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Isolation and identification of phyllosphere bacteria from three different crops

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ABSTRACT

The phyllosphere represents the habitat provided by the aboveground parts of plants. There have been few studies on the phyllosphere of plants. A large number of microbes are associated with the phyllosphere, where bacteria are the most dominant. The main objective of the present study was to isolate phyllosphere bacteria from three different crops cultivated in the Jaffna district. Phyllosphere bacteria were isolated by washing the host leaf and plating on nutrient agar media. Two different phyllosphere bacteria, namely, Pseudomonas putida and Staphylococcus equorum and two different species of the genus Bacillus (B. cereus and B. pumilus)were isolated from Zea mays. Staphylococcus epidermis and two different species of Bacillus (B. cereus and B. subtilis) were isolated from Solanum lycopersicum. Two different phyllosphere bacteria, namely, Lactobacillus brevis and Micrococcus luteus, and three different species of Bacillus (B. subtilis, B. mycoides and B. licheniformis) were isolated from Brassica oleracea var. capitata. The isolates were identified based on colony morphological characteristics and biochemical tests.

Keywords: *Phyllosphere, Bacteria, Zea mays, Solanum lycopersicum, Brassica oleracea var. capitata*

Introduction

The aerial habitat colonized by microbes is known as the phyllosphere, and the inhabitants are called epiphytes. There are several studies on the inhabitants of flowers and buds [1]. Microbes can be found in different segments of plants as epiphytes on the surface and as endophytes inside plant tissues [2-4]. Most of the research on phyllosphere microbiology has focused on leaves. The phyllosphere environment is commonly occupied by diverse microbes, such as bacteria, filamentous fungi, and yeast, where bacteria are the dominant microorganisms. Bacteria are the most abundant microorganisms on leaves, ranging from 106 to 107 cells/cm^2[2,5]. The phyllosphere microbes facilitate carbon dioxide fixation and the release of molecular oxygen and thus facilitate primary productivity. They can also fix N, absorb minerals,and decrease disease severity [6]. Phyllosphere microbial communities are significantly different from airborne microbial communities, although they are in direct contact with the atmosphere surrounding plants. Bacterial endophytes normally complete their life cycle within host plants without causing damage to the plant, but their multiplication might be limited by the innate immune system of host plants [7]. The phyllosphere can be further subdivided into caulosphere (stems), phylloplane (leaves), anthosphere (flowers), and carposphere (fruits). Various types of microorganisms are found on plant surfaces and in the surrounding environment. Some are beneficial, while others are harmful to plants as plant pathogens [8]. Each leaf creates a specific environment in which only some microorganisms can thrive. This selective effect of leaves on microbial communities can be termed the phyllosphere effect. Phyllosphere interactions depend on environmental factors, host genotypes, and microbial communities. Factors such as water availability, leaf surface topology, nutrient availability, leaf age, plant species, and leaf physiology. On the surface of the leaves, different morphological parts, such as trichomes, veins, cell wall junctions of the epidermis and stomata, are abundant in the microbial community. Therefore, these regions are protected by phyllosphere bacteria from UV radiation as well as other environmental extremes [8-10].

Most phyllospheric bacteria act as commensals without any known effect on their plant host, but multiple bacteria establish a mutualistic relationship with plants. Host plants supply nutrients and provide shelter to bacteria, and bacteria can promote the growth of host plants and confer resistance against insects, pests, and pathogens. The phyllosphere environment is commonly occupied by diverse microbes, such as bacteria, filamentous fungi, and yeast, where bacteria are the dominant microorganisms. Plant growth is promoted by phyllosphere bacteria. Phyllosphere bacteria can suppress and stimulate the colonization and infection of tissues by plant pathogens [4,11]. The global surface area of the phyllosphere has been estimated to be more than 4 x 10^8 km^2, with bacterial populations in this region of 10^{26} cells [12]. Different bacteria have been isolated from different plant sources, such as carrots, tomatoes, soybeans, cabbage, citrus, pineapple, clover, maize, and wheat [13].Epiphytic bacterial populations differ significantly in size within the plants of the same species [14].These variations are caused by fluctuations in the leaf environment and nutritional conditions.The leaf bacteria differs from the root bacteria. Previous studies showed that pigmented bacteria which are rarely found in the rhizosphere are observed to dominate the leaf surfaces [15].

Materials and Methods

Collection of samples

Sample collection was performed in two seasons: summer (August) and rainy (January). Three healthy leaves of maize (Zea mays), tomato (Solanum lycopersicum),and cabbage (*Brassica oleracea* var. *capitata*) were collected in sterile UV bags from separate fields at the Thirunelvely Agricultural Research Station and Thinnai Organic Farm using a random sampling method. Some parameters of the field, such as temperature, humidity, longitude, and latitude, were measured.

The collected samples were brought to the laboratory in ice bags.

Isolation of phyllosphere bacteria

The collected leaves were cut into equal sizes by using a sterile scalpel and imprinted on nutrient agar (NA) media. Replicates were also prepared for each sample. Then, the plates were labeled and incubated at 37°C for 24-48 hours to observe bacterial growth.

Subculturing of phyllosphere bacteria

Surface and hand sterilization were performed using a spirit homogenizer. Spirit lamps wereused.The individual bacteria that grew from the edges of the leaf segments were removed by using a sterile inoculating loop and streaked on fresh NA media. Then, the plates were incubated at 37°C for 24-48 hours. Single colonies of isolates were purified by subculturing on nutrient agar media, and the process was repeated until pure bacterial isolates were obtained, as confirmed by microscopic examination.

Storage of bacterial cultures

After the growth of pure bacterial isolates on plated nutrient agar media, they were streaked on nutrient agar slants and stored in a refrigerator at 4°C-5°C. These stored bacterial cultures were sub-cultured in NA media and used whenever they were required for the assay.

Identification of phyllosphere bacteria by colony morphological characteristics and biochemical tests

Colony Morphological study was done on the basis of shape, elevation, texture, margin, colour, size and pigmentation of colonies. The phyllosphere bacteria were grown on NA medium and incubated at 37°C for 24-48 hours.

Preliminary tests were done, such as Gram staining, endospore staining, capsule staining, motility test, catalase test, and oxidase test.

Biochemical tests such as starch hydrolysis, sugar fermentation tests, citrate utilization tests, gelatin hydrolysis, nitrate reduction test, indole test, and MR-VP test were carried out.

Results and Discussion

The selection of representative colonies was based on their growth on NA media, staining, and microscopic examination. Phyllosphere bacteria were identified through culture and morphological tests (Fig. 1, Table 1, and Table 2), and their biochemical properties were also studied (Table 3).

B1 was isolated from *Zea*mays and *Solanum lycopersicum*. B2 was isolated from *Zea mays.* B3 was isolated from *Solanum lycopersicum* and *Brassica oleracea* var. *capitata.* B4 and B5 were isolated from *Brassica oleracea* var. *capitata.* P1 was isolated from *Zea mays.* S1 was isolated from *Zea mays.* S2 was isolated from *Solanum lycopersicum.* L1 was isolated from *Brassica oleracea* var.*capitata*, and M1 was isolated from *Brassica oleracea* var. *capitata.*

The results from the biochemical characterization are presented in Table 3. The microbial communities of the phyllosphere are diverse, supporting numerous genera of bacteria, filamentous fungi, yeast, algae, and, in some situations, protozoans and nematodes [4]. Bacteria are the most numerous and diverse colonists of leaves, with culturable counts ranging between $10^2$and 10^{12} cells/g leaf. Ten isolates from the phyllosphere were identified based on biochemical tests and colony morphology as described in Bergey's Manual of Determinative Bacteriology.

The identified phyllosphere microorganisms are shown in table 1, table 2, and table 3.

B1- *Bacillus cereus*
B2-*Bacillus pumilus*
P1-*Pseudomonas putida*
S1-*Staphylococcus equorum*
B3-*Bacillus subtilis*
S1-*Staphylococcus epidermidis*
L1-*Lactobacillus brevis*
B4-*Bacillus mycoides*
M1-*Micrococcus luteus*
B5-*Bacillus licheniformis*

UV bags were used to collect the leaf samples to prevent contamination. An ice box was used to prevent dryness of the samples. Healthy leaves were selected for the isolation of beneficial phyllosphere bacteria. A random sampling method was used to collect samples to confirm the uniform distribution of particular isolates. To assess seasonal variation, samples were collected in the summer and rainy seasons. Organic fields were selected to obtain the natural microbial population.

The leaf imprint technique was used to isolate phyllosphere bacteria. This approach is a favorable method for isolating epiphytic microorganisms. *Bacillus cereus, Bacillus pumilus, Pseudomonas putida,*and *Staphylococcus equorum* were isolated from *Zea mays. Bacillus cereus, Bacillus subtilis,* and *Staphylococcus epidermidis* were isolated from *Solanum lycopersicum*. *Bacillus subtilis, Lactobacillus brevis, Bacillus mycoides, Micrococcus luteus* and *Bacillus licheniformis* were isolated from *Brassica oleracea* var. *capitata.*

These ten isolates can be confirmed at the species level by molecular identification in future studies.

The phyllosphere represents a group with immense agricultural and environmental significance. Many studies have investigated the important interactions of phyllosphere microorganisms that may affect the fitness of plant populations and the productivity of crops. Phyllosphere bacteria can promote plant growth and may suppress the colonization of tissues by plant pathogens. Further studies should be performed to confirm whether phyllosphere bacteria protect plants from herbivores and whether they can promote drought tolerance in plants. If this study is performed, it would be beneficial to tropical countries that have long-term dry climates.

Acknowledgments
Not applicable.

Table 1: Pure cultures obtained from Zea mays, Solanum lycopersicum and Brassica oleracea var. capita

Plant	Pure culture
Zea mays	Isolate B1 Isolate B2 Isolate P1 Isolate S1
Solanum lycopersicum	Isolate B1 Isolate B3 Isolate S2
Brassica oleracea var. *capitata*	Isolate B3 Isolate L1 Isolate B4 Isolate M1 Isolate B5

B1, B2, B3, B4 and B5: Bacillus species. P1: Pseudomonas species. S1 and S2: Staphylococcus species. L1: Lactobacillus species. M1: Micrococcus species.

Table 2: Colony morphology and culture characteristics of phyllosphere bacteria

Isolate	Colony morphology and culture characteristics
B1	Large, Feathery, Dull, White, Granular with less wavy edge, Opaque with a rough surface, Irregular margin, Convex.
B2	Opaque, Off-white, Roughly circular with irregular margin, Slightly filamentous appearance, Convex.
P1	Translucent and cream colour, Convex and round with smooth, entire margin, appear shiny but not mucoid.
S1	White, Opaque, entire margin, Convex, Circular colony.
B3	Fuzzy white, rough Opaque, Circular colony, flat elevation, Swarming edge.
S2	White, raised, opaque, smooth colony. Spindle form, entire margin.
L1	Rough/intermediate, flat, white, smooth, opaque colony. Entire margin, Convex.
B4	Hairy colony with characteristic swirls, white, opaque, rhizoid colony.
M1	Bright yellow colony, circular, convex, smooth with dull/glistening surface.
B5	Irregular shape, irregular (undulate fimbriate) margin, Rough and wrinkled, hair like growth, white and opaque.

Table 3: Biochemical test results for phyllosphere bacteria

Biochemical test	B1	B2	P1	S1	B3	S2	L1	B4	M1	B5
Gram's stain	+	+	-	-	+	+	+	+	+	-
Endospore stain	+	+	-	-	+	-	-	+	-	+
Catalase	+	+	+	+	+	+	-	+	+	+
Motility	+	+	+	-	+	-	-	-	-	+
Oxidase	-	-	+	-	+	-	-	-	+	+
Shape	R	R	R	S	R	S	R	R	S	R
Starch hydrolysis	+	-	-	-	+	-	+	-	-	+
Glucose	+	-	+	+	+	+	+	-	+	+
Lactose	-	-		+	+	+	+	-	-	+
Mannitol	-	+	-	+	+	-	-	-	+	+
Sucrose	+	-	+	+	+	+	+	-	+	+
Arabinose	-	+	+	+	+	-	+	-	-	+
Citrate	+	+	+	-	+	-	-	-	-	+
Gelatin hydrolysis	+	+	+	-	+	-	-	-	+	+
Nitrate reduction	+	-	+	+	+	+	-	+	-	-
Indole	-	-	+	-	-	-	-	-	-	-
Methyl red	-	+	-	-	-	-	-	-	-	+
Voges-Proskauer test	+	+	-	-	+	+	-	-	-	+

+ and – in the above table indicate positive and negative results, respectively, for the biochemical tests. R: rod shape. S: spherical shape.

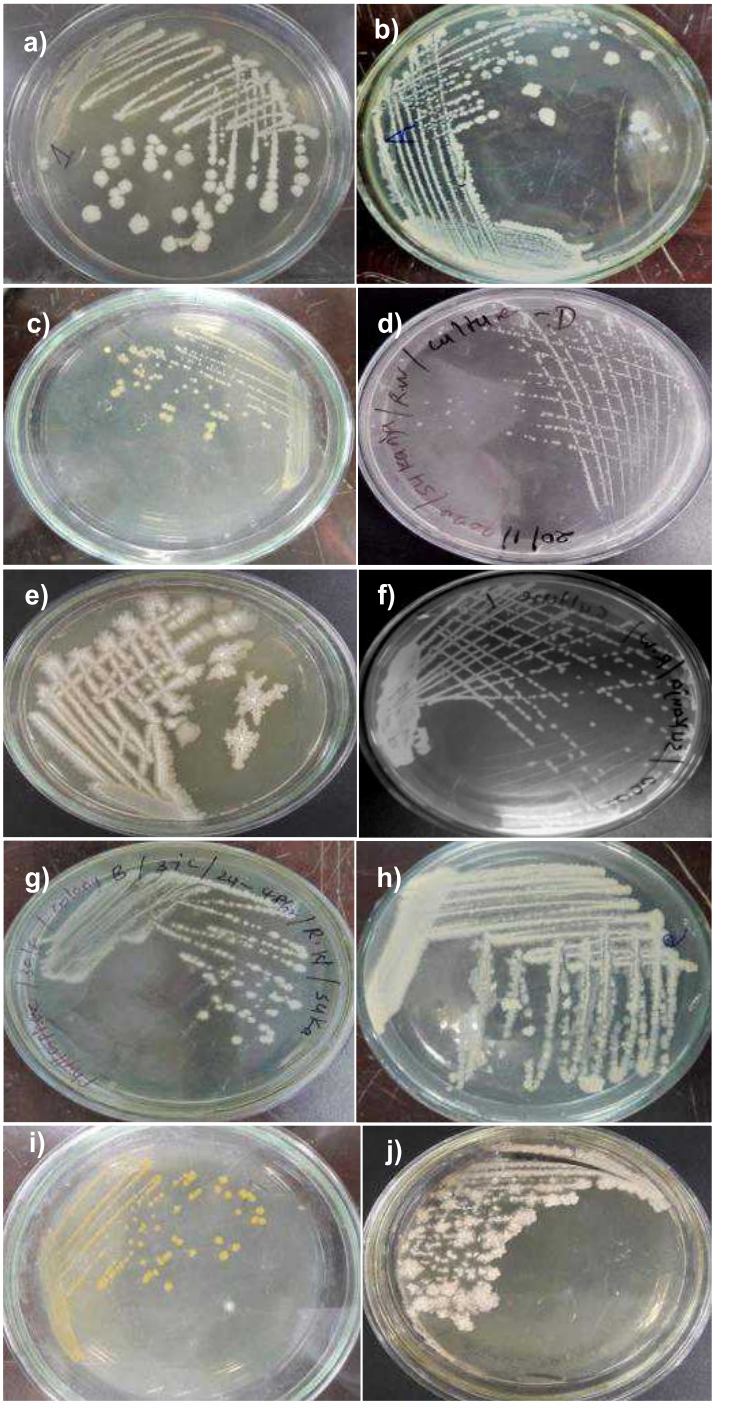

*Fig. 1: Culture and morphological characteristics of the isolates: **a)**Isolate B1; **b)**Isolate B2; **c)**Isolate P1; **d)**Isolate S1; **e)**Isolate B3; **f)**Isolate S2; **g)**Isolate L1; **h)**Isolate B4; **i)**Isolate M1; **j)**Isolate B5.*

Table 2: Colony morphology and culture characteristics of phyllosphere bacteria

Isolate	Colony morphology and culture characteristics
B1	Large, Feathery, Dull, White, Granular with less wavy edge, Opaque with a rough surface, Irregular margin, Convex.
B2	Opaque, Off-white, Roughly circular with irregular margin, Slightly filamentous appearance, Convex.
P1	Translucent and cream colour, Convex and round with smooth, entire margin, appear shiny but not mucoid.
S1	White, Opaque, entire margin, Convex, Circular colony.
B3	Fuzzy white, rough Opaque, Circular colony, flat elevation, Swarming edge.
S2	White, raised, opaque, smooth colony. Spindle form, entire margin.
L1	Rough/intermediate, flat, white, smooth, opaque colony. Entire margin, Convex.
B4	Hairy colony with characteristic swirls, white, opaque, rhizoid colony.
M1	Bright yellow colony, circular, convex, smooth with dull/glistening surface.
B5	Irregular shape, irregular (undulate fimbriate) margin, Rough and wrinkled, hair like growth, white and opaque.

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