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Original Article

Distribution pattern analysis of epiphytic bacteria on ethnomedicinal plant surfaces: A micrographical and molecular approach

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ABSTRACT

The epiphytic bacterial community prevalent on ethnomedicinal plant surfaces were studied for their diversity, niche localization and colonization using the micrographical and molecular approaches. Scanning electron microscopy (SEM) revealed the presence of large aggregates of bacterial communities. The bacterial localization was observed in the grooves along the veins, stomata and near the trichomes of leaves and along the root hairs. A total of 20 cultivable epiphytes were characterized which were analyzed for richness, evenness and diversity indices. Species belonging to the genera *Bacillus* and *Pseudomonas* were the most abundant. *Bacillus thuringiensis* was the most prevalent epiphyte with the ability to form biofilm, as a mode of adaptation to environmental stresses. Biofilm formation explains the potential importance of cooperative interactions of epiphytes among both homogeneous and heterogeneous populations observed under SEM and influencing the development of microbial communities. The study has revealed a definite pattern in the diversity of culturable epiphytic bacteria, host-dependent colonization, microhabitat localization and biofilm formation which play a significant role in plant–microbe interaction.

1. Introduction

Plants maintain a complex system where bacterial communities interact continuously supporting the development of large microbial population. Epiphytic bacteria are capable of living (i.e., multiplying) on plant surfaces [1] and their colonization is controlled by biological factors such as the host plant growth and the life cycle of epiphytes [2,3]. Epiphytes occupy a narrow ecological niche because of their existence at the interface of vegetation and atmosphere, a variation in climatic conditions including moisture, humidity, temperature, wind speed, radiation and rainfall may influence epiphyte diversity [33]. Exploitation of ethnomedicinal plants for their natural products has resulted in loss of epiphytic diversity harboured on novel ethnomedicinal plants. The purpose of this investigation is to determine the sites and extent of growth of epiphytic microflora on selected ethnomedicinal plants on the leaf and root surfaces and to characterize the diversity of culturable epiphytic bacteria associated with these plants using microscopic (SEM) and molecular techniques.

Epiphytic bacterial aggregates on plant surfaces may vary in size and composition depending on nutrient availability at a given site [4,5]. These aggregates have characteristics similar to those cells in biofilms that are described in aquatic and medical environments [6]. Biofilm consists of multilayered cell clusters embedded in a

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complex matrix comprising of a variety of extracellular polymeric substances (EPS) [7] which facilitates the adherence of epiphytes to other microbial cells and to plant surfaces. Plants incorporate epiphytes in the strategy to survive under stressed conditions [8] and thus, it is more likely that the phytoepiphytes play an eminent role in plant defence and other environmental stresses. The knowledge of plant associated bacteria is important for the study of the effect of pathogens, symbionts and commensals on their hosts.

2. Materials and methods

2.1. Plant sample collection

Healthy plants used by different traditional medicinal practitioners (TMPs) were collected from different parts of Meghalaya (N – 25°26.737′, E – 091°44.737′; N – 25°36.904′, E – 091°54.121′) based on their ethnomedicinal usages. *Rubia cordifolia*, *Centella asiatica*, *Potentilla fulgens*, *Acmella oleracea* and *Houttuynia cordata* are used by the tribal people of Meghalaya, India as folk remedies for treating a variety of ailments [9]. The taxonomic identity of the plants was confirmed with the help of Herbarium Curator of the parent University. All samples were collected in sterile polythene bags and brought to the laboratory and used for isolation within 24 h of collection.

2.2. Sample preparation for scanning electron microscopy

The occurrence of bacteria on the surface of plants was observed using a scanning electron microscope (JSM-6360, Jeol). The plant material for observation was prepared according to a modified version of Baker's method. Fresh plant fragments were cut into approximately 1 cm^2 pieces and fixed by immersion in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for at least 1 h. The samples were drained and placed in three consecutive 1 h washes of 0.1 M cacodylate buffer. Samples were then stored in fresh cold cacodylate buffer for transport to the electron microscopy laboratory. Samples were dehydrated in a series of acetone–water washes for 15 min each and critical-point dried with liquid CO_2. Finally, samples were sputter coated with a thin layer of gold–palladium and the distribution of bacteria on the plants and their morphological differentiation were observed at several magnifications under the scanning electron microscope.

2.3. Isolation of epiphytic bacteria

To analyze the epiphytic microbiota, plant samples were washed thoroughly with tap water followed by sterile double distilled water [10]. Each plant sample was cut aseptically into 1 cm long segments using a sterile blade under the laminar flow hood and allowed to dry. The cut surfaces of plant segments were placed on Petri plates containing Nutrient Agar (NA) media (Himedia, India). Each plant segment was inoculated in triplicate. Plates were then incubated at 32 °C for 48 h. Colonies with different morphology and pigmentation were randomly selected from each plate and streaked on fresh NA plates as described above. Simultaneously, the pure isolates were preserved in 20% glycerol at −20 °C for further studies.

2.4. Molecular characterization of the isolates

Total genomic DNA was extracted using HiPurA™ bacterial and yeast genomic DNA Isolation Kits (Himedia, India). PCR amplification and sequencing of 16S rRNA gene was carried out in a 25 μl reaction mixture. Using general primers 27F 5′-AGAGTTTGATCCTGGCTGAG-3′ and

Table 1
Epiphytic bacteria isolated from ethnomedicinal plants of Northeast India showing biofilm production.

Host plants	Epiphytes isolated	Isolate name	GenBank Accession no.	% Similarity	Biofilm formation
Rubia cordifolia	*Citrobacter youngae*	ME5	JX390623	99.2	+
	Bacillus thuringiensis	MEA7	JN418875	100	+
	Raoultella ornithinolytica	ME11	JX390624	99.8	–
	Enterobacter soli	MEA10	JN680692	99.8	–
Centella asiatica	*Bacillus tequilensis*	CEN3E	JN628288	99.9	–
	Bacillus aryabhattai	CEN5E	JN628290	100	–
	Bacillus thuringiensis	CEN6E	JN628291	100	+
	Pantoea eucalypti	CEN7E	JN628292	99.3	+
Potentilla fulgens	*Bacillus thuringiensis*	POT1	JQ281538	99	+
	Pseudomonas palleroniana	POT2	JQ281539	99	+
	Serratia nematodiphila	POT3	JQ281540	99	+
	Stenotrophomonas maltophilia	POT5	JQ281541	99	+
Acmella oleracea	*Pseudomonas mosselii*	Y9	JQ446443	100	+
	Pantoea eucalypti	Y5	JQ446440	99.6	+
	Pseudomonas putida	Y6	JQ446441	99.5	+
	Bacillus thuringiensis	Y7	JQ446442	99.9	+
Houttuynia cordata	*Lysinibacillus xylanilyticus*	F1	JN418870	100	–
	Bacillus thuringiensis	F41	JX390622	99.9	+
	Enterobacter asburiae	F7	JN418868	100	–
	Acinetobacter johnsonii	F8	JN418869	100	–

+, Capable of biofilm formation; –, incapable of biofilm formation.