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Morphological and biochemical profiles of bacterial endophytes from *Leucas aspera* and *Mimosa pudica*

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Abstract
The bacterial endophytes are present within the host plants and confer beneficial effects in plants. In the present study, 12 bacterial endophytes were isolated from *Leucas aspera* and *Mimosa pudica*. Further, were characterized based on colony morphology and biochemical tests. Bacterial endophytes colony morphology varied with different coloration, margin, elevation, and translucency. Of 12 endophytic bacterial isolates, 33.33% were Gram-positive and 66.66% were Gram-negative with shapes varied between cocci and rod. Fifty *percent* of the isolates were endospore formers. Biochemical tests for the isolates found positive results for methyl red (33.33%), Voges-Proskaeur (75%), H_2S production (41.67%), carbohydrate fermentation (Glucose, 75%; Sucrose, 70.83%), catalase (83.33%), indole production (54.17%), and citrate utilization (50%). The present study explored the diversity of the bacterial endophytes of *Leucas aspera* and *Mimosa pudica* species and has led to the creation of their biochemical profiles.

Keywords: Bacterial endophyte, diversity, growth promotion, *Leucas aspera*, *Mimosa pudica*, biochemical tests

1. Introduction
Plants harbor the indigenous microorganisms within them, which have evolved their own biochemical pathways that aid in plant growth and development. Over time many biomolecules that aid in human well-being have been explored from these endophytes that harbor plant systems without causing any symptoms of the disease. Bacterial endophytes live in a relatively privileged niche within the host plants and benefit their hosts (Ullah *et al.*, 2019) [17]. In this context, the interaction between plants and endophytic bacteria has emerged as a fascinating area of research that can be used for novel agricultural techniques. Pseudomonas, Arthrobacter, Bacillus, Flavobacterium, Stenotrophomonas, Enterobacter, and others are the bacterial genera found within plant tissues (Hallmann and Berg, 2006) [6].
Microorganisms are known to produce various enzymes that are exploited industrially for application in biotechnology, bioremediation, pharmaceutical fields, and others through fermentation techniques in large amounts (Singh *et al.*, 2016) [15]. Bacterial endophytes act as plant growth-promoting organisms directly or indirectly through the production of phytohormones, accessibility of mineral nutrients, production of primary and secondary metabolites along with activation of the plant immune system against biotic stress and tolerance to abiotic stress (Kandel *et al.*, 2017) [18]. Though this is a predominant technology to obtain many biomolecules and aid in promoting plant growth and development, the ability and usage of bacterial endophytes are still not explored.
Weeds are predominantly present under both favorable and adverse environmental conditions. And this may be due to endophytic bacteria, known for their plant growth promotion. The bacterial endophytes from weed species are poorly explored, but as a natural bioresource possess tremendous potential for application in eco-friendly sustainable agriculture to a new dimension. Bacteria from diverse taxonomic genera such as Sinorhizobium, Bacillus, Pseudomonas, Marinorhizobium, Sphingomonas, Sphingobium, Herbaspirillum, Micrococcus, Microbacterium and Rhodococcus are associated with weed species (Fatema *et al.*, 2019) [4].
Hence, due to the numerous applications of bacterial endophytes in agriculture, industry, and the medical field, the aim of the present work was to isolate bacterial endophytes from *Leucas aspera* and *Mimosa pudica* of Lamiaceae and Fabaceae family, respectively, that are usually present in all environmental conditions, and creation of their profiles based on colony morphology, microscopic observations, and biochemical tests.

2. Materials and Methods

2.1 Collection of weed species

The healthy weed species *viz.*, *Leucas aspera* (13°08'75.14", 77°56'97.93") and *Mimosa pudica* (13°08'74.18", 77°56'70.11"), which are known for their drought tolerance were collected in polyethylene bags from the dry regions of UAS, GKVK Campus, Bangalore, and carried to the laboratory.

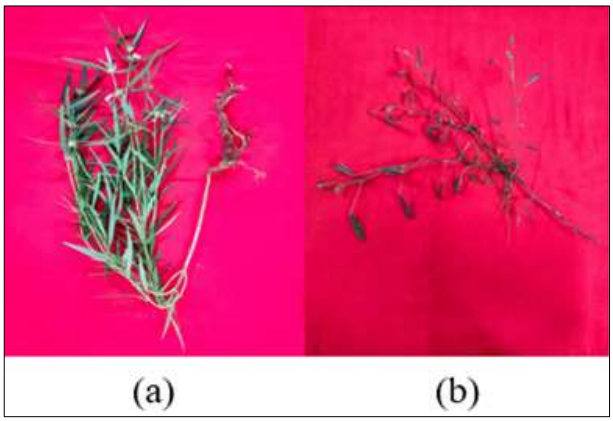

Fig 1: Weed plant species (a) *Leucas aspera* and (b) *Mimosa pudica*

2.2 Isolation of bacterial endophytes

The roots and stem of each plant were washed under tap water to remove the adhering soil particles and the majority of epiphytes. The roots and stem were cut into approximately 1 cm lengths and were surface sterilized by treating with 70% ethanol for 1 min followed by sodium hypochlorite solution (2%) for 3 min (root) and 1 min 30 sec (shoot). Later were repeatedly washed using sterile water. The surface sterilized root and stem bits were impregnated on nutrient agar by giving a gentle press and incubated at 30 °C for 48 h (hour) (Anjum and Chandra, 2015) [2].

After incubation, bacterial growth around the cut ends of root and stem bits was observed. The bacterial growth was picked up and streaked on a nutrient agar medium which was incubated at 30 °C for 48 h to obtain the pure culture. Then the pure cultures were maintained in slants, broth, and glycerol stock (70%) at 4 °C under refrigerator conditions. Further, studies were carried out through sub culturing the maintained cultures.

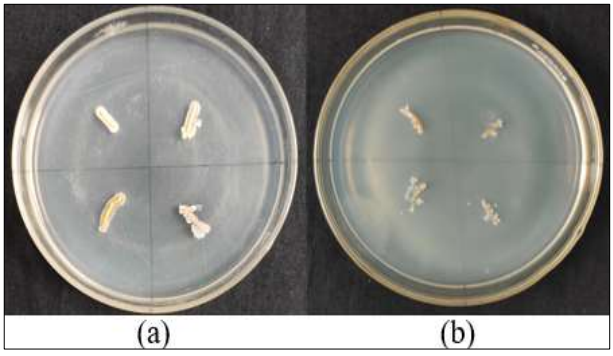

Fig. 2. Isolation of bacterial endophytes from (a) stem and (b) root

2.3 Morphological characterization

The colony characters of bacterial isolates *viz.*, color, margin, elevation, and translucency of the isolates were observed as described by Kalaivanan and Mohan (2017) [7].

2.4 Biochemical characterization

2.4.1 Gram staining

The Gram staining for bacterial isolates was performed per Smith and Hussey's method (2005) [16].

2.4.2 Endospore staining

Schaeffer-Fulton method was used for endospore staining as described by Gerhardt *et al.* (1981) [5] for all the 12 endophytic bacterial isolates.

2.4.3 Methyl red

The methyl red test was performed by inoculating isolates into 5 mL of glucose phosphate broth (Safary *et al.*, 2013) [14] and incubated at 28 ± 2 °C for 2 - 5 d (day). As growth occurs, five drops of 0.04% solution of alcoholic methyl red were added and observed for bright red color formation which indicated a positive result and yellow color indicated a negative result (Van Thuoc *et al.*, 2019) [18].

2.4.4 Voges-Proskauer

The test organisms were inoculated into 5 mL glucose phosphate broth (Safary *et al.*, 2013) [14] and incubated at 28 ± 2 °C for 48 h. As growth occurred about 1 mL of potassium hydroxide containing 0.3% creatine and 3 mL of α-naphthol solution were added. A positive reaction was indicated by the development of pink color within 2-5 min (Van Thuoc *et al.*, 2019) [18].

2.4.5 H_2S production

Bacterial isolates were stab inoculated into the sulfide indole motility (SIM) agar and incubated for 48 h at 30 °C. Blackening along the line of inoculation is confirmed as positive for H_2S production (Aneja, 2020) [1].

2.4.6 Carbohydrate fermentation

Bacterial isolates were inoculated into nutrient broth containing phenol red and Durham's tube with reducing sugar source as D-glucose and sucrose separately and incubated at 28 ± 2 °C for 48 h. The change in broth color from red to yellow and the gas bubble in Durham's tube indicated acid and gas production, respectively (Li *et al.*, 2019) [10].

2.4.7 Catalase activity

Bacterial endophytes were streaked on the nutrient agar plates and incubated for 48 h at 30 °C. Then a drop of 3% H_2O_2 was placed on colonies. The formation of effervescence indicated catalase positive reaction (Van Thuoc *et al.*, 2019) [18].

2.4.8 Indole production

The bacterial isolates were inoculated into tryptone broth (Aneja, 2020) [1] and incubated at 28 ± 2 °C for 48 - 96 h. After incubation 0.5 mL of Kovac's reagent was added and shaken. The development of pink or red color in the alcohol layer indicated a positive reaction (Li *et al.*, 2019) [10].

2.4.9 Citrate utilization

A citrate utilization test was performed by streaking bacterial isolates on Simmon's citrate agar slants (Safary *et al.*, 2013) [14]. And were incubated at 28 ± 2 °C for 48 h and observed for the color change in the medium from green to blue which indicated a positive result (Das *et al.*, 2019) [3].

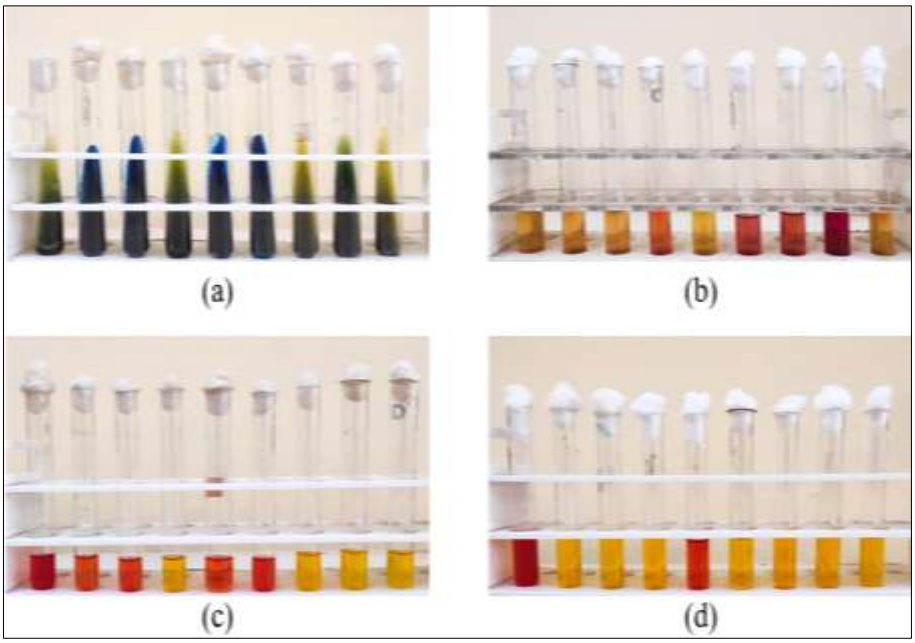

Fig 3: Biochemical tests (a) Citrate agar test, (b) Methyl red (c) Sucrose Fermentation, and (d) Glucose fermentation

3. Results and Discussions

3.1 Isolation of endophytic bacteria from weeds

Root and stem samples of weed plant species collected from the dry regions of UAS, GKVK campus, Bangalore were used for the isolation of bacterial endophytes, and based on the colony morphology 12 isolates (Table 1) were selected to characterize on the basis of colony morphology, microscopic observations and production of various enzymes.

3.2 Morphological characterization of endophytic bacterial isolates

The colony morphology of the endophytic bacterial isolates had varied characteristics. Colony color varied between white, cream, creamy white, and light pink. The colony margin was entire in appearance. Whereas, the elevation was observed to be convex, raised, and flat. And based on the opacity the cultures were opaque and translucent in their nature (Table 2).

3.3 Biochemical characterization

All the 12 endophytic bacterial isolates were observed microscopically (Table 3) and tested for various biochemical activities such as methyl red test, Voges-Proskauer, H_2S production, carbohydrate fermentation, catalase activity, indole production and citrate agar test as presented in table 4.

Microscopic observations revealed endophytic bacterial isolates belong to both Gram-positive (33.33%) and Gram-negative (66.66%) with shapes varied between cocci (58.33%) and rod (41.66%). And through endospore, staining isolates were categorized as endospore producers (50%) and non-endospore producers (50%).

About 25% of endophytic bacterial isolates showed positive test results for methyl red test through the formation of bright red color by the addition of alcoholic methyl red and 75% of the endophytic bacterial found positive for the Voges-Proskauer test through the formation of pink color. Similarly, Novero and Labrador (2014) [11] found all 8 bacterial endophytes as positive for Voges- Proskauer, citrate utilization, catalase test, and negative for methyl red and indole production. Based on the results obtained, 50% of the endophytic bacterial isolates were confirmed to produce H_2S through blackening along the line of inoculation. Pandey *et al.* (2015) [12] stated to isolate PAK6 and PAK8 as H_2S producers out of the selected 7 endophytic bacterial isolates from *Mussaenda roxburghii*.

Table 1: Source of endophytic bacterial isolates from weed plant species

Sl. No.	Isolates	Part used	Weed species
1	LAR1A	Root	*Leucas aspera*
2	LAR1B		
3	LAR2A		
4	LAR2B		
5	LAS1A	Stem	
6	LAS2A		
7	MPR1A	Root	*Mimosa pudica*
8	MPR1B		
9	MPR2A		
10	MPR2B		
11	MPR2C		
12	MPS1A	Stem	

*The first two and the third letters of the isolates represent the scientific name of the weed species and the plant part used to isolate those, respectively, and the last two digits are a reference to a specific isolate.

Table 2: Morphological characters of endophytic bacterial isolates colony

Sl. No.	Isolates	Color	Margin	Elevation	Opacity
1	LAR1A	Creamy White	Entire	Convex	Opaque
2	LAR1B	Cream	Entire	Raised	Opaque
3	LAR2A	White	Entire	Convex	Translucent
4	LAR2B	Cream	Entire	Raised	Opaque
5	LAS1A	Cream	Erose	Convex	Translucent
6	LAS2A	White	Entire	Convex	Opaque
7	MPR1A	Cream	Entire	Convex	Opaque
8	MPR1B	Light Pink	Entire	Flat	Translucent
9	MPR2A	White	Entire	Flat	Opaque
10	MPR2B	Cream	Entire	Raised	Opaque
11	MPR2C	Cream	Entire	Raised	Translucent
12	MPS1A	Cream	Entire	Convex	Opaque

Endophytic bacterial isolates grown in the fermentation broth supplemented with different sources of carbohydrates were observed for color change and gas production. The pattern of sugar utilization by bacterial isolates revealed nearly 58.33% of the endophytic bacterial isolates as acid producers with or without gas production and the remaining 41.66% of endophytic bacterial isolates showed negative test results for carbohydrate fermentation when supplemented with glucose. Whereas, 41.66% of the isolates were acid producers with or without gas production and the remaining 58.33% of isolates showed negative test results for carbohydrate fermentation when supplemented with sucrose. Even Khanam and Chandra (2015) [9] reported bacterial endophyte *Serratia marcescens* isolated from *Beta vulgaris* showed a positive result for sugar fermentation with supplementation of both glucose and sucrose.

Table 3: Morphological characters of endophytic bacterial isolates

Sl. No.	Isolates	Gram staining		Endospore formation
		Gram reaction	Shape	
19	LAR1A	+	Cocci	-
20	LAR1B	-	Cocci	-
21	LAR2A	-	Cocci	-
22	LAR2B	-	Cocci	-
23	LAS1A	+	Cocci	+
24	LAS2A	+	Rod	+
25	MPR1A	-	Rod	-
26	MPR1B	-	Rod	+
27	MPR2A	+	Cocci	+
28	MPR2B	-	Rod	+
29	MPR2C	-	Cocci	+
30	MPS1A	-	Rod	-

*Gram staining: (+) Gram positive (-) Gram negative
* Endospore staining: (+) endospore formation (-) no endospore formation

The majority of the endophytic bacterial isolates were found to be positive for the catalase test through the production of effervescence except for 16.66% of the isolates. Phuong *et al.* (2021) [13] reported out of 77 endophytic bacterial strains isolated from lowland rice roots, 51 isolates (66.24%) were able to produce catalase. While 50% of the isolates produced pink to red color in the alcoholic layer of the test tube and were proven to produce indole. Through the change of medium color in the incubated slants from green to blue, 50% of the bacterial isolates were found to utilize citrate, but the other 50% of the isolates were found negative for citrate utilization. Khanam and Chandra (2015) [9] also found positive for the citrate test by *Serratia marcescens*.

Table 4: Biochemical characters of endophytic bacterial isolates

Isolates	Methyl Red	Voges-Proskauer	H_2S production	Carbohydrate fermentation		Catalase activity	Indole production	Citrate agar test
				Glucose	Sucrose			
LAR1A	-	-	+	AG	-	+	-	+
LAR1B	-	+	-	-	-	+	-	-
LAR2A	-	+	-	AG	-	+	-	+
LAR2B	+	-	-	AG	-	+	+	+
LAS1A	-	+	+	AG	A	+	+	+
LAS2A	-	-	+	A	-	+	-	+
MPR1A	-	+	+	-	AG	+	+	-
MPR1B	-	+	-	-	AG	+	-	-
MPR2A	-	+	-	-	-	+	-	-
MPR2B	+	+	-	A	AG	+	+	-
MPR2C	-	+	+	-	-	-	+	-
MPS1A	+	+	+	AG	A	-	+	+

*Methyl red: (+) bright red colour formation; Voges-Proskauer: (+) pink colour formation; H_2S production: (+) blackening along the line of stab inoculation; Indole production test: (+) development of cherry red colour; Citrate agar test: (+) formation of blue medium.
*Carbohydrate fermentation: (AG) both acid and gas production (A) only acid production (-) neither acid nor gas production.
*Catalase activity: (+) production of effervescence (-) absence of effervescence.

4. Conclusion

Microorganisms are the ones that provide us the most if we know the way to approach those through biotechnological ways. In that context, bacterial endophytes are the ones with the potential to aid mankind. The present study has revealed the diversity of bacterial endophytes present within the *Leucas aspera* and *Mimosa pudica* species. The characterization of these endophytic bacterial isolates has exposed the variable biochemical attributes and the role of those, which in turn explains the usage of these microorganisms in industrial, biotechnological, and agricultural applications. Hence, bacterial endophytes are effective tools of science and if they could be used in a righteous manner, it may lead to a sustainable ecosystem and hence a healthy lifestyle.

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