Characterization of endophytic bacteria, *Solibacillus silvestris* DDBU6 and *Kocuriaassamensis* DDBU9 isolated from the leaves of a medicinal plant, *Phlogacanthusthyrsiformis*

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KEYWORDS
Endophytic bacteria; *Phlogacanthusthyrsiformis*; plant growth promotion activity; salt stress.

ABSTRACT
Endophyte's symbiotic association with host plants can be immensely valuable to agriculture because it promotes plants's growth and development. Endophytic bacteria present in medicinal plants play a highly essential role in the fields of pharmacology as well as agriculture, since therapeutic plants themselves are reservoirs for a variety of significant bioactive substances. However, the study of the endophytic bacteria presents in the medicinal plant, *Phlogacanthusthyrsiformis*, remains unknown. The primary goal of this research is to isolate, characterize, and molecular identification of endophytic bacteria present in the leaves of *P. thyrsiformis*. Two endophytic bacteria were isolated from theleaves: *Solibacillus silvestris*DDBU6 (PTL-1) and *Kocuriaassamensis*DDBU9 (PTL-2). In this study, *K. assamensis* was identified as an endophytic bacterium for the first time. Gram staining revealed that both leaf isolates were Gram-positive. In biochemical tests, both isolates tested negative for indole production but positive for citrate utilization and catalase activity PTL-1 and PTL-2 tested negative for oxidase test. *S. silvestris* and *K. assamensis*were able to tolerate 5% NaCl. These bacteria, under conditions of salt stress, hold potential to support plant growth. Moreover, in plant growth promotion assays, both the isolates exhibited positive results for ammonia production, negative for phosphate solubilization. For IAA production ability, highest production was shown by PTL-1 (81 ± 1.24 µg/ml) followed by PTL-2 (45 ± 0.47 µg/ml). All isolates showed promise as a viable endophytic bacterium for application in agriculture, possibly helping to produce ecologically friendly biofertilizers due to its positive results for some plant growth promotion activities and its tolerance of high salt concentrations.

1. **Introduction:** The improvement of agriculture is imperative to ensure food safety for the world's growing population. Conventional methods to enhance soil fertility and crop yields involve the application of manures, herbicides, and chemical fertilizers. Unfortunately, these practices have detrimental environmental impacts, including fertilizer adsorption, runoff, and the accumulation of toxins like cadmium in the soil (Shah D, 2022). It is vitally needed to replace these chemical fertilizers with environmentally friendly microorganism-based biofertilizer (Ali et al., 2021). The plant-microbe relationship has been shown to improve plant growth and development by facilitating the synthesis of food, fibre, biofuels, and essential bioactive compounds (Wu et al., 2009).In recent decades, endophyte-plant host communication has garnered substantial attention across industries, including healthcare and agriculture (Aleynova& Kiselev, 2023; Singh et al., 2022). Endophytes are microorganisms (bacteria or fungi) that live inside the plant tissue intracellularly or intercellularly without causing visible signs of disease (Nair & Padmavathy, 2014).

Previous studies showed the ability of endophytic bacteria to help plant for their growth and development by solubilizing inorganic phosphate into soluble form, producing phytohormones and siderophore, fixing nitrogen and also have 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Yan et al., 2018). Endophytic bacteria can boost plant growth by producing phytohormones, as well as help them to survive biotic and abiotic stresses.*Bacillus licheniformis*, *Bacillussp*., *Bacillus subtilis*, and *Lysinibacillus fusiformis* were isolated from the roots of a medicinal plant, *Momordica charantia* L. These bacterial endophytes showed several plant growth promotion activities like phosphate solubilization, IAA and siderphore production. Some showed high salt tolerance ability like *B. subtilis* and *B. licheniformis* able to tolerate upto 10% NaCl (Singh et al., 2022). The endophytic bacteria were also found to produce

extracellular enzymes and bioactive compounds. Some bioactive compounds can help the plants to protect themselves against several phytopathogens (Fouda et al., 2021). *Bacillus halotolerans,* isolated from *Lilium davidii var.* unicolor roots, was found to have antagonistic activity against three important lily plant diseases like *Botrytis cinereal*, *Botryosphaeria dothidea*,and *Fusarium oxysporum* (Gao et al., 2022).

Phlogacanthusthyrsiformis, an evergreen shrub that grows up to 2.4 metres tall, is a member of the Acanthaceae family. Its leaves are 13 to 35 cm long, and its flowers are up to 30 cm long and elongated thyrsoid panicles with a tubular, curving corolla that is orange or brick red in villous (A. et al., 2014). Indian tribes in upper Assam utilize *P.thyrsiflorus* to cure helminthiasis and it showed to have poisonous effects on mice and rats, therefore its traditional use as an anthelmintic can go on. There have been reports of using its leaves to cure fever, rheumatism, gout, and allergies. Furthermore, its leaf extract yielded the first bioactive compounds, including ß-sitosterol, lupeol, and botulin (Deori et al., 2023). In Assam, the plant holds a role in traditional medicine. Its distribution spans the Himalayan region, encompassing Bhutan, the north-eastern provinces of India, Indo-China, southern China, and Sulawesi. Furthermore, the leaf extract exhibits efficacy in hindering the proliferation of HeLa cells and demonstrates antibacterial properties (Baro & Das, 2022).Previously, research on the diversity of fungal endophytes found in *P. thyrsiformis* was conducted in Arunachal Pradesh, in north-eastern India (Sharma et al., 2020). Potential antibacterial and antioxidant properties were demonstrated by *Colletotrichum gloeosporioides*, which was isolated from *P. thyrsiformis* (Nirjanta Devi &Shyamkeso Singh, 2014). However, the study of the endophytic bacteria present in *P. thyrsiformis* remains an untapped area of research.

The main objective of this study is to isolate, biochemical characterization, and molecular identification of isolated bacterial endophyes from the leaf, of medicinal plant *Phlogacanthusthyrsiformis*. This study also reports on the plant growth promotion activities along with salt tolerance ability of the isolated endophytic bacteria.

2. Methods and methodology:

2.1. **Collection and Authetication of the sample plant:** Healthy and visibly disease-free leaves and stems of *P. thyrsiformis* were collected during February to March 2023 from Bodoland University, Kokrajhar, Assam, India (26.469239°N, 90.296309°E, altitude: 27m). At the Bodoland University Botanical Herbarium, the plant was identified.

2.2. **Isolation of Endophytic bacteria:** Surface sterilization of plant tissue was performed to isolate endophytic bacteria by following the method of Das & Das, 2022, with certain modifications. The leaves of the *P. thyrsiformis* plant were properly washed with running tap water before being placed under laminar flow. The samples were at first treated with 70% C_2H_5OH for 1 min then 1% NaClO for 7-10 mins. After treating with NaClO, using sterilized distilled water, the samples were cleaned three to four times. After that the samples were dried using sterilized filter paper (Whatman filter paper). And then cut into small pieces using sterilized blade and inoculated on Nutrient broth (NB) and Luria Bertani (LB) Broth as well as Nutrient agar (NA) and Luria Bertani Agar (Duhan et al., 2020) (**All media are procured from Himedia**) then incubated at 33°C for 96 hours for the highest recovery of endophytic bacteria from leaf and stem samples.

The final sample washed water was inoculated onto NA media to perform sterility test. The sterility test revealed no bacterial growth after 96 hours of incubation.

2.3. **Sub-culture and Pure-culture:** Once bacterial growth was observed in the stem and leaf samples; the samples were sub cultured until a pure culture was obtained. All of the isolates were then stored at 4°C for the future experiment. During sub-culturing, it was observed that LB agar showed better results than NA. For the rest of the experiment, LB agar media was used.

2.4. **Colony morphology and biochemical characterization:** There were four biochemical tests carried out: catalase, oxidase, indole, citrate. For Catalase test, a 3% H_2O_2 drop was added to a glass slide along with the bacterial colony to determine whether catalase was present in the isolates. The appearance of bubbles was considered positive, but their lack or a few dispersed bubbles were considered negative (Shah et al., 2022). As a positive control, *Staphylococcus aureus* was employed. For the citrate test, endophytic bacterial isolates were incubated at 37°C for 24 hours following inoculation onto Simmons citrate agar slants. A positive result was indicated by the media's color changing from green to blue and the presence of growth (Salo & Novero, 2020). *Bacillus subtilis* was used as positive control. Oxidase test was performed using standard oxidase disc procured from Himedia. For citrate, isolates were inoculated in the simmon's citrate agar slants. The color changes of the media from green to blue after 48 to 72 hours of incubation at 35℃ indicate positive result.

2.5. **Molecular identification and Phylogenetic analysis of isolated endophytic bacteria:**

The genomic DNA was extracted from endophytic bacteria using CTAB protocol (Salo & Novero, 2020).16S rDNA gene amplification was done using universal primer 27F (AGAGTTTGATCCTGGCTCAG), 1492R (CGGTTACCTTGTTACGACTT) (Senthilraj et al., 2016) and reaction mixture contain final volume 25µl containing: 12.5 µl of 2X concentration of PCR master mix (Genei), Forward primer (10 pmol), Reverse primer (10pmol), template 90 ng concentration and rest of the volume was made up by adding nuclease free water PCR reaction was carried out in 2720 Thermal Cycler of applied biosystem by Thermo Fisher Scientificand the conditions were as follows: initial denaturation at 94 ℃ for 4 minutes; 35 cycles of denaturation at 94 ℃ for 1 minute, annealing at 55 ℃ for 1 minute, extension at 72 ℃ for 2 minutes, and final extension at 72 ℃ for 10 minutes. After PCR gel electrophoresis was done, a single distinct PCR amplicon band of 1500 bp was detected on a 1.5% agarose gel. Gel Extraction Kit by Genetix Brand was used for the purification of the samples.The BDT v3.1 Cycle sequencing kit was used on an ABI 3730xl Genetic Analyzer to run the forward and reverse DNA sequencing procedures for the PCR amplicon.We used aligner software to construct a consensus sequence for the 16S rDNA gene from forward and reverse sequence data.

BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed for the 16S r DNA gene sequences against the NCBI genbank database. The top 10 sequences were picked based on their highest identity score and aligned using Clustal W, a multiple alignment software application.MEGA version 7 was used to generate a maximum likelihood phylogenetic tree based on the Kimura 2 parameter model. 1000 bootstrapping repetitions were used to determine the reliability of the branching and clustering patterns (Beiranvand, 2017). To obtain their accession numbers, the sequences of all the bacterial isolates were submitted to GenBank.

2.6 Isolated endophytic bacteria's ability to promote plant growth:

Phosphate solubilization: Isolates were inoculated on Pikovskaya's agar medium (Himedia) and incubated for 5 to 7 days at 28 °C to conduct the phosphate solubilization test. The formation of clear zone around the colonies indicated as positive result for phosphate solubilization. Phosphate solubilization index (SI) was also calculated by using the formula, SI = (colony diameter + halo zone diameter) / colony diameter (Shah et al., 2022).

Ammonia Production: After inoculating the endophytic bacteria in peptone medium (comprising 10 gL−1 peptone, 5 gL−1 NaCl, and 1 L distilled water) and incubated for 72 hours at 35 ± 2 °C, we assessed their ability to produce NH3. A control group utilized peptone water that had not been inoculated with bacteria. Ammonia production was confirmed by adding 1 mL of Nessler's reagent to the peptone liquid medium. The colour changes from dark yellow to brownish indicated the highest ammonia production, whereas a change to a pale-yellow colour suggested minimum ammonia production **(**ALKahtani et al., 2020).

IAA production: To confirm the ability of isolated endophytic bacteria produce IAA, the method of (Singh et al., 2022) was followed with minor modifications. For IAA production test, all isolated

endophytic bacteria were inoculated in Luria bertani broth supplemented with L-tryptophan (400 µg/mL) and incubated at 25 ± 2°C. After 48 hours of incubation, the cultures were centrifuged at 8000 rpm for 10 minutes. The supernatant (2 ml) was then mix with two drops of orthophosphoric acid and 4 mL of Salkowski reagent (50 mL of 35% perchloric acid and 1 mL of 0.5 M FeCl3). A pink hue appeared, indicating the production of IAA. IAA was quantified using a standard curve containing known amounts of pure commercial IAA. IAA was measured calorimetrically at 530 nm. As negative control, un-inoculated broth was used, and the experiment was conducted three times for each endophytic bacterium.

Salt tolerance: Salt tolerance of the isolated endophytic bacteria was tested by introducing different amounts of sodium chloride (from 0% to 10%) into LB agar media. Following the inoculation of plates with endophytic bacterial strains, the plates underwent incubation for five to six days at 35±2°C. Regular observations were conducted at 24-hour intervals throughout this incubation period (Sharma &Mallubhotla, 2022).

Data analysis: Experiments were performed in triplicates and data analysis was done using SPSS software. And for sequence analysis, MEGA 7, BLAST, Clustal W was used.

3. Results:

Morphological and Biochemical characterization:

Endophytic bacterial growth was observed from leaves following 48 hours of incubation at 33°C. To maximize the recovery of endophytic bacteria, both tissue samples were further incubated for 96 hours. Subsequent to multiple sub-culturing, pure cultures were obtained and subsequently stored at 4°C for further analysis. Two different endophytic bacteria were identified from the leaves. Initial characterization of all isolates involved analyzing their morphological and biochemical properties. In terms of biochemical characterization, all isolates showed positive results in the catalase and citrate production test; negative resultin the indole test and oxidase test. Comprehensive details regarding the morphological and biochemical properties of the isolates are presented in **Table 1**.

Table 1: Morphological and biochemical characterization of isolated endophytic bacteria

SL NO	Isolates	Plant tissue used in isolation	Colony morphology	Microscopic characteristics	Catalase	Oxidase	Citrate	Indole
1	PTL-1	Leaves	Irregular, raised, creamy color colony	Gram +ve, bacilli	Positive	Negative	Positive	Negative
2	PTL-2		Small, round, raised, yellow color colony	Gram +ve, cocci	Positive	Negative	Positive	Negative

Molecular identification:

Identification of isolated endophytic bacteria was done using 16S rDNA gene amplification and sequencing.The isolates were identified as *Solibacillus silvestris* (PTL-3), and *Kocuriaassamensis* (PTL-4), based on nucleotide homology and phylogenetic analysis. Molecular identification involved conducting BLAST analysis against the NCBI GenBank database and, after that, using Clustal W to align the top ten sequences, which were selected based on the maximum identity score.The Maximum Likelihood method was used to infer evolutionary history using the Kimura 2 parameter model and a bootstrap consensus tree produced based on 1000 replicates.Branches that were less than 50% bootstrap replicates collapsed.The percentage of replicated trees that showed related taxa clustering in the bootstrap test is shown next to branches. The analysis included eleven nucleotide sequences, with evolutionary

analysis performed using MEGA7. Sequence of all isolates were deposited in GenBank and accession number was obtained for all isolates (**Table 2**).

Table 2: Based on nucleotide homology and phylogenetic analysis, highly similar organisms were identified and obtained accession number

Isolates	Tissue of isolation	Identified organisms	Accession number
PTL-1	Leaves of *P. thyrsiformis*	*Solibacillus silvestris* strain DDBU6	PP412075
PTL-2		*Kocuriaassamensis*strain DDBU9	PP412522

Plant growth Promotion activity

All the isolated endophytic bacteria showed different plant growth promotion activity which showed in Table. 4.

Phosphate solubilizing ability: After inoculating on the Pikovskaya's agar medium, both the isolates were unable to show clearing zone around the colony after 7 to 10 days incubation at 28℃.

Ammoniaproduction: After adding Nessler's reagent to the peptone medium containing the endophytic bacterial isolates that had been cultured for 72 hours, all of the isolates demonstrated positive ammonia production, which was confirmed by the production yellow colour. The details of the plant growth promotion abilities of endophytic bacteria were showed in the Table 3.

Table 3: Plant growth promotion activities of isolated endophytic bacteria

Isolates	IAA production (µg/ml)	Phosphate solubilization	Ammonia production
PTL-3	81 ± 1.24	**Negative**	Positive
PTL-4	45 ± 0.47	**Negative**	Positive

Salt tolerance: Significant salt tolerance was exhibited by both the isolated endophytic bacteria, since they were able to grow in media with high salt concentrations. Both the strains PTL-1 and PTL-2 demonstrated robust growth capabilities, tolerating up to 5% NaCl concentration. The detailed result is shown in **Table 4**.

Table 4: Salt tolerance ability of isolated endophytic bacteria

Isolates	1% NaCl	2% NaCl	3% NaCl	4% NaCl	5% NaCl	6% NaCl
PTL-3	Resistant	Resistant	Resistant	Resistant	Resistant	-
PTL-4	Resistant	Resistant	Resistant	Resistant	Resistant	-

4. Discussions:

Microorganisms isolated from medicinal plants have a promising function in producing important and novel bioactive compounds (Singh et al., 2017). There are various medicinal plants that have not yet been studied for endophytic bacteria. In this study, we identified four endophytic bacteria from leaf and stem tissues of *P. thyrsiformis* of Kokrajhar, Northeast, India. *Solibacillus silvestris* (PTL-3) and *Kocuriaassamensis* (PTL-4) isolated from leaves.

S. silvestris, an endophytic bacterium, has shown promise as a multi-stress reliever, bioremediation agent, and crop growth stimulator in important crops (Kaur &Karnwal, 2023). In this study, *S. silvestris* was isolated from *P. thyrsiformis* leaves and demonstrated plant growth promotion activities like ammonia production. It also tolerated 5% NaCl concentration. This endophytic bacterium can also be used in agriculture to help plants grow under salt stress.

Previously, several *Kocuria*species, such as *K. arsenatis* and *K. palustris*, were found as endophytic bacteria in various plant species (Román-Ponce et al., 2016; Zacaria Vital et al., 2019). This study indicates the first identification of *K. assamensis* as an endophytic microbe. A water sample taken from the Brahmaputra river in Assam, India, led to the discovery of *K. assamensis*sp.nov (Kaur et al., 2011). In this study, it was isolated from *P. thyrsiformis* leaves and demonstrated tolerance up to 5% NaCl concentration. It also showed ability to produce ammonia.

In this study, both isolates showed high salt tolerance ability, thus, we can use bacteria in agriculture to grow plants under salt stress condition.

5. Conclusions:

This work represents the first report on *Phlogacanthusthyrsiformis*'s bacterial endophytes. It provides information on the molecular, biochemical, and plant growth-promoting properties of endophytic bacteria that have been isolated from leaves of *P. thyrsiformis*. Both the isolates from both leafs exhibited positive results in ammonia production and IAA production. Both isolates showed high salt tolerance ability. Consequently, these isolates hold potential for utilization as potent biofertilizers in agriculture.

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Credit authorship contribution statement: Debajani Das: Design the experiments, developed the structure, carried out the experiments and prepared the manuscript;**Tikendrajit Baro**: assisted in media preparation, molecular analysis and data analysis. **Sandeep Das:** Participated in supervisions, made critical revisions and approved the final version. All authors reviewed and approved the final manuscript.

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Data availability statement: All the data from the findings and experiments during the study are included in this article. All the four sequences of isolated endophytic bacteria were deposited in NCBI GenBank having accession number: PTL-1: **PP412075**, PTL-2: **PP412522.**

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