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Isolation and characterisation of endophytic bacteria present in the leaves of *Glycosmis pentaphylla* (Retz.) Correa

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Abstract

Endophytes are an endosymbiotic group of microorganisms that dwell in plant tissues and are reservoirs of bioactive compounds. However, the researches on this area are limited. The present investigation was undertaken to isolate and characterise endophytic bacteria from the leaves of *Glycosmis pentaphylla* (Retz.) Correa. An effective surface sterilisation procedure was developed from the experiment to isolate endophytic bacteria from the leaves of the candidate species. There was no bacterial growth observed in the sterility test. A total of 3 endophytic bacteria were isolated from leaves of *G. pentaphylla*. Isolates showed distinct morphological and biochemical characteristics. Biochemical characterisation of the isolates was performed by following Bergey's manual of systematic bacteriology. Two isolates were found gram-positive, and one was gram-negative. All three isolates were found positive for the catalase test and negative for the indole test; two isolates (GP-1, GP-3) were positive for the oxidase test; 2 isolates (GP-1, GP-2) were found positive for both citrate and methyl red test. Some plant growth -promoting activities of the isolates were also performed. All the 3 isolates (GP-1, GP-2, and GP-3) were found positive for the ammonia production test; isolate GP-2 was found positive for phosphate solubilisation test; isolate GP-3 was found positive for both IAA production and lipase activity test. The isolated endophytic bacteria survived in different salt concentrations.

Keywords

medicinal plants, plant growth promotion activity, salt tolerance

Introduction

Globally medicinal plants are used directly or indirectly to treat several diseases as these plants are the storehouse of several bioactive compounds. The microorganisms present inside these plants are not fully explored, so a systematic study is necessary in this area (1). The bacterial species which live inside the plant tissue without causing any harm to the host plants are known as endophytic bacteria (2). Endophytic bacteria can be isolated from surface sterilised plant tissues like roots, stems, leaves and sometimes from flowers, fruits and seeds (3). It was reported that leaves contain more endophytic bacteria than other organs like roots and stems (4). Almost all plants have endophytic bacteria; however, the isolation methods can affect the diversity of the isolated endophytic bacteria (5).

Endophytic bacteria show several functions, including plant growth activities like Indole acetic acid (IAA) production, phosphate solubilisation, siderophore production (6), 1-aminocyclopropane-1-carboxylic acid (ACC)

deaminase production, zinc and potassium solubilisation (7). Some endophytic bacterial isolates sometimes also show extracellular enzymes like amylase, esterase, lipase, protease, pectinase, xylanase and cellulase activities (4). Endophytes can also help the plants to survive under environmental stress (8). Endophytic bacteria isolated from medicinal plants were found to produce antibiotics (9). *Bacillus* sp. and *Pseudomonas* sp. isolated from medicinal plant *Plectranthus tenuiflorus* (Vatke) Agnew, showed antibacterial activity against pathogenic bacteria *Salmonella typhi* (ATCC-51812), *Staphylococcus aureus* (ATCC 29213, *Escherichia coli* (ATCC 9637), *Klebsiella pneumonia* (ATCC 37853), *Streptococcus agalactiae*, *Proteus mirabilis*, *Candida albicans* (ATCC 10231) (4). Medicinal plants are expected to be source of isolating rare and interesting endophytes with novel bioactive compounds (10).

Glycosmis pentaphylla (Retz.) Correa is a shrub belonging to the family Rutaceae. Traditionally this plant is used to treat jaundice, fever, cough, rheumatism, anaemia in different places (11). Phytochemical, antioxidant, antibacterial properties of the leaf extract of *G. pentaphylla* were already reported. Ethyl acetate extraction of leaf sample showed antibacterial activity against multi-drug resistant *Staphylococcus aureus*, *E. coli*, *Streptococcus pnemouniae* (11). Ethyl acetate extraction of the isolated endophytic fungi was from *G. pentaphylla* plant showed antibacterial property against pathogenic *Escherichia coli*, *Corynebacterium diphtheria* and *Proteus mirablis* (12). Satyajit Roy Rony reported five endophytic fungi isolated from *G. pentaphylla* and able to taxonomically identified one isolates as *Colletotricum* sp. (13). The endophytic bacteria present in this plant are still not explored. The main objectives of this study were to isolate and characterise endophytic bacteria from the leaves of *G. pentaphylla* with biochemical, salt tolerance and some plant growth-promoting activity.

Materials and Methods

Collection and Identification of plant material

Healthy and disease-free leaves from *G. pentaphylla* were collected from Titaguri (26.444751°N, 90.287123°E, Altitude; 54.8m), Kokrajhar district, Assam, India. The plant was identified at Bodoland University Botanical Herbarium and a voucher specimen (Accession number: BUBH0000143) is also deposited at Bodoland University Botanical Herbarium, Department of Botany, Bodoland University.

Isolation, purification and sub-culture of endophytic bacteria

The leaf samples of *G. pentaphylla* were first washed under running tap water to remove dirt from the surface of the leaves. It was then treated with mild detergent for 15-30 seconds and again washed with running tap water until the complete removal of detergent from the samples. After this, the samples were surface sterilised with the help of laminar airflow (LAF). Surface sterilisation of the samples was performed following the methods of Duhan (14) with some modifications. Inside the LAF, the leaves were treated with 70 % ethanol for 3 min, followed by 2% sodium hypochlorite for 5 to10 min. Then, the samples were washed with sterile distilled water three times. And then, samples were introduced into 10% sodium bicarbonate for 10 min to inhibit endophytic fungal growth. The samples were then taken in the sterile filter paper (Whatman) to drain excess moisture from the leaf sample and then cut into pieces of 0.5-1 cm. Finally, the samples were inoculated onto tryptic soy agar (TSA) media in aseptic condition then incubated at 37 °C for 72 hrs for the maximum recovery of endophytic bacteria from the leaves. The sterility test was done by inoculating the last sample washing water onto nutrient agar (NA) media, and incubating at 37 °C for 10 days. After 72 hrs of incubation, isolates were purified and sub-cultured onto TSA media, and finally, pure culture isolation was done, and all the plates were stored at 4 °C for further experiments.

Characterisation of the isolates

Morphological and biochemical characterisation of the isolates

Initial differentiation of the isolates was performed by analysing morphological and biochemical characteristics of the isolates such as Gram reaction, catalase, oxidase, indole, citrate and methyl red test following the standard methods of Bergay's Manual of systematic bacteriology (15).

Salt tolerance

Isolates were inoculated at the nutrient agar media containing NaCl at various concentrations from 1-7% (6).

Plant Growth promoting activity

Phosphate solubilisation

Bacterial isolates were spot inoculated on the Pikovskaya's medium (16) and incubated for 5-7 days at 28 °C. The plate without isolate was kept as a control plate. Clearing zone formation after incubation indicated a positive result.

Ammonia production

Ammonia production ability of endophytic bacteria was performed by growing the isolates in nutrient broth for 24 hrs, and then 20 µl of the cultures were taken and mixed with 5 ml of 1% peptone medium and incubated at 30 °C for 24 to 48 hrs. After that, 0.5 ml Nesseler's reagent was added to the culture, and yellow colouration indicated the positive result (17).

IAA production

The ability of the isolated endophytic bacteria to produce IAA was tested by growing bacterial isolates in a nutrient broth for 24 hrs, then transferring 20 µl of culture to a medium containing 5 ml of TSB with and without tryptophan (500 $\mu g ml^{-1}$) and incubated at 30 °C for 24 hrs. Then, the cultures were centrifuged at 3000 RPM (REMI C-24 PLUS) for 30 min and 2 ml of supernatant was mixed with 2 drops of orthophosphoric acid and 4 ml of Salkowski reagent (50 ml of 35 % perchloric acid, 1 ml 0.5M $FeCl_3$ solution) and pink colour indicates positive for IAA production (6, 17).

Lipase production

Lipase production test was performed by inoculating the isolates in a medium containing 10 gm peptone, 0.1 gm calcium chloride, 5 gm NaCl, 15 gm Agar, 1000 ml distilled water, 10 ml sterile Tween 20 and incubated at 27 °C for 48 hrs. Depositions around the colonies indicated the lipase enzyme activity (18).

Results and Discussion

Morphological and Biochemical Characterisation

The surface sterilisation method plays an important role in isolating endophytic bacteria. The method used for isolating the endophytic bacteria from the leaves of *G. pentaphylla* showed effective results and no bacterial growth was observed in the sterility test. Three endophytic bacteria were isolated. These bacteria showed different morphological characteristics (Fig. 1), such as GP-1 was white in colour, small size, sticky, round filamentous colony; GP-2 was creamy colour, small, round, raised and filamentous colony and GP-3 was watery colour, irregular shape and slightly raised colony. The gram nature of the isolates was also evaluated; two isolates were gram-positive, rod-shaped and one isolate was Gram-negative rod-shaped (Table 1).

Table 1. Results for colony morphology and gram nature of the isolates

Isolates	Colony morphology	Gram nature	Micro-scopic nature
GP-1	White colour, small, sticky, round, filamentous colony.	Gram positive	Rod shaped
GP-2	Creamy colour, small, round, raised, filamentous colony.	Gram positive	Rod shaped
GP-3	Watery colour, irregular shape, slightly raised colony.	Gram negative	Rod shaped

Some biochemical tests of the isolates were also performed following the standard protocol of Bergay's Manual of systematic bacteriology (15). GP-1 isolate was found positive for catalase, oxidase, citrate and methyl red test whereas negative for indole test. GP-2 isolate showed positive for catalase, citrate and methyl red test; on the other hand, negative for oxidase and indole test. GP-3 isolate showed positive for catalase and oxidase test, whereas negative for indole, citrate and methyl red test (Table 2).

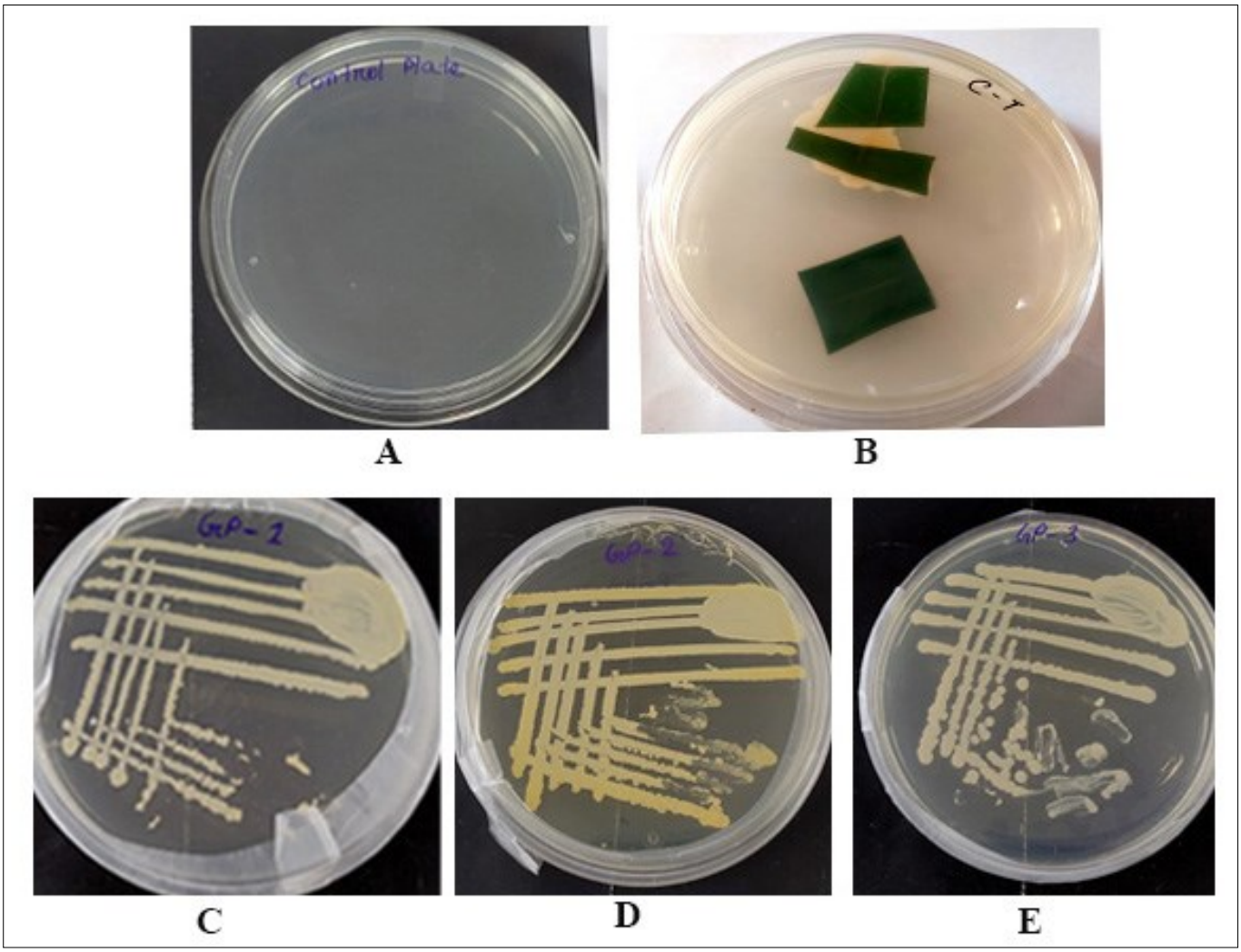

Fig. 1. **A**) Control plate with last sample washed water; **B**) Isolation of endophytic bacteria from the leaves of *Glycosmis pentaphylla*; **C**) Pure culture of GP-1; **D**) Pure culture of GP-2 and **E**) Pure culture of GP-3

Table 2. The biochemical test results of the isolates

Isolates	Catalase	Oxidase	Indole	Citrate	Methyl red
GP-1	+	+	–	+	+
GP-2	+	–	–	+	+
GP-3	+	+	–	–	–

Salt tolerance ability of bacterial endophytes

Isolates were grown on NA media containing NaCl at different concentrations (1% to 7%) to observe their salt tolerance ability (Table 3). Two isolates (GP-2 and GP-3) were found to survive up to 6% NaCl, and GP-1 was found to survive up to 5% NaCl. Microbial endophytes present inside the different tissues helped the plants to survive under stressed environment (14). It was found that leaves contain highest number of endophytic bacteria than the stems and roots (4). Some high salt tolerant endophytic bacteria isolated from the leaves were previously reported. *Bacillus foraminis* and *Bacillus gibsonii* able to tolerated upto 7.5% NaCl; *A. baumannii* and *Paenibacillus xylanisolvens* able to tolerated upto 2.5% NaCl; *P. fluorescence* able to tolerated upto 5% NaCl (19). If these kind of stress tolerant endophytes get restored in agricultural crops, it could lead to the generation of crops which can tolerate biotic and abiotic stresses (14). Our results showed that three endophytic bacterial isolates from the leaves of the sample plant are able to survive under high salt concentration which is already mentioned. However, there exist minimal data on salt tolerance ability of the endophytic bacterial isolates from the leaves of the referred plant.

Table 3. The results of the salt-tolerant ability of the isolates

Isolates	1% NaCl	2% NaCl	3% NaCl	4% NaCl	5% NaCl	6% NaCl	7% NaCl
GP-1	+	+	+	+	+	_	_
GP-2	+	+	+	+	+	+	_
GP-3	+	+	+	+	+	+	_

Plant growth promoting activity

Endophytic bacteria show several plant growth-promoting activities by producing ammonia, IAA or solubilising phosphate. Phosphorus is the second important macronutrient after nitrogen for plant growth and development. It is abundantly present in soil but mostly in insoluble form. As a supplement/ crop nutrient P is added to the soil mostly as chemical P fertilizer which is not eco-friendly. There are some phosphate solubilising microorganisms which are known as the best eco-friendly source of P (20). Phosphate solubilising endophytic bacteria can be present in roots, stem and leafs of different plants. These endophytic bacteria are able to solubilise insoluble phosphate, enhance soil quality and plant growth and development (21). It was previously reported that *Bacillus cereus*, *Bacillus* sp., *Bacillus pumilis* and *Pseudomonas putida* could solubilise phosphate (21).

The endophytic bacterial isolates from *G. pentaphylla* showed some plant growth-promoting activity, including phosphate solubilisation, IAA production, ammonia production and lipase enzyme activity (Table 4). It was found that all three isolates can produce ammonia, and only the isolate GP-2 showed phosphate solubilising ability. Further, it was found that the isolate GP-3 can produce IAA and show activity for lipase enzyme.

Table 4. Plant growth-promoting activity of the isolates

Isolates	Ammonia production	Phosphate	IAA production	Lipase production
GP-1	+	_	_	_
GP-2	+	+	_	_
GP-3	+	_	+	+

Conclusion

Medicinal plants and endophytic bacteria both are potent sources of bioactive compounds. It is possible to extract some novel compounds that can be used as medicine, bio fertiliser or antibiotics from the endophytic bacteria isolated from medicinal plants. Thus, systematic research in this field is indispensably required. This present study is mainly focused on the isolation and characterisation of endophytic bacteria from *G. pentaphylla.* In plant growth promotion activity of the isolates, the isolate GP-2 showed the ability to solubilise insoluble phosphate into a soluble form. All three isolates can produce ammonia. Further, the isolate GP-3 can produce IAA and also showed lipase enzyme activity. Since *G. pentaphylla* is reported to have antimicrobial activity, the anti-microbial study of its endophytic bacteria holds strong prospects to add to the extended spectrum of a comprehensive study of the isolated endophytic bacteria.

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Authors contributions

DD carried out the experiments and manuscript writing. SD participated in experimental design, coordination and supervision.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

Supplementary data

Supplementary Figure. **A**) *Glycosmis pentaphylla* plant habitat**; B**) Inflorescence; **C**) Leaves and fruits.

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