



# Isolation and identification of phloem sap endophytes from the lac host plants, *Flemingia* spp.

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**Keywords:** endophytes; *Flemingia semialata*; *Flemingia macrophylla*; lac host plant; phloem sap

## Abstract

**Background and purpose:** Plants harbour mutualistic endophytes which are important for their growth and development. Lac host plant is the most important factor determining the lac yield because lac insects depend on the host for their nutrition and shelter throughout their life. Lac host endophytes could be postulated to impact lac insects positively. Hence, this study was attempted to explore the diversity of endophytic bacteria in the phloem sap of the lac host plants, *Flemingia semialata* and *Flemingia macrophylla*, and study their role in nutrient supplementation.

**Methods:** Phloem sap was extracted from *F. semialata* and *F. macrophylla* and cultured on different nutrient-rich media. The colonies obtained were subjected to molecular identification by 16S rRNA gene sequencing. The colonies were also tested for IAA production, urea and zinc solubilization abilities.

**Results:** It revealed that the isolates from *F. semialata* phloem sap to be *Agrobacterium larrymoorei*, *Rhizobium* sp., *Sphingomonas* sp., and *Priestia aryabhatai* whereas, the isolates from *F. macrophylla* to be *Brevundimonas albigilva* and *Bacillus australimaris*. The isolates also showed varied growth-promoting substance production and nutrient-solubilizing ability.

**Conclusion:** For the first time, a few growth promoting bacterial endophytes were isolated from phloem sap of *Flemingia* spp.

## INTRODUCTION

De Bary introduced the term endophytes for the microbes living inside the plant parts, which are different from epiphytes that are living on the plant surface. Endophytes belong to diverse taxa such as bacteria, fungi, protists, and archaeal, which are generally considered mutualists. Their association can be obligatory or facultative and cause no harm to the host plants. Endophytes are ubiquitous and have been isolated from almost all plants. However, their population varies from plant species to plant species. Even within the same species, it not only varies from region to region but also differs with changes in the climatic conditions of the region (1). Plants acquire essential elements such as C, H, O, N, P, and K from the soil, atmosphere, and water. Endophytes play a vital role in the uptake of these essential nutrients. They also produce phytohormones, such as cytokinins, auxins, and gibberellic acids. Hence, endophytes are proven to be essential for plant growth and development. Endophytes also can synthesize bioactive compounds that are useful in plant defense against pathogens, and a few of these compounds are useful for novel drug discovery (2). Endophytes can

colonize the internal tissues and improve the quality and physiology of the host, which makes them very important for the agricultural sector. These microbes may promote plant growth in terms of increased germination rates, leaf area, root and shoot length, nitrogen content, protein content, chlorophyll content, biomass, yield, and tolerance to pathogen attack and abiotic stresses like drought, flood, temperature, salinity, *etc* (3).

Microorganisms enter the root zone through cuts, and other natural openings or by dissolving the plant cell wall with enzymes such as cellulase, xylanase, endoglucanase, and pectinase. Besides these enzymes, endophytes produce lipase, phenoloxidase, proteinase, and lignin catabolic enzymes for their establishment. Few bacteria enter the xylem tissues by crossing the endodermal barrier and spread to almost all tissues and organs, including reproductive organs, and developing seeds. The count of endophytic bacteria decreases with an increase in the distance from roots. The increase in fitness in plants is achieved by endophytes through the conferring of abiotic and biotic stress tolerance, increasing biomass, decreasing water consumption, and/or decreasing fitness by altering the allocation of resources from the soil to the plants (4).

Lac insects are phloem-feeders and complete their life cycle on only one host plant. They thrive on the twigs of certain plant species (host plants), suck the plant sap, and grow, all the while secreting lac resin from their bodies (5). *Schleichera oleosa* (*kusum*), *Butea monosperma* (*palas*), and *Ziziphus mauritiana* (*ber*) are well-known hosts of lac insects, and recently, the bushy host plant *Flemingia semialata* has been used for commercial lac cultivation. *Flemingia macrophylla*, though does not yield commercial lac, can sustain all the known species of lac insects. The host plant is the most important factor for lac yield since the entire life of lac insects depends on the host for their nutrition and shelter. Both host and insect share the same mode of nutrition i.e., phloem sap. It is well established that the endophytes from one plant get transmitted to other plants via the phloem feeders. Besides being lac host plants, *Flemingia* spp. help in improving soil fertility by biological nitrogen fixation through their symbiotic rhizobial association due to their leguminous nature. *F. semialata* is suitable for mountain agroforestry due to its perennial nature, fast leaf litter decomposition, and nutrient release. *F. macrophylla* is used for mulching to control weeds, as a sod and erosion control.

Endophytes play important roles in plant nutrition by enhancing the availability of nutrients as in the case of zinc and nitrogen. Zinc solubilization ability of the endophytes is essential to make unavailable form of zinc in the soil to plant available form; urease activity helps in hydrolyzing urea so that the host plants can utilize urea in a better way. At the same time, by producing plant hormones such as IAA (Indole Acetic Acid) endophytes promote host plant growth (6). Since lac insect - host plant

interaction is mandatory for lac insects, it postulates that the plant endophytes might have a certain role in this relationship. Hence, the objective of this study was to isolate endophytic bacteria from the phloem sap of the bushy lac host plants, *F. semialata* and *F. macrophylla* and to investigate the capability of endophyte isolates from the two plant species to solubilize zinc and urea and produce IAA thus evidencing bacterial traits functional to plant growth and, indirectly, to lac insects.

## MATERIALS AND METHODS

### Sample collection and culturing of endophytes

The study was carried out at the Agri-bioresources Augmentation Division, ICAR-National Institute of Secondary Agriculture, Ranchi, Jharkhand, India. Plant barks were collected for phloem sap collection from the two different bushy lac host plants, *viz.*, *Flemingia semialata* and *Flemingia macrophylla*. For surface sterilization, they were washed with absolute ethanol for 2 min, followed by 2% sodium chlorite for 1 min, and 0.1% mercuric chloride for 30 sec under sterile condition in a laminar air-flow cabinet. Then the barks were washed thoroughly with autoclaved double-distilled water 4-5 times. Washed water was collected to check for contamination (as a control). The bark was cut into 1 cm pieces and 2-3 pieces of the bark were vertically placed in a 0.5 ml microcentrifuge tube. A small hole was made at the bottom of the tube using a sterile razor blade, and the tube was immersed into a 1.5 ml microcentrifuge tube. Then the sample was centrifuged at 12000 rpm for 15 min at 4 °C, and then the oozing out portion was collected from the 1.5 ml microcentrifuge tube, which is the phloem sap (7). Phloem sap was then inoculated in the nutrient broth at 28 °C. Phloem sap-inoculated nutrient broth showed growth 72 hai (hours after inoculation). The bacterial culture grown for 72 hours in nutrient broth was subsequently plated on three different enriched media, tryptic soy agar (TSA), blood agar (BA) and brain heart infusion agar (BHI) to support also the growth of those bacteria with more complex or particular nutritional requirements. Colony-forming units (CFU) were observed on all three different media after 72 h incubation.

Washed water was also plated on nutrient agar plates to confirm the absence of surface contaminants. All the CFUs obtained on different media were re-streaked to isolate single colonies and preserved as glycerol stocks.

### DNA extraction of isolates and amplification of 16S rRNA

Bacterial DNA was isolated by TE method. Bacterial colonies were inoculated in nutrient broth and grown overnight. The culture was pelleted down by centrifugation and re-suspended in TE buffer [100 mM Tris and 10

mM EDTA (pH 8.0)] containing 10% SDS and 20 µg/ml proteinase K and incubated for 1-2 hours at 55 °C. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed well by inverting the tubes until the phases were completely mixed. After phase separation, the tubes were centrifuged at 12,000 x g for 10 min. The aqueous phase was collected in a fresh tube and precipitated with ethanol to pellet the DNA. The pellet was suspended in 200 µl of TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA). After measuring the absorbance of DNA at 260 nm, pure DNA was used for PCR (Polymerase Chain Reaction). PCR was performed to amplify 1200 to 1400 bp of the 16S rRNA gene from all the DNA samples by using universal 16S rRNA primers 8F (5'-AGAGTTTGATCTGGCTCAG-3') and 1492R (5'-ACGGCTACCTTGTTACGACTT-3'). The master mixes of 25 µl contained, 25 ng of template DNA, 0.25 mM of dNTPs (Fermentas Inc, MD, USA), 10 pico moles of each primer, 1 unit of *Taq* DNA polymerase (Fermentas Inc., MD, USA). All the PCR reactions were carried out in a thermal cycler (Sensoquest, Germany) programmed with the following cycling conditions. Initial denaturation of template DNA was carried out at 95 °C for 3 min followed by 35 cycles programmed for denaturation step at 95 °C for 30 sec, primer annealing step at 57.3 °C for 30 sec, and DNA extension step at 72 °C for 1 min. The final extension of the PCR products was carried out at 72 °C for 10 min.

### Sequencing, sequence analysis, and sequence submission to NCBI

The purified PCR products were sequenced using 16S rRNA primers in a sequencing reaction on ABI 3130 automated sequencer (Applied Bio systems Inc., Carlsbad, CA, USA) at the sequencing facility of the AgriGenome Labs Pvt Ltd., Kochi, India. Raw Sequence was imported to Geneious (6.1.8) software (8) in order to identify sequence quality having at least 25 Phred quality scores. The good quality reads were extracted. Extracted forward and reverse sequences were aligned to generate a consensus sequence with the help of *de novo* assembly. Preliminary identification of the 16S rRNA gene sequences was done using BLASTn ([http:// blast.ncbi.nlm.nih.gov/Blast.cgi](http://blast.ncbi.nlm.nih.gov/Blast.cgi)) against 16S ribosomal RNA sequence (Bacteria and Archaea) database at NCBI. Sequences were subsequently submitted to GenBank ribosomal RNA database. The presence of chimeras was checked using DECIPHER v.2.8.1 (9).

### Phylogenetic tree construction

Phylogenetic trees were constructed using the Neighbor-Joining (NJ) algorithm in MEGA version 11 (10) with the Tamura-Nei multiple base substitution models used to correct for multiple hits. The robustness of the trees was evaluated by bootstrap analysis with 1000 replications, providing a measure of support for the inferred relation-

ships. This approach enabled the reconstruction of a reliable phylogeny, which was used to investigate the evolutionary relationships among the endophytic microbes.

### Test for Zn solubilization activity

To check the Zn solubilization ability of selected strains, bacteria were subjected to grow on modified Bunt & Rovira media containing Peptone 1 g/l, Yeast extract 1 g/l, Glucose 20 g/l, Ammonium sulphate 5 g/l, Dipotassium hydrogen phosphate 0.4 g/l, Magnesium chloride 0.01 g/l, Ferric chloride 0.01 g/l, Zinc oxide 1g/l, Agar 20g/l and final pH 6.6-7.0. Experiments were done in triplicates. Bacterial cultures were spot inoculated on the media using a sterile loopful of bacterial culture. The petri plates were incubated at 28±1 °C for 5 days in the dark to observe clear halo zone formation around the colonies.

### Production of Indole Acetic Acid (IAA)

IAA production was detected as described by Brick et al., 2004 (11). Bacterial cultures were grown on Luria Bertani (LB) broth amended with 100 mg/l Tryptophan as the precursor of IAA and incubated in a shaker at 110 rpm at 28 °C for 3 to 5 days. The tests were replicated thrice. Fully grown cultures were centrifuged at 10,000 rpm for 10 min. One ml of the supernatant was mixed with 200 µl of the Salkowski reagent (50 ml, 35% of HClO<sub>4</sub>, 1 ml 0.5 M FeCl<sub>3</sub> solution). The appearance of pink colour in test tubes indicated a positive result for IAA production. The absorbance was measured at OD<sub>530</sub> nm after 25 minutes of incubation at room temperature and the concentration of IAA produced by bacteria in 1 ml of culture was calculated by using a standard curve equation.

### Urease Activity Test

The strain phenotypic urease activities were tested using Christensen's urea agar. The agar plates were prepared according to the manufacturer's instructions. Colonies sub-cultured on nutrient broth were inoculated onto prepared urea agar tubes and incubated at 28 °C for 2- 3 days, and the colour change from yellow to purple-red was monitored.

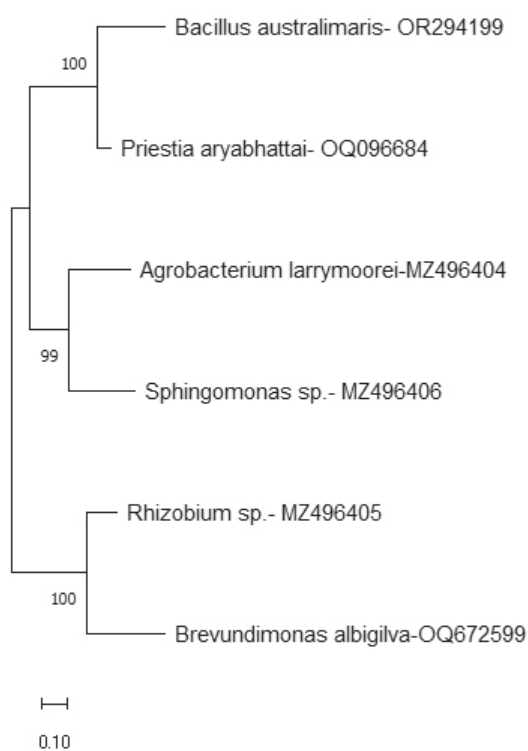
## RESULTS

Endophytic bacterial isolates were obtained from all the enriched media used in the study although the number of CFUs differed between them. Isolates ESH1, ESH2, ESA3 were obtained on BHI, EMB1 and EMB2 were obtained on BA and only EST3 was obtained on TSA.

Chimera detection results showed that there is no chimera present in the obtained 16S rDNA sequences of the isolates. The 16S rRNA sequences of all the isolates were submitted to GenBank and the accession numbers ob-

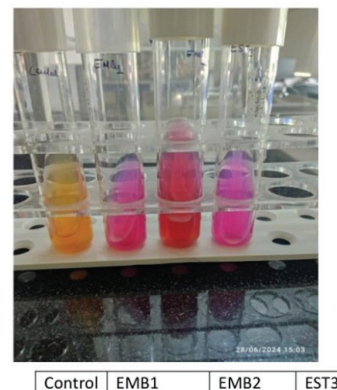
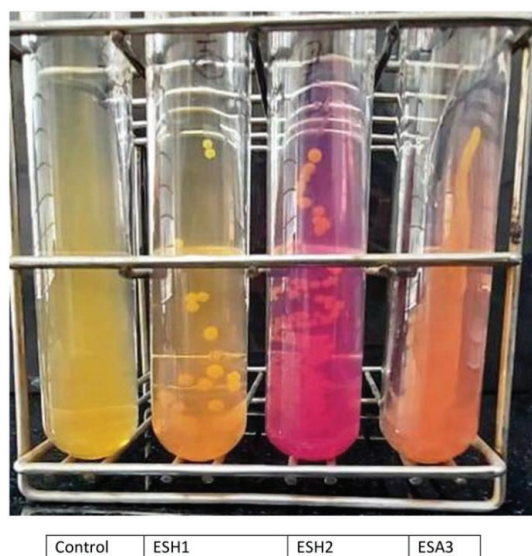
**Table 1.** Details of bacterial endophytes isolated from the phloem sap of lac host plants *Flemingia* spp.

S. no.	Isolate	Species Identity	Sequence length	Accession Number	Closest match in GenBank	Accession no. of match	Percent identity
From <i>Flemingia semialata</i>							
1.	ESH1	<i>Agrobacterium larrymoorei</i>	1151 bp	MZ496404	<i>Agrobacterium larrymoorei</i> strain Os_Ep_VSA_68	MN932337.1	99.74
2.	ESH2	<i>Rhizobium</i> sp.	1226 bp	MZ496405	<i>Rhizobium</i> sp.S3-160	JQ660121.1	99.92
3.	ESA3	<i>Sphingomonas</i> sp.	1088 bp	MZ496406	<i>Sphingomonas</i> sp. strain OsEp_Plm_15B2	MT367777.1	99.72
4.	EST3	<i>Priestia aryabhatai</i>	1277 bp	OQ096684	<i>Priestia aryabhatai</i> strain LW-5	PP506468.1	100
From <i>Flemingia macrophylla</i>							
5.	EMB1	<i>Brevundimonas albigilva</i>	1067 bp	OQ672599	<i>Brevundimonas albigilva</i> strain EP55	MG778752	99.81
6.	EMB2	<i>Bacillus australimaris</i>	1410 bp	OR294199	<i>Bacillus australimaris</i> strain ROA027	MT510169.1	100

**Figure 1.** Dendrogram of the phloem sap endophytes of *Flemingia* spp. using Neighbor-Joining method.

tained, and BLAST results are mentioned in Table 1. Twenty-three and nineteen colonies were obtained from *F. semialata* and *F. macrophylla*, respectively. Based on the distinct colony morphology, five and seven colonies were selected from *F. macrophylla* and *F. semialata*, respectively for sequencing. A total of six different endophytic bacteria were identified based on 16S rRNA sequencing (Table 1). *Priestia aryabhatai*, *Sphingomonas* sp., *Rhizobium* sp., *Agrobacterium larrymoorei* were found in the phloem sap of *F. semialata*; whereas, *Brevundimonas al-*

### Urease production test

**Figure 2.** Urease production assays of phloem sap endophytic bacteria isolated from *Flemingia* spp. (a change in colour from yellow to purple-red is indicative of urease activity, while the intensity of the colour is indicative of the quantity of enzyme produced).

**Table 2.** Biochemical analyses of endophytic bacteria isolates from phloem sap of lac host plants (-ve means negative; +ve means positive, +, ++ and +++ indicate the intensity of the reaction).

Code	Isolate	Production of growth-promoting substances		Nutrient solubility test
		IAA test( $\mu\text{g/ml}$ )	Urease test	Zinc solubilization test
ESH1	<i>Agrobacterium larrymoorei</i>	338 $\pm$ 7	-ve	Negative
ESH2	<i>Rhizobium</i> sp.	441.3 $\pm$ 2.8	+++ ve	Negative
ESA3	<i>Sphingomonas</i> sp.	255.7 $\pm$ 10.4	+ ve	Negative
EST3	<i>Priestia aryabhatai</i>	247 $\pm$ 10.6	+++ ve	Positive
EMB1	<i>Brevundimonas albigilva</i>	251 $\pm$ 8.5	+ ve	Negative
EMB2	<i>Bacillus australimaris</i>	175 $\pm$ 7.1	++ ve	Positive

*albigilva* and *Bacillus australimaris* were found in the phloem sap of *F. macrophylla*.

According to the Neighbor-Joining method dendrogram analysis, the isolates were divided into three clusters (Figure 1). In the first cluster, *B. australimaris* and *P. aryabhatai* are aligned. The second cluster comprises of *A. larrymoorei* and *Sphingomonas* sp. and the third cluster is formed with *Rhizobium* sp. and *B. albigilva*.

### Biochemical analysis of the isolates

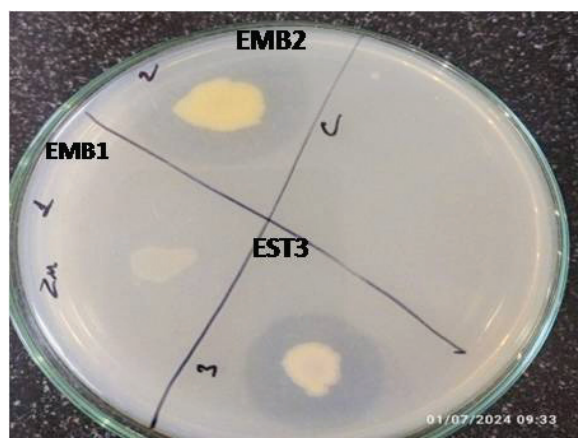
The isolates were tested for the production of the growth promoting substances IAA and urease and also tested for nutrient solubilization activity for zinc (Table 2). *B. albigilva* produced a larger amount of urease enzyme as it showed purple red colour in the assay. On the other hand, *A. larrymoorei* was found to be urease-negative, suggesting that it may not have the same ability to hydrolyze urea and affect soil pH (Figure 2). All the isolated bacterial strains were subjected to *in vitro* nutrient supplementation activity by evaluating their ability to solubilize essential element zinc to plant-available form. Only *B. australimaris* and *P. aryabhatai* which were in the same cluster were observed to be efficient Zn solubiliz-

ing bacteria based on the formation of halo clearing zones around bacterial colonies when incubated in media containing an insoluble form of zinc (Figure 3).

### DISCUSSION

Endophytes are important constituents of the plant micro-ecological system, providing resources to the plant that promote growth and plant disease prevention, protect against predators, and help the hosts adapt to different abiotic and biotic stresses (12). Recent studies revealed that host and or insect associated symbionts are influential in their interactions on plant defense as well as utilization of plants by insects. Lac host plants are the unique entities that can sustain the lac insect though lac insects are apparently not beneficial to them. Endophytes present in the phloem sap of the lac host plants need to be explored as a part of understanding this sustenance.

In this study, we isolated several bacterial strains from the phloem sap of two bushy lac host plants, *F. semialata* and *F. macrophylla* (Table 1). Six distinct endophytes were identified among the isolates: *Agrobacterium larrymoorei*, *Sphingomonas* sp., *Rhizobium* sp., *Priestia aryabhatai*, *Brevundimonas albigilva*, and *Bacillus australimaris*. The bacterial diversity obtained in the study from the phloem sap of *Flemingia* spp. seems to be lower. An earlier study also recorded a lower bacterial diversity for phloem endophytes (13). It was observed that the phloem restricted bacteria actively multiplied within sieve tubes of the phloem. However, they could not be readily cultured under *in vitro* conditions. This could be due to the fact that culture media nutrients are not as rich as phloem sap to support the growth of the phloem endophytes and still some phloem sap nutrients which are crucial for the growth of phloem sap endophytes are not yet identified (13). It is expected that the roots would harbour more bacterial diversity compared to other plant parts due to the movement of bacteria from soil to roots. Since the flow of the phloem contents is from leaves to other sink organs, the chance of more bacterial diversity in phloem is less when compared to roots. As the present study followed

**Figure 3.** Zinc solubilization assay of phloem sap endophytic bacteria isolated from *Flemingia* spp.

only culture-based method for isolation and identification of phloem sap endophytes, it might have missed the non-culturable bacteria. On the other hand, approaches such as 16S rDNA metabarcoding would have revealed the identity of even the non-culturable bacteria present in phloem sap.

The identified endophytes most likely have important roles for their host plants. *Agrobacterium larrymoorei*, a Gram-negative bacterium, produces robust cellulase enzymes, making it valuable in enzyme biotechnology and plant biotechnology. Its cellobiase gene has been cloned, expressed, and characterized for potential biotechnology applications (14). *Sphingomonas* is a Gram-negative bacterium that produces yellow-pigmented colonies and thrives in diverse environments. *Sphingomonas* genus is metabolically versatile, utilizing various natural compounds and environmental contaminants, and contributes to the degradation of persistent environmental pollutants. It possesses genes for carbazole degradation and regulates pesticides like dibenzo-p-dioxins, remediates heavy metals, and accumulates intracellular zinc (15). It may be hypothesized that the *Sphingomonas* sp. obtained in our study may be involved in the degradation of pesticides sprayed on the studied host plants. *Priestia aryabhatai* is a Gram-positive, rod-shaped bacterium that was first discovered in the Earth's stratosphere and has since been found to reside inside and outside various host plants. It synthesizes growth-promoting metabolites and tolerates various abiotic stress factors (16). It exhibits anti-phytopathogenic activity, promotes crop growth, and has been shown to solubilize phosphorus in soil and increase available phosphorus for plants. Overall, *P. aryabhatai* is a multi-stress-tolerant plant growth promoter with significant potential in agriculture and soil fertility management. *P. aryabhatai* was also found as an endosymbiont in lac insects (unpublished data from our lab) suggesting it could have been transmitted from the host phloem sap to lac insect and might have a vital role such as abiotic stress tolerance both in host plant and in lac insect.

*Rhizobium* sp. is a diverse group of endophytic bacteria that have a positive impact on plant growth and nutrient status. They are soil bacteria that can form symbiotic associations with legume plants, residing in root or stem nodules and fixing atmospheric nitrogen (17). This symbiosis is well-studied and has significant agricultural importance, as it can enhance crop productivity, reduce the need for inorganic fertilizers, and involve diverse microorganisms that assist in nodulation and promote plant growth (18). Since *Flemingia* spp are leguminous plants, they are expected to harbour *Rhizobium* sp. In our earlier studies on lac insect endosymbiont study, we could observe *Rhizobium* in the lac insects (19). Hence, it is reasonable to assume that the *Rhizobium* sp. might have got transmitted from the phloem sap to the lac insect through feeding.

*Brevundimonas albigilva* is a novel bacterium, found to be present as an endophyte in the fruits of an invasive plant *Xanthium italicum* (20) and genome sequences of different strains of this bacteria are reported (21). *Bacillus australimaris* is a catalase-positive bacterium that produces hydrolytic enzymes, exopolysaccharides, and ammonia and promotes plant growth, as measured by increased shoot and root lengths. Its hydrolytic enzymes can break down organic matter, making nutrients available to plants, while exopolysaccharides can improve soil structure and water retention (22). The ammonia produced can also serve as a nitrogen source for plants. Overall, *B. australimaris* has beneficial effects on plant growth and soil health. *B. australimaris* identified from this study could produce IAA and urease and be able to solubilize zinc (Table 2). Since it is positive for urease activity, we can expect it to produce ammonia as a nitrogen source for the host plant. Another species under the *Bacillus* genus, *Bacillus pumilus* was identified from the phloem of pine trees (23).

*Bacillus* sp., *Rhizobium* sp., *Priestia* sp., isolated from the plant *Isoetes cangae* showed plant growth promoting activities such as phosphorous mineralization and IAA production (6). The isolates obtained in our study showed IAA producing ability, from 175 µg/ml by *B. australimaris* to 441.3 µg/ml by *Rhizobium* sp. The IAA produced by the isolates may be utilized for housekeeping activities of the plant, especially root growth. Out of the six bacterial isolates, five were found to be urease-positive although at different rates, indicating their potential to produce ammonia which is the nitrogen source for plants. The urease activity is the highest in the isolates *Rhizobium* sp. and *P. aryabhatai*. These results show that the soil dwelling bacteria, *Rhizobium* sp. has good root growth promoting activity through IAA production and urea utilizing capability by urease production. *P. aryabhatai* and *B. australimaris* were found to be positive for zinc solubilization ability showing that these isolates would have a role in nutrient supplementation of lac host plants.

The phloem sap endophytes not only supplement nutrition to the host plants but also to lac insects indirectly because lac insects depend solely on the host plant phloem sap for their nutrition. It may be postulated that the phloem sap endophytes would have contributed to the lac insect host plant interaction. Knowledge of the diversity and roles of these endophytes could be enhanced further by 16S rRNA metabarcoding besides the culture-based method used in this study.

## CONCLUSION

The study identified the bacterial isolates *Agrobacterium larrymoorei*, *Rhizobium* sp., *Sphingomonas* sp., and *Priestia aryabhatai* from the phloem sap of *F. semialata* and *Brevundimonas albigilva* and *Bacillus australimaris* from the phloem sap of *F. macrophylla*. The bacterial iso-

lates identified in the study exhibited varied levels of IAA production, zinc solubilization and urease activity. These phloem sap endophytes might be involved in growth and development of the lac host plants, *Flemingia* spp.

**Acknowledgment:** The authors are thankful to the Indian Council of Agricultural Research, New Delhi for funding this study under the Network Project on Conservation of Lac Insect Genetic Resources.

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