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Isolation and Characterization of Plant Growth-Promoting Rhizobacteria from Root Nodules of *Arachis hypogaea*

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Abstract

In recent years, sustainable agriculture has gained increasing attention, emphasizing the practice of eco-friendly substitutes for conventional chemical inputs. Among these, bioinoculants derived from rhizobacteria play a crucial role in enhancing soil fertility, promoting plant growth, and improving crop productivity, all while minimizing hostile effects on the environment and human health. In the present investigation, root nodules of Arachis hypogaea were explored as a source of potential plant growth-promoting rhizobacteria (PGPR). Ten isolates, designated KS1 to KS10, were obtained and characterized based on their Gram reaction, morphology, and growth-promoting attributes. Of these, isolate KS10 demonstrated superior plant growth-promoting traits. Molecular identification through 16S rRNA sequencing revealed its affiliation with Providencia stuartii. This strain holds promise as an effective bioinoculant capable of improving yield and crop quality, reducing dependency on chemical fertilizers and pesticides, and supporting the development of sustainable farming practices. Its broad applicability also highlights potential for commercialization and global use in eco-friendly agriculture.

Keywords: *Sustainable agriculture, Bioinoculants, Eco-friendly farming, Rhizobacteria, plant growth.*

Introduction

Arachis hypogaea L. (groundnut) is an ancient and economically significant legume crop that originated in South America and is now cultivated extensively across tropical and subtropical regions. The major producers include China, India, Nigeria, and the United States, together contributing to global annual production estimates of 4.7–107 million tons [1]. Groundnut is often cultivated on marginal lands in resource-limited settings and intercropped with cereals, reflecting its nutritional, medicinal, and economic value. Nutritionally, it contains ~30% protein, 20% carbohydrates, 5% fiber, and 45% fat, and is primarily cultivated for oil production. Its by-products further serve as sources of proteins, polyphenols, antioxidants, vitamins, and minerals. Recent studies also highlight its richness in bioactive compounds such as resveratrol, phenolic acids, flavonoids, and phytosterols, which are known to reduce cholesterol absorption and promote human health [2]. Despite its global importance, groundnut cultivation is constrained by inconsistent yields, largely due to poor soil fertility, disease incidence, and environmental stress. In many cases, farmers rely heavily on chemical fertilizers to overcome nutrient deficiencies, but this approach is unsustainable as it increases production costs, degrades soil health, and contributes to environmental pollution. With the rising global population and food demand, there is an urgent need for sustainable strategies to improve productivity.

In recent decades, bioinoculants have increased attention as eco-friendly alternatives to chemical inputs. These inoculants, particularly plant growth-promoting rhizobacteria (PGPR), colonize root surfaces and enhance plant growth by improving nutrient acquisition, producing phytohormones, protecting against phytopathogens, and inducing systemic resistance [3,4]. PGPR exert their effects over direct mechanisms (potassium solubilization, nitrogen fixation, phosphate and phytohormone synthesis, and ammonia production) and indirect mechanisms (antagonism against pathogens via hydrogen cyanide, siderophores, antibiotics and induction of systemic resistance) [5,6].

While genera such as *Bacillus*, *Pseudomonas*, and *Burkholderia* are well documented as PGPR, emerging studies have reported the plant-associated potential of *Providencia* species. *Providencia stuartii*, traditionally known as an opportunistic bacterium, has recently been identified in the rhizosphere and nodule environments, where it contributes to nutrient cycling, phosphate solubilization, and indole acetic acid (IAA) synthesis. Some isolates also exhibit siderophore production and antagonistic activity against soilborne pathogens, indicating their potential as dual-function inoculants enhancing growth while conferring protection to plants. Isolation of *P. stuartii* from groundnut root nodules in the present study therefore highlights a novel association, expanding the diversity of PGPR candidates for sustainable agriculture.

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Given its observed plant growth-promoting traits, *P. stuartii* may serve as a bioinoculant capable of enhancing yield, reducing dependency on chemical fertilizers, and improving resilience under stress conditions. This adds to the growing body of evidence that lesser-studied genera can play important roles in sustainable crop production systems [7].

2. Materials and Methods

2.1. Source of Sample

*Arachis hypogea r*oot nodules with whole plants were collected from Keeranur village in the Pudukkottai district of Tamil Nadu, India. The collected samples placed in the bag, were taken to Rhizosphere Biology Laboratory and stored in the refrigerator for further analysis.

2.2. Isolation and Identification of PGPR

Bacterial isolates were obtained from *Arachis hypogea* plant root nodules through serial dilution [8], whereas root nodules were crushed to produce a suspension, which was serially diluted to acquire bacterial isolates. For bacterial isolation, dilutions of 10^{-3}, 10^{-4}, and 10^{-5} were utilized. The nutrient agar medium was covered with a 0.1ml sample and the plates were incubated at 30°C for up to 48 hours. Based on the colony's appearance and results of biochemical testing [9,10].

2.3. Screening of Plant Growth Promoting Rhizobacteria

2.3.1. Production of Indole Acetic Acid

The bacterial isolates were incubated at 28 °C for approximately two weeks in nutrient broth enriched with 0.1% tryptophan. At specific intervals (from the 2nd to the 10th day, with samples collected every two days), 1 ml of culture was withdrawn and centrifuged at 6000 rpm for 30 minutes. The indole acetic acid (IAA) content was determined following the method described by [8]. For the assay, 1mL of the resulting supernatant was mixed with a drop of orthophosphoric acid, followed by the addition of 2 ml of Salkowski's reagent (prepared using 50 ml of 35% perchloric acid and 1 ml of 0.5 M $FeCl_3$). The developed colour was measured using a spectrophotometer at 530 nm.

2.3.2. Siderophore Production

Cetrimide agar plates were spot-infected for 72 hours at 28°C with the test organism before being incubated with the test organism. The presence of a yellow-orange halo around the developing organism after adding the blue dye in the surrounding organism was intended to help in siderophore production [10].

2.3.3. Phosphate Solubilization

The modified Pikovskaya's medium was prepared using 10 g of glucose, 5 g of calcium phosphate ($Ca_5HO_{13}P_3$), 0.5 g of ammonium sulfate ($(NH_4)_2SO_4$), 0.2 g of potassium chloride (KCl), 0.1 g of magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), 0.0001 g each of manganese sulfate ($MnSO_4$) and ferrous sulfate ($FeSO_4$), along with 0.5 g of yeast extract and 15 g of agar per 1000 ml of distilled water. Bacterial isolates were spot-inoculated on the medium, and after four days of incubation, the formation of clear halo zones indicated phosphate solubilization. The diameter of these zones was recorded, and the solubilization index and efficiency were calculated using the formulas described by [11]. SE= Solubilization diameter/growth diameter

PSI = Colony diameter + zone of clearance
Colony diameter

2.3.4. Hydrogen Cyanide Production

Bacterial isolates were streaked onto a modified agar medium prepared with 0.5 g peptone, 0.2 g yeast extract, 0.5 g NaCl, 0.4 g glycine, and 2 g agar per 100 ml of nutrient broth enriched with glycine. The surface of each agar plate was then covered with a Whatman filter paper previously soaked in a mixture containing 2% sodium carbonate and 0.5% picric acid. The plates were sealed and incubated at 28 °C for four days. A shift in the colour of the filter paper from orange to red signified hydrogen cyanide (HCN) production, as described by [12].

2.3.5. Ammonia Production

Freshly grown cultures were inoculated into 5 ml of peptone water prepared with 1 g peptone and 0.5 g NaCl per 100 ml, and incubated at 28 °C. After incubation, 0.5 ml of Nessler's reagent was added to each tube, and the appearance of a brown to yellow colour indicated ammonia production [13].

2.3.6. Biological Nitrogen Fixation

The bacterial strain was evaluated for its nitrogen-fixing capability using Glucose Nitrogen-Free Minimal Medium (G-NFMM). A single colony was inoculated into G-NFMM broth supplemented with bromothymol blue (BTB) indicator. Following 24 hours of incubation, the culture was examined for any change in colour, which signified nitrogen fixation activity [14].

2.4 Molecular Identification

2.4.1 DNA Isolation

The most efficient bacterial strain was subjected to genomic DNA extraction using the CTAB protocol described by [15]. A 2 mL culture was centrifuged at 10,000 rpm for 15 minutes, after which the supernatant was discarded and the pellet collected. The pellet was resuspended in 500 µl of Solution I and 50 µl of lysozyme-C, vortexed, and incubated at 37 °C for 15 minutes, followed by another brief vortex. Subsequently, 50 µl of 10% SDS and 5 µl of Proteinase K were added, and the mixture was incubated at 65 °C for 30 minutes. After incubation, 40 µl of 5 M NaCl and 32 µl of CTAB/NaCl solution were incorporated, mixed thoroughly, and incubated again at 65 °C for 30 minutes. An equal volume of freshly prepared chloroform: isoamyl alcohol (24:1; approximately 677 µl) was then added, mixed until the solution turned milky, and centrifuged at 10,000 rpm for 10 minutes. About 300 µl of the aqueous phase was transferred to a fresh tube, and pre-chilled ethanol was added (18 µl increments) until the total volume reached 1 ml. The mixture was gently mixed and stored at –20 °C for 1 hour or overnight to precipitate DNA. The sample was then centrifuged at 10,000 rpm for 30 minutes, the supernatant discarded, and the pellet air-dried. The dried DNA was dissolved in 50 µl of TE buffer. For quality assessment, 0.8% agarose gel was prepared in 1X TAE buffer containing ethidium bromide. Once solidified, the comb was removed, and 10 µl of sample prepared by mixing 6 µl of DNA with 4 µl of loading dye, was loaded into the wells. Electrophoresis was performed at 60 V for 35 minutes using a DNA ladder as reference. DNA bands were finally visualized under UV illumination to confirm integrity and purity.

2.4.2 16S rRNA Gene Sequencing, NCBI Submission, and Phylogenetic Tree Construction

The bacterial 16S rRNA gene was amplified using universal primers described by [16]: UB16SF (5′-AGAGTTTGATCCTGGCTCAG-3′) as the forward primer and UB16SR (5′-ACGGCTACCTTGTTAGCACT-3′) as the reverse

primer. The PCR reaction mixture consisted of 5 µl of Master Mix, 1 µl each of the forward and reverse primers, 2 µl of Milli-Q water, and 1 µl of genomic DNA template, prepared in a sterile PCR tube. Amplification was carried out with an initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute, and extension at 72 °C for 2 minutes, with a final extension step at 72 °C for 10 minutes. The PCR products were resolved on a 1.2% agarose gel containing ethidium bromide (approximately 8 µl), and the bands were visualized under UV light. The amplified fragments were purified and sequenced. The obtained sequences were analyzed using BLAST to compare them with available sequences in the NCBI database. Phylogenetic and evolutionary analyses were performed using MEGA 11 software [17].

3. Results and Discussions

3.1. Isolation and identification of Rhizobacteria

This study aimed to promote eco-friendly and sustainable agriculture by exploring the role of potent plant growth-promoting rhizobacteria (PGPR) as bioinoculants for enhancing plant growth and improving crop yield. PGPR serve as a biological alternative to synthetic fertilizers, helping to meet the increasing demand for food production while addressing unstable agricultural productivity and reducing environmental pollution. Soil samples were collected from Keeranur Village, Pudukkottai district, Tamil Nadu, India. Root nodule suspensions were serially diluted and cultured on Congo Red YEMA plates, which revealed colonies with varied morphologies. Based on colony characteristics and biochemical analyses, ten Gram-negative bacterial isolates (designated KS1 to KS10) were obtained. These isolates displayed distinct colony features, including pink, yellow, pale pink, and predominantly white mucoid or flat morphologies. All isolates were screened for plant growth-promoting traits. Among them, the isolate KS10 demonstrated superior performance and was identified as the most efficient strain for further study.

3.2. Screening for Plant Growth Promoting Rhizobacteria

3.2.1. Production of Indole Acetic Acid

The isolates were capable of producing indole acetic acid (IAA) in varying amounts at different incubation periods. IAA production was confirmed by the development of a pink coloration upon the addition of Salkowski's reagent, with colour intensity subsequently quantified spectrophotometrically. Among the isolates, strain KS10 exhibited the highest IAA production on the second day of incubation. Elevated IAA levels are known to enhance shoot development in plants, thereby contributing to improved growth performance (Figure 1). Similar results have been reported for several rhizobacteria in earlier studies [18,19,20]. The enhanced synthesis of IAA by PGPR has been attributed to the presence of tryptophan as a precursor molecule [21,22].

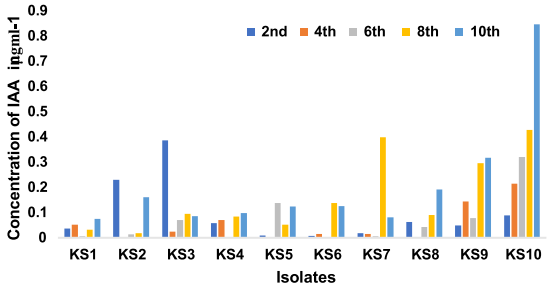

Figure 1: Indole acetic acid Production

3.2.2. Siderophore Production

Under iron-deficient conditions, one of the important biocontrol strategies employed by plant growth-promoting rhizobacteria (PGPR) is the synthesis of siderophores. These compounds chelate iron acquired from the immediate surroundings, thereby restricting the availability of this essential nutrient to pathogenic microorganisms and inhibiting their growth [10,23].In this study, siderophore production was detected using the Fe-CAS-hexadecyl trimethyl ammonium bromide assay. The sequestration of iron by siderophores resulted in the release of CAS dye from the complex, producing a visible colour change from blue to orange around the bacterial colonies, which indicated positive activity. Among the ten isolates tested, strains KS4 and KS10 were identified as siderophore producers.

3.2.3. Phosphate Solubilization

Phosphorus is the second most essential nutrient required for plant growth, yet it is often unavailable to plants due to its presence in insoluble forms within the soil. Although synthetic P fertilizers are commonly used, their high cost and environmental impact make biological alternatives increasingly important. Bioinoculants with phosphate-solubilizing activity offer a sustainable option, as certain rhizobacteria can convert insoluble phosphates into plant-available forms. The ability of these bacteria to solubilize mineral phosphate enhances the uptake of both phosphorus and iron, thereby promoting overall plant development. Phosphate-solubilizing rhizobacteria release phosphorus from insoluble inorganic and organic complexes in the soil, making it accessible to plants [22]. This process is also influenced by the production of plant growth-regulating hormones, which further support plant development [11]. In the present study, three isolates demonstrated phosphate-solubilizing activity. Among them, KS5 exhibited the smallest halo zone, whereas KS10 produced the largest zone of clearance, indicating superior phosphate solubilization ability. Based on these results, KS10 was identified as the most efficient phosphate solubilizer compared to the other strains (Figure 2).

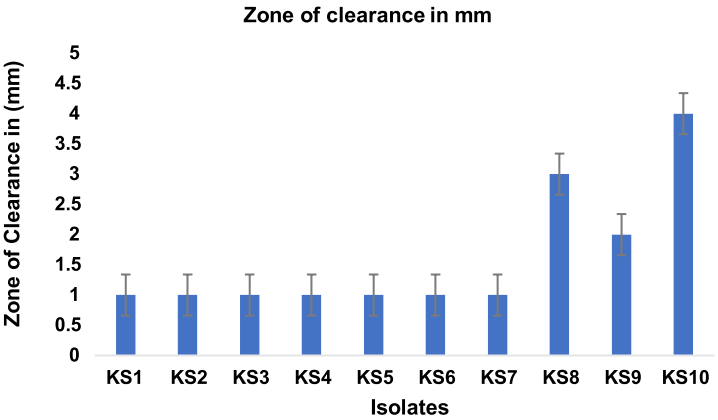

Figure 2: Zone of clearance of phosphate solubilization

3.2.4. Hydrogen Cyanide Production

Hydrogen cyanide (HCN) is a volatile secondary metabolite produced by several rhizobacteria, and it plays a dual role in promoting plant growth and functioning as a biological control agent (BCA) against various plant pathogens [24]. HCN production is considered an important plant growth-promoting (PGP) trait, as it contributes to enhanced plant development and improved disease resistance. Rhizobacteria capable of synthesizing HCN help suppress soilborne pathogens, thereby supporting the biological control of plant diseases [25].

In the HCN assay, picric acid in the reagent reacts with HCN to form an isopurpuric acid complex, resulting in a shift from yellow to an orange or red-orange colour, which indicates positive HCN production. In the present study, five out of the ten isolates tested showed HCN production. Among them, KS10 exhibited a strong positive reaction, producing a distinct dark orange colour, whereas KS1 showed a moderate positive response.

3.2.5. Ammonia Production

Ammonia production by PGPR contributes indirectly to plant growth by supplying a readily available nitrogen source [22]. Diazotrophic bacteria convert atmospheric nitrogen into ammonia, which not only supports plant nutrition but also enhances biocontrol activity against soilborne pathogens [26,27]. In the present study, ammonia production was detected through the reaction between potassium tetraiodomercurate and the ammonia released by the isolates, resulting in the development of a brown precipitate. Most of the ten isolates exhibited the ability to produce ammonia. Among them, KS10, KS2, KS6, and KS9 produced an intense brown coloration, indicating strong ammonia-generating potential (Table 1).

Table 1: Ammonia production

Isolates	Ammonia production
KS1	+
KS2	++
KS3	+
KS4	+
KS5	+
KS6	++
KS7	+
KS8	+
KS9	++
KS10	+

+ weakly positive (yellow colour change), ++ moderately positive (light brown), +++ strongly positive (Deep brown)

3.2.6. Biological Nitrogen Fixation

Nitrogen is a fundamental nutrient required for plant growth and metabolic processes. Despite its abundance in the atmosphere, molecular nitrogen (N_2) is not directly accessible to plants, especially in nutrient-poor tropical soils. Many associative and free-living rhizosphere bacteria possess the ability to fix atmospheric nitrogen, converting it into plant-available forms, and are therefore recognized for their significant contribution to plant development [28]. During nitrogen fixation, atmospheric N_2 is reduced and combines with hydrogen ions to form ammonia (NH_3). This activity can be visually detected by a colour shift from green to blue, indicating the presence of active nitrogen-fixing microorganisms. In the present study, five isolates showed positive nitrogen-fixing ability when grown in glucose nitrogen-free mineral medium. Among these, strains KS1 and KS10 demonstrated the highest nitrogen fixation potential.

3.3 Molecular Identification

3.3.1 Genomic DNA Isolation and 16S r RNA Gene Sequence Analysis

Genomic DNA was extracted from the most efficient PGPR isolate, KS10, and subsequently amplified using universal 16S rRNA primers. The resulting PCR products yielded an amplicon of approximately 1000 bp. The amplified fragments were sequenced, and the obtained sequences were analyzed using the BLAST algorithm to determine their closest taxonomic matches. Isolate KS10 showed 99% similarity to previously reported bacterial sequences (Table 2). The sequence corresponding to KS10 was identified as *Providencia stuartii* and was submitted to the NCBI database under the accession number PQ585096. Phylogenetic analysis using the neighbour-joining method further confirmed that isolate KS10 clustered closely with other *Providencia stuartii* strains (Figure 3), demonstrating strong evolutionary relatedness.

Table 2: GenBank submission

Bacterial strains	Isolation source (Root Nodule)	Identification similarity	Closest match in GenBank	Accession Number
KS10	***Arachis hypogea* L.**	**99%**	*Providencia stuartii*	PQ585096

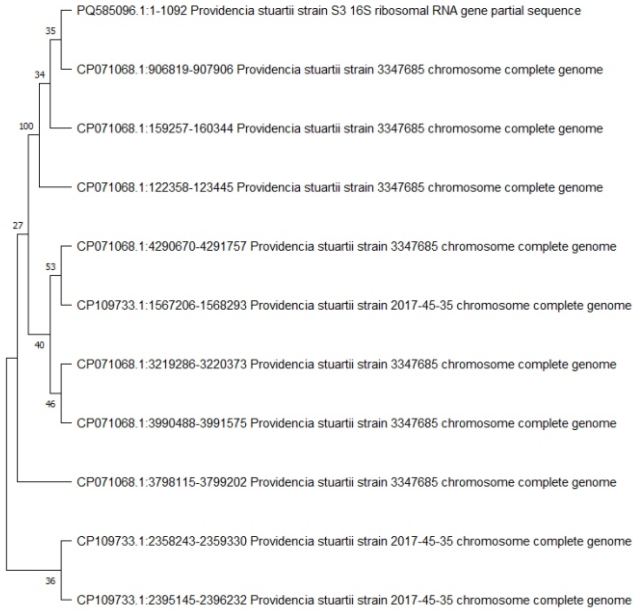

Figure 3: Neighbor-joining phylogenetic tree of efficient strain Ks10

Note: The phylogenetic relationship of PGPR efficient strain KS10 was performed by Neighbor joining method using MEGA 11.0 software and evolutionary distances of sequences were calculated by Bootstrap method.

4. Conclusion

The present study successfully isolated and characterized a tolerant PGPR strain from root nodules of *Arachis hypogaea*. This strain (KS10) demonstrated efficient plant growth-promoting traits and was identified as a potential bioinoculant that can improve plant growth while simultaneously enhancing soil health. The findings suggest that KS10 significantly influenced the growth of *Arachis hypogaea*, highlighting its potential as an eco-friendly alternative to chemical fertilizers. PGPR isolates may be further evaluated for their ability to function as biofertilizers under diverse environmental conditions. Future research at the molecular level is essential to unravel the specific enzymes or genes responsible for their growth-promoting capacity. The application of bioinoculants and biofertilizers, owing to their efficiency and sustainability, provides a promising strategy to achieve higher crop yields without environmental hazards, thereby contributing to global food security.

Author contribution
All authors contributed equally to the manuscript M. Geetha: Writing – original draft, Software, Resources, Data curation, Methodology, Conceptualization and Methodology. V. Rajesh Kannan: Resources, Software, Validation All authors have read and approved the final manuscript.

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Data availability is Not applicable.

Declarations
Ethics approval and consent to participate: Neither ethical approval nor informed consent was required for this study.
Consent for publication All authors gave consent for publication in this scientific journal.
Competing interests The authors declare no competing interests.

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