i>Suaeda maritima</i>
1	Kattumavadi	BMBT1, BMBT2, BMBT3	BMBT2, BMBT3	BMBT1, BMBT5
2	Kottaipattinam	BMBT4, BMBT5,	BMBT1, BMBT4, BMBT5	BMBT6, BMBT7
3	Sethubavachatram	BMBT6, BMBT7, BMBT9	BMBT6, BMBT8, BMBT1	BMBT2, BMBT9, BMBT1

The grown cultures were subjected to a series of biochemical assays to identify the taxonomic classification of the isolated endophytic bacteria. The outcomes of those biochemical assays are listed in Table 3.

Table 3. Identification of endophytic bacteria from marine - associated plants leaves

Biochemical test	BMBT1	BMBT2	BMBT3	BMBT4	BMBT5	BMBT6	BMBT7	BMBT8	BMBT9
Gram staining	+	+	+	-	+	+	+	-	+
Motility	+	+	+	+	-	+	+	+	-
Catalase test	+	+	+	+	+	+	+	-	+
Urease test	-	+	-	-	-	-	±	+	-
Citrate test	±	+	-	+	+	±	+		+
Indole test	+	-	-	-	-	-	±	+	-
Utilization of Glucose	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	-	+		-	+	+
Maltose	+	+	+	-	+	+	+	+	+
Sucrose	-	-	±	-	+	+	+	+	+
Methyl red test	+	-	-	-	-	+	-	+	-
Voges Proskauer	+	-	±	-	-	+	-	+	-

Notations: (+) Positive; (-) Negative; (±) invalid

Based on the above results, the isolated endophytic bacterial strains were identified as BMBT1 - *Bacillus amyloliquefaciens*, BMBT2 - *Bacillus megaterium*, BMBT3 - *Bacillus subtilis*, BMBT4 - *Pseudomonas aeruginosa*, BMBT5 - *Corynebacterium sp.*, BMBT6 - *Bacillus licheniformis*, BMBT7- *Citrobacter sp.*, BMBT8 - *Halomonas sp.*, BMBT9 - *Microbacterium sp.*,

The Diversity indices were computed based on equations (1) to (6) for the endophytic bacteria obtained from three distinct halophytes and were documented in Tables 4, 5 and 6. The Simpson diversity for *Avicennia marina*, *Salicornia brachiata* and *Suaeda maritima* leaves were found to be 0.345, 0.368 and 0.341 respectively.

The use of endophytes to mitigate abiotic challenges like salt and drought is a field of study that has not been thoroughly investigated. Despite extensive research on endophytes over the past three decades^(11,12), little is known about how they contribute to plant resilience to salinity and drought⁽¹³⁾. Endophytes from halophytic plants should be isolated, identified, and studied in order to create bio inoculants that will help with salt soil phytoremediation and reduce stress on the plants that are thriving in these salt-affected settings.

Numerous studies have described endophytic bacteria from the genera *Bacillus*, *Serratia*, *Pantoea*, and *Stenotrophomonas* as present in diverse plant tissues⁽¹⁴⁻¹⁷⁾. All of the strains found in this study were related to previously identified species that were known to promote plant growth⁽¹⁸⁾. These findings are substantially in conformity with the bacterial diversity found in the outcomes of our research. *Bacillus sp*, *Pseudomonas sp.*, *Corynebacterium sp*, *Citrobacter sp*, *Halomonas sp*, and *Microbacterium*

Table 4. Diversity indices of Endophytic Bacteria from *Avicennia marina* leaves

Places	No. of. Genera	No. of. Species	Richness Indices		Evenness Indices		Diversity Indices		
			d	I	J	E	DMg	H'	D
Kattumavadi	29	3	5.968	2.777	0.006	0.400	25.486	0.011	0.070
Kottaipattinam	33	2	6.821	3.160	0.006	0.483	46.166	0.004	0.091
Sethubavachatram	47	4	9.805	4.501	0.006	0.330	33.181	0.007	0.184
TOTAL	109	9	22.595	10.44031	0.018217	1.213312	104.8349	0.021618	0.345

Notations: d - Margalef's Richness Index; I - Menhinick's Richness Index; J - Pielou's Evenness Index; E - McIntosh's Evenness Index; DMg - Margalef's Diversity Index; H' - Shannon's Diversity Index; D - Simpson's Diversity Index.

Table 5. Diversity indices of Endophytic Bacteria from *Salicornia barchiata* leaves

Places	No. of. Genera	No. of. Species	Richness Indices		Evenness Indices		Diversity Indices		
			d	I	J	E	DMg	H'	D
Kattumavadi	10	2	2.277	1.386	0.036	0.583	12.984	0.040	0.036
Kottaipattinam	18	3	4.302	2.496	0.029	0.426	15.474	0.028	0.119
Sethubavachatram	24	3	5.820	3.328	0.026	0.409	20.935	0.016	0.213
TOTAL	52	8	12.401	7.211103	0.0912	1.418911	49.39382	0.083403	0.368

Notations: d - Margalef's Richness Index; I - Menhinick's Richness Index; J - Pielou's Evenness Index; E - McIntosh's Evenness Index; DMg - Margalef's Diversity Index; H' - Shannon's Diversity Index; D - Simpson's Diversity Index.

Table 6. Diversity indices of Endophytic Bacteria from *Suaeda maritima* leaves

Places	No. of. Genera	No. of. Species	Richness Indices		Evenness Indices		Diversity Indices		
			d	I	J	E	DMg	H'	D
Kattumavadi	15	2	3.447	1.969	0.018	0.538	20.197	0.018	0.066
Kottaipattinam	23	2	5.418	3.020	0.015	0.504	31.739	0.008	0.157
Sethubavachatram	20	3	4.679	2.626	0.016	0.420	17.294	0.023	0.118
TOTAL	58	7	13.545	7.615773	0.048892	1.461394	69.23157	0.047839	0.341

Notations: d - Margalef's Richness Index; I - Menhinick's Richness Index; J - Pielou's Evenness Index; E - McIntosh's Evenness Index; DMg - Margalef's Diversity Index; H' - Shannon's Diversity Index; D - Simpson's Diversity Index

sp are among the many bacterial species recognized by the current study.

Endophytic bacterial diversity was quantified using Shannon (H) and Simpson (1-D) diversity indices, which showed differences in species richness between cultivated and wild rice. Shannon and Simpson indices with higher values are indicative of more varied populations. Cultivated (H = 2.718, 1-D = 0.930) and wild (H = 1.946, 1-D = 0.857) rice roots had high indices. This might be explained by the fact that most endophytic bacteria come from the soil. Bacteria can live and obtain nutrients from the host plant in the rhizosphere of the root⁽¹⁹⁾. The mangrove ecosystem's halophytes were found to have Shannon and Simpson diversity indices of H' = 0.315 and 1-D = 0.91 for the *Avicennia marina*.

It's possible for bacteria in the rhizosphere to invade and colonize plant roots. The species richness of the microbial population in the rhizosphere plays a significant role in the quantity and diversity of endophytes found in a host plant⁽²⁰⁾. Some rhizoplane-colonizing bacteria have a lower bacterial density than those colonizing the roots of plants, and some of these strains may migrate to the stem and leaves of plants⁽²¹⁾.

In various literature study, a decline in the endophytic population was seen from the root through the stem to the leaf. The cause may be because most endophytes enter plant tissue through the root, while only a small number can enter xylem vessels through the Casparian strip. The bacterial population diminishes from root to stem and leaf as the few microbes that enter the xylem vessels migrate slowly toward the apical regions of the plant⁽²²⁾. According to a study by Prakamhang⁽²³⁾, rice roots have the highest density of endophytic bacteria compared to other regions of the plant. Most often, endophytic and rhizospheric bacteria enter the plant through root hairs using active or passive mechanisms. But only a limited part of the root endophytes is able to penetrate the endoderm and colonize other plant compartments using xylem vessels. Here in the present research work, mangrove leaves showed the richness in microbial diversity and hence this would open doors to employ microorganisms for various biotechnological applications and such bacteria can carry special genetic and metabolic determinants that are more suitable for an endophytic lifestyle⁽²⁴⁾. It is also possible that at the stage of colonization, the plant is able to select endophytic strains that have certain useful properties, including growth stimulation. *Bacillus sp.* strains had strong

antifungal and plant growth-promoting properties, making them potential candidates for bio-inoculants that could help with salt soil phytoremediation and reduce the stress that salt exposure causes to plants in salt-affected habitats. This will create new possibilities for the efficient application of grown types of growth-promoting bacteria isolated from halophytic plants to deal with biotic and abiotic stresses on plants and improve their salt resistance^(25,26).

4 Conclusion

In this study, we present the results of the first in-depth analysis of the endophytic community composition in halophytic plants from the Tamil Nadu mangrove forests of Kattumavadi, Kottaipattinam, and Sethubhavachathiram. Our findings suggested that the endophytic microbial populations vary in samples obtained from various geographical locations. The leaves of *Salicornia brachiata* showed a comparatively high diversity of microbiota. It was discovered that *Bacillus sp.* predominated in the microbial population. To identify their unique traits, pathogenicity, and benefits in the synthesis of bioactive substances, more research is required. For future investigation of the microbial diversity and technological applications, our findings provide new insight into the microbiota of mangrove leaves. On investigation with the bacterial communities associated with halophytic plants as *Suaeda maritima*, *Salicornia brachiata*, and *Avicennia marina* and the halophyte, *Suaeda maritima* showed richness in diversity of species. The results of this study could lay the groundwork for future research into the role of numerous growth-stimulating activities. This research also demonstrates that halophytes in salterns and other high-salinity environments represent a vast and untapped source of new microorganisms that may be useful in high-salinity environments. Our research indicates that halophytes are useful for discovering microorganisms that can improve host plant growth and health in salt-affected soils.

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