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Exploration of Plant Growth-Promoting Rhizobacteria Associated with Root Nodules of *Arachis hypogaea*

Murugaiya Geetha and Velu Rajesh Kannan *

Rhizosphere Biology Laboratory, Department of Microbiology, Bharathidasan University, Tiruchirappalli, 620 024, Tamil Nadu, India

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Abstract

In the last two decades, there has been a growing interest in environmentally friendly sustainable agricultural practices, thus increasing the role of bioinoculants in rhizobacteria. Bioinoculants were used to fertilise the soil to scale up plant growth and quality crop yield without generating environmental threats and health risks in the eco-friendly aspects. The present study focused on characterising the efficacy of potent plant growth-promoting rhizobacteria (PGPR) isolated from *Arachis hypogaea* root nodules. Eleven isolates were identified as Gram-negative with distinctive morphological characteristics and plant growth-promoting traits deputed as *A.hypogaea* isolates with the number appended from VS1 to VS11. One of the thirteen isolates namely VS11 was efficient in plant growth promoting PGP traits, the 16S rRNA sequencing analysis revealed that the isolate belonged to the genus *Bacillus pumilus*. This isolate can be harnessed as a bioinoculant significantly providing an environment-friendly approach on a broad spectrum to increase crop production and quality; reduce expenses for fertilizers and pesticides; mitigate environmental issues; meet food demand and healthy lifestyle, development of sustainable agriculture and commercialization by using plant growth promoting rhizobacteria with global applicability.

1. Introduction

Arachis hypogaea L. (groundnut) is a primordial and essential cash crop that originated from South America and it was cultivated worldwide in tropical and subtropical regions. The four major producers of peanuts include China, India, Nigeria, and the United States, with global production of approximately 47 million metric tonnes per year (USDA Foreign Agricultural Services, 2020). In many impoverished countries, it is commonly grown on marginal lands with depreciated resources and intercropped with cereals, owing to their economic importance, nutritional values, and medicinal benefits for both humans and livestock such as 30% protein, 20% carbohydrates, 5% fibres and 45% fat rich crop grown primarily for oil production, however, its by-products contain several useful constituents that may be utilized as bioactive components in a range of processed foods, including as proteins, fibres, polyphenols, antioxidants, vitamins and minerals. Recently, it was discovered that peanuts are particularly rich in compounds including resveratrol, phenolic acids, flavonoids, and phytosterols, which help to reduce the absorption of cholesterol from food (Ahmed et al., 2022).

The agroeconomically important crop *Arachis hypogaea* L. was cultivated in several remote areas throughout the world due to heavy population there is an obligation to enlarge the yield of crops and to meet food demand, one of those remote areas was chosen for this study.

Arachis hypogaea was cultivated as a dual crop one year giving a high yield and a lower yield in another year; low availability of nutrients in soil is the most prevalent barrier to increasing agricultural productivity. Therefore producers ineluctably fertilize the soil to scale up production and quality of crops. Due to the impressive advancements made in the research of the relationship between microorganisms and plants over the past two decades paved the way for developing bioinoculants (Anirban et al., 2021).

Bioinoculants comprise microorganisms that use a wide range of strategies and exert influence on plant growth and crop yield through a plethora of processes. Plant growth-promoting rhizobacteria is a diverse group of bacteria known to synthesize and release several regulatory compounds in proximity to plant roots that strengthen plant growth and productivity (Khosravi et al., 2018; Khoshru et al., 2020) via aiding in improved nutrient uptake by host plants, defending against phytopathogenic microorganisms and fostering tolerance to a variety of biotic and abiotic stressors (Backer et al., 2018; Singh et al., 2019). PGPR strains can boost crop yields, display biocontrol, improve induced systemic disease resistance, encourage nodulation in legumes, and improve the sprouting of the seed, as well bioinoculants elicit action in two ways direct action involve contributing phytonutrients like fixed nitrogen and solubilized minerals like phosphate and potassium, ammonia production, phytohormones controlling and indirectly through impeding phytopathogens, inducing antagonistic substances like hydrogen cyanide, antibiotics and siderophore, parasitism, and induced systemic resistance against a wide range of pathogens (Nazir et al., 2018; Kalam et al., 2020; Swarnalakshmi et al., 2020).

Hence, the current study was designed to collect legume plant root nodules and from that isolation, identification, and screening of PGPR had done,

PGPR effectiveness for an isolate to stimulate the development of legume plants and improve crop production, to undertake molecular characterization of isolated PGPR strains and to conduct *In vitro* and *In vivo* investigations to evaluate the PGPR efficiency to increase the yield of crops as well as enhance the growth of legume plants.

2. Materials and methods

2.1. Source of Sample

*Arachis hypogaea r*oot nodules with whole plants were collected from Vamban 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 hypogaea plant root nodules through serial dilution (Albdaiwi et al., 2019), whereas root nodules were crushed to produce a suspension, which was then combined with 1 mL of sterile water and 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 0.1 mL sample and the plates were incubated at 30ºC for up to 48 hours. Based on the colony's appearance and results of biochemical testing (Aditi et al., 2017; Tirry et al., 2018). The isolates were kept for further analysis.

2.3. Screening of Plant Growth Promoting Rhizobacteria

2.3.1. Production of Indole Acetic Acid

The bacterial strains were incubated at 28ºC for around two weeks; while being cultivated in nutrient broth supplemented with tryptophan (0.1%). Amounts of cultures equal to 1 ml were taken out at various times (2nd – 10th day with a 2-day break) and centrifuged for 30 minutes at 6,000 rpm. Indole acetic acid content was estimated (Lwin et al., 2012), 1 ml of the supernatant and a drop of orthophosphoric acid were combined and 2 ml of Salkowski's reagent (50 ml of 35% Perchloric acid and 1 ml of 0.5 M FeCl3) added and combined. The colour was measured spectrophotometrically at 530 nm.

2.3.2. Siderophore Production

Cetrimide agar plates were spot-infected for 72 hours at 28ºC with the test organism before being incubated with the test organism. The presence of a yellow-orange halo around the development after adding the blue dye in the surrounding organism was intended to help in siderophore production (Tirry et al., 2018).

2.3.3. Phosphate Solubilization

Pikovskaya's medium that has been modified contains 10 g of glucose, 5 g of tribasic phosphate (Ca5HO13P3), 0.5 g of (NH4)2SO4, 0.2 g of KCl, 0.1 g of MgSO47H2O, 0.0001 g of MnSO4 and FeSO4, 0.5 g of yeast extract and 15 g of agar in 1,000 mL of distilled water. Spot inoculations of bacterial isolates produced a zone of clearance after 4 days of incubation. The diameter of the halo clear zones was measured, and the following calculations were used to calculate the solubilization index and efficiency (Garcia et al., 2017).

SE= Solubilization diameter/growth diameter

PSI= $\frac{\text{Colony diameter + zone of clearance}}{\text{Colony diameter}}$

2.3.4. Hydrogen Cyanide Production

Bacteria were streaked on modified agar consisting of Peptone - 0.5g, Yeast extract - 0.2g, NaCl - 0.5g, Glycine - 0.4g, and Agar-agar - 2g per 100 mL of nutrient broth supplemented with Glycine/L. The top of the agar plate was covered with a Whatman filter paper that had been soaked in a solution of 2% sodium carbonate and 0.5% picric acid. For 4 days, the plates were incubated at 280C and remained sealed. Hydrogen cyanide production was indicated by the orange-to-red colour transformation (Pandey & Gupta, 2019).

2.3.5. Ammonia Production

Freshly developed cultures were added to 5 ml of peptone water (Peptone - 1 g), (NaCl - 0.5 g) per 100 mL and incubated at 28ºC. Each tube was filled with 0.5 ml of Nessler's reagent and the development of a brown-to-yellow colour was observed (Nathiya et al., 2019).

2.3.6. Biological Nitrogen Fixation

The Bacterial strain was tested for nitrogen-fixing ability with Glucose Nitrogen Free Minimal Medium (G-NFMM). The single colony was transferred to GNFMM broth containing Bromothymol blue solution (BTB). After being incubated for 24 hours, the broth was observed for colour change (Win et al., 2012).

2.4 Molecular Identification

2.4.1 DNA Isolation

The screened efficient strain was directed to genomic DNA isolation by following the CTAB method (Moeller & Cross, 1962). 2 mL of culture was centrifuged at 10,000 rpm for 15 minutes. Remove the supernatant and collect the pellet. Resuspend pellet in 500 µl of solution I and 50 µl of lysozyme-C. Vortex the sample and incubate at 370C for 15 minutes, vortex again after incubation. Then add 50 µl of 10% SDS and 5 µl Proteinase K and incubate at 650C for 30 minutes. Add 40µl of 5M NaCl and 32 µl of CTAB/ NaCl; incubate at 650C for 30 minutes and vortex it. Add an equal volume of freshly prepared chloroform: Isoamyl alcohol (24:1) proportion (~677 µl). Mix well until changed to milky white and centrifuge at 10,000 rpm for 10 minutes then mix it lightly without vortexing. Take 300 µl supernatant and add 18 µl of prechilled ethanol until the volume becomes 1 mL. Mix and keep at 70ºC for 1 hour or overnight. Centrifuge at 10,000 rpm for 30 minutes, the supernatant was discarded and air dried the pellet. Air-dried DNA samples were reconstituted in 50 µl of TE buffer. 0.8% agarose was prepared by adding 1X TAE buffer and ethidium bromide. The prepared agarose was poured into the electrophoresis apparatus with a comb to create wells on a gel. After solidification comb was removed. Then 4 µl of gel loading buffer was mixed with 6µl of sample. After that 10 µl of sample is loaded on the well. The sample was allowed to run on the gel and was employed in electrophoresis at 60V for 35 minutes, using a DNA ladder to confirm the purity of DNA. The bands were then visualized under UV light to make the band visible.

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

The following universal 16S rRNA gene primers (Weisberg *et al*., 1991) were used to amplify the bacterial 16S rRNA gene from isolated genomic DNA: UB16SF -5'AGAGTTTGATCCTGGCTCAG3' and UB16SR – 5'ACGGCTACCTTGTTAGCACT – 3' are the forward and reverse primers for 16S rRNA. In a sterile PCR tube, combine 5µl Master mix, 1µl Forward primer, 1µl Reverse primer, 2µl Milli Q water, and 1µl DNA template to make the reaction mixture. The PCR profile included an initial denaturation of 5 minutes at 95°C, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 2 minutes, and final extension at 72°C for 10 minutes. The final products were separated on a 1.2% agarose gel and stained with ethidium bromide (about 8 µl ethidium bromide). Purified and sequenced PCR products were obtained.

BLAST was used to align the sequence obtained with previously published sequences in the NCBI Database. MEGA 11 software was used to perform the phylogenetic and evolutionary studies (Tamura et al., 2011).

3. Results and Discussions

3.1. Isolation and identification of Rhizobacteria

The present study was mainly focused on the nurturing of eco-friendly sustainable agriculture through understanding the role of potent PGPR as a bioinoculant to increase plant growth and quality crop yield. The PGPR is a biological tool to combat the demand for food supply caused by heavy population, unstable productivity, and mitigating environmental pollution as an alternative for synthetic fertilizers. The sampling site was located at Puddukottai district, Vamban Village of Tamil Nadu, India. Root nodule sample suspension was serially diluted and spread on Congo Red YEMA Media plates showing different colonies. Based on the colony morphology and biochemical characterization, 11 Gram-negative different bacterial isolates (VS1 to VS11) were acquired with distinct colony morphology such as pink-coloured, yellow-coloured, pale pink-coloured, and majorly white-coloured mucoid and flat colonies were observed. All the isolates were screened for PGP traits. One of them VS11 was found to be an efficient strain through screening of PGPR traits.

3.2. Screening for Plant Growth Promoting Rhizobacteria

3.2.1. Production of Indole Acetic Acid

All thirteen isolates produced varying amounts of IAA on different days and were identified by the formation of a pink colour followed by the addition of Salkowski's reagent. The colour intensity was measured spectrophotometrically. According to that maximum levels of IAA were produced on the 2nd day by VS11. The plant grows its shoots better when IAA levels are at their highest. Similar outcomes were shown by several rhizobacteria (Lwin et al., 2012; Islam et al., 2016; Luziatelli et al., 2020). The higher degree of IAA generation in PGPR has been linked to tryptophan as a precursor (Naz & Bano, 2009; Yadav et al., 2010).

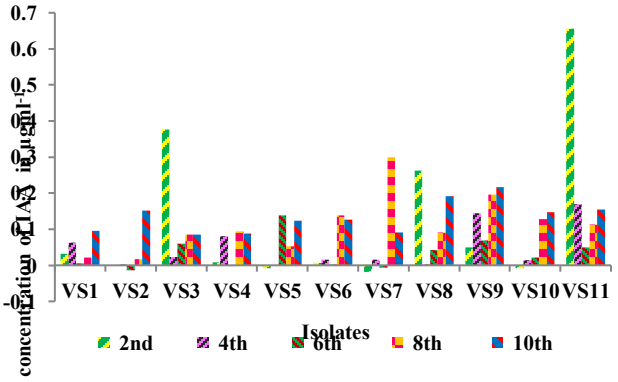


Figure 1: Indole acetic acid Production

3.2.2. Siderophore Production

Plant growth-promoting rhizobacteria groups in iron-deficient conditions used one of the biocontrol strategies is the synthesis of siderophore. As a result, the environment's iron limitation would stop the development of hazardous microorganisms (Miller et al., 2008). Produced siderophore sequestered iron from Fe-CAS-hexadecyl trimethyl ammonium bromide

complex, and following the release of CAS dye from complex leads to colour change from blue to orange zones around the growth of an organism indicates positive for siderophore production. In this study the PGPR isolates VS5 and VS11 were siderophore producers out of the thirteen isolates tested.

3.2.3. Phosphate Solubilization

The second most important nutrient for plants is phosphate, which might reduce the need for expensive P fertilizers. Bioinoculants are another option that has P solubilization activity, but plants are unable to absorb phosphorus from the soil in the form of insoluble phosphates. Agricultural researchers are very interested in bacterial capacity to solubilize the mineral phosphate because it increases the availability of phosphorus and iron for plant development. Rhizobacteria that encourage plant development solubilize precipitated phosphate, increasing the phosphate availability to plants. Free-living bacteria that solubilize phosphate release phosphate from leftover soluble inorganic and organic phosphate complexes in soil and make it available to plants (Duarah et al., 2011). (Yadav et al., 2010; Duarah et al., 2011). Plant hormones produce phosphate solubilization, which increases plant growth (Albdaiwi et al., 2019). In this present study, three isolates showed positive for phosphate solubilization in that VS10 showed the lowest zone of clearance and VS11 showed the highest zone of clearance indication of phosphorus solubilization. According to experimental findings, VS11 acts as an effective phosphate solubilizer that other strains.

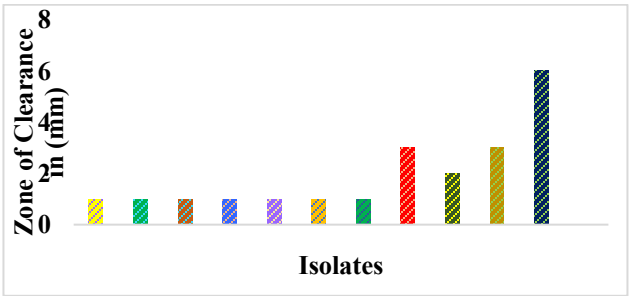


Figure 2: Zone of clearance of phosphate solubilization

3.2.4. Hydrogen Cyanide Production

Hydrogen cyanide is a volatile metabolite produced by rhizobacteria that promotes plant growth and functions as a biocontrol agent (BCA) against plant diseases (Jayaprakashvel et al., 2010). One of the PGP traits that supported plant development and disease resistance was the production of hydrogen cyanide (HCN). Rhizobacteria that generate hydrogen cyanide are responsible for the biological control of pathogens (Deshwal & Kumar, 2013). Yellow color picric acid reacted with HCN to form an isopurpuric acid complex that resembles a red-orange color indicating the ammonia-producing ability. In this present study the production of HCN, which induces resistance and promotes plant growth, and among 13 isolates seven isolates have shown positive in that VS11 was substantially positive by producing a dark orange colour and VS1 was moderately positive.

3.2.5. Ammonia Production

Plant growth was indirectly influenced by PGPR's ammonia production (Kaur & Sharma, 2013). For plants to use nitrogen, the diazotrophic bacterium converts it to ammonia, in addition, this plays an important role in biocontrol activity (Goswami et al., 2014). The potassium tetraidomecurate reacted with produced ammonia to form a brown precipitate indicating positive for ammonia producing activity. The majority of the ten isolates were capable of producing ammonia. Among them, VS11, VS3, VS5, and VS6 displayed deep brown colour indicating strongly positive results.

Table 1: Ammonia Production

Isolates	Strength of Ammonia
VS1	+
VS2	+
VS3	+++
VS4	+
VS5	++ ,
VS6	++
VS7	+
VS8	+
VS9	++
VS10	+
VS11	+++

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

3.2.6. Biological Nitrogen Fixation

The primary source for plants that are required for their growth and development is nitrogen. Even though the element N is plentiful in the earth's atmosphere, most tropical soils are easily accessible. Numerous associative and free-living rhizosphere bacteria may fix nitrogen, and they are well-recognized for playing a vital role in plant development (Win et al., 2012). The N2 combines with hydrogen ions and forms NH3 leading to a color change from green to blue indicating positive for nitrogen fixers. In this current study, five isolates have shown nitrogen-fixing activity, when the isolated rhizobacteria were tested for their capacity to fix nitrogen in the glucose nitrogen-free mineral medium. Among them, VS1, VS11, and VS12 were found to show high nitrogen fixation activity.

3.3 Molecular Identification

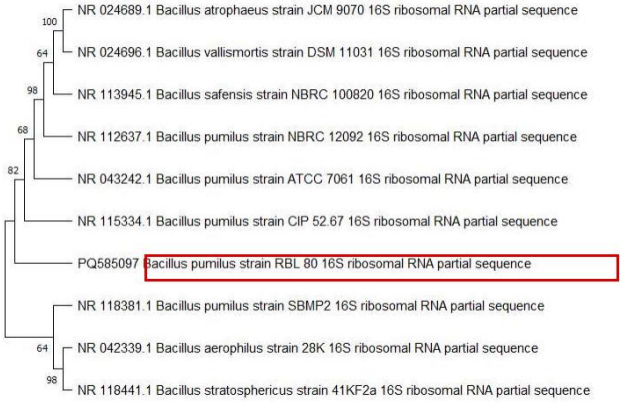
3.3.1 Genomic DNA Isolation and 16S rRNA Gene Sequence Analysis

Genomic DNA was isolated from the efficient PGPR isolate VS11. The isolated DNA sample was amplified by using universal primers. The amplified gene product size was approximately 1,000 bp for both isolates. The amplified 16S rRNA products were sequenced and the sequence was verified in BLAST, species names of the isolates were identified based on their similarity with other bacterial sequences. Isolate VS11 has shown 99% with (Table 2). The sequence of the isolate of VS11 Bacillus pumilus was obtained and submitted to NCBI under the accession number PQ585097. The bacterial strain VS11 isolated from the Arachis hypogaea root nodule has shown phylogenetic relatedness with Bacillus pumilus strains (Figure 3). The genetic relationship of the bacterial isolates was confirmed through phylogenetic analysis by the neighbour-joining method (Tamura et al., 2011).

Table 2: GenBank submission

Bacterial strains	Isolation source (Root Nodule)	Identification similarity	Closest match in GenBank	Accession Number
VS11	***Arachis hypogaea* L.**	**99%**	*Bacillus pumilus*	PQ585097

Figure 3: Neighbor-joining phylogenetic tree of efficient strain VS11



Note: The phylogenetic relationship of PGPR efficient strain VS11 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 purposefully isolated and characterized tolerant PGPR strain from root nodules of the *Arachis hypogaea* plant. That promotes plant growth and is beneficial to soil health by acting as an alternative for biofertilizers. (VS11) were identified as efficient strains that were applied on plants as bioinoculants. The results of the present study suggest that (VS11) influenced *Arachis hypogaea* growth. In conclusion, these PGPR characteristic isolates may be evaluated for their ability to convert it into biofertilizing activity under other multifarious traits, and future research on interaction at the molecular level is needed to identify which enzymes or genes are responsible for the PGPR ability. Also suggested to using bioinoculants and biofertilizers to get higher plant growth and crop yield because of their efficient PGPR traits without causing environmental hazards offers the potential to sustainably feed the growing population.

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