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Isolation and Determination of Plant Growth Promotion Traits of Non nodulating Root Nodule Associated Bacteria in Vegetable Legumes

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ABSTRACT

The present investigation was conducted to determine the plant growth promotion traits *viz.,* ammonia production and phosphate solubilization of eight non nodulating nodule associated bacterial isolates obtained from the root nodules of cowpea and garden pea grown in different locations of Andhra Pradesh and Karnataka. These isolates were selected from a collection of one hundred and two bacterial isolates, based on a rapid preliminary screening. The isolates *Enterobacter sp* CPH64., *Enterobacter* sp CPK42., *Chryseobacterium* sp. CPM11, *Stenotrophomonas* sp. CPH62 originating from cowpea nodules and *Enterobacter* sp. GP44, *Enterobacter sp.* GP71, *Enterobacter sp.* GP84, *Bacillus* sp. GP102, from garden pea nodules were able to solubilize phosphates and produce ammonia, which are considered the most important traits for the growth and development of crop plants.

Key words: *Ammonia production,Cowpea, Garden pea, Non nodulating bacteria, Phosphate solubilization,*

Vegetable legumes form a diverse and nutritionally rich category of plants that play a crucial role in both our dietary choices and agricultural practices. These remarkable vegetables are renowned for their ability to fix nitrogen in the soil, making them not only a valuable food source but also essential for sustainable farming practices. Legumes encompass a broad array of plants, including peas, beans, lentils, chickpeas, and soybeans etc. Cowpea (*Vigna unguiculata*) and garden pea (*Pisum sativum*) are the two most important food and vegetable legumes. The total global cowpea production in 2019 was 8.9 million tonnes (FAO STAT, 2021)(http://www.fao.org/faostat) and the area under garden pea has been estimated at 2.18 million hectares with a production of 21.77 million tonnes and productivity of 9.99 mt ha^{-1} (FAO STAT, 2021) (http://www.fao.org/faostat).

The root nodules of leguminous crops are colonised by both nodulating and non nodulating bacteria. While the nodulating bacteria are generally classified as rhizobia, the non nodulating types may belong to a wide range of bacteria genera and species. The rhizobial and non-rhizobial bacteria represent two distinct groups within root nodules of legumes having 16S rRNA gene sequence and physiological characteristics of the 31 isolates revealed the presence of *Agrobacterium radiobacter, A. tumefaciens, Azospirillum lipoferum, Bradyrhizobium elkanii, Burkholderia cepacia, Frateuria aurantia, Klebsiella oxytoca, K. pneumoniae, Rhizobium gallicum, Rhizobium sp., Starkeya novella and Xantobacter flavus*. Lee *et al.* (2005) isolated *Bacillus* strain DLA from bean (*Phaseolus vulgaris* L.) nodules. Kan *et al.* (2007) characterised the symbiotic and endophytic bacteria from root nodules of herbaceous legumes grown in Qinghai–Tibet plateau and in other zones of China. They confirmed that the rhizobia associated with test herbaceous legumes grown in Qinghai–Tibet plateau and in surrounding regions. They reported that *Medicago archiducis-nicolai* and *Oxytropis* spp were new host plants for *R.leguminosarum* while *Oxytropis glabra* and *Medicago lupulina* were newly recorded hosts for *S. fredii*. Li *et al.* (2008) isolated ninety eight non-symbiotic endophytic bacterial strains from soybean root nodules. The phylogenetic analysis of 16S *rDNA* identified them as *Pantoea, Serratia, Acinetobacter, Bacillus, Agrobacterium* and *Burkholderia*. Most of the strains produced Indole Acetic Acid (IAA), could solubilize mineral phosphate

and fix nitrogen. Stajkovic *et al.* (2009) isolated 15 endophytic non-rhizobial strains from alfalfa (*Medicago sativa* L.) out of which five strains were identified as *Bacillus* sp., which improved nodulation and plant yield. Several bacterial isolates were recovered from surface-sterilized root nodules of *Arachis hypogaea* L. (peanut) plants growing in soils from Cordoba, Argentina by Ibanez *et al.* (2009). The 16S rDNA sequences revealed that these isolates belonged to the phylum Proteobacteria, class γ-proteobacteria and included *Pseudomonas* sp., *Enterobacter* sp., and *Klebsiella* sp. Ngamau *et al.* (2014) isolated 43 bacterial isolates using five different isolation media and characterised them on the basis of their morphology, biochemical and molecular characteristics. Using 16S rRNA gene sequencing, they were identified as *Serratia* sp. (17 strains), *Pseudomonas* sp. (12 strains), *Enterobacter* sp. (4 strains), *Rahnella* sp. (4 strains), *Raoultella* sp. (2 strains), *Bacillus* sp. (1 strain), *Klebsiella* sp. (1 strain), *Yersinia* sp. (1 strain) and *Ewingella* sp. (1 strain). They found that all the strains showed varied levels of nitrogenase activities as measured by the acetylene reduction assay and 37 strains were observed to solubilize phosphates. Saidi *et al.* (2013) carried out experiments on characterization of root-nodule bacteria isolated from *Vicia faba* and concluded that bacteria isolated from root-nodules of *V. faba* grown in different Tunisian soils were highly diverse and could be affiliated to 12 genera, including *R. leguminosarum* and a high proportion of putative nodule endophytes. Leite *et al.* (2017) conducted an experiment on diversity of bacterial communities associated with root nodules of cowpea (*Vigna unguiculata*). They concluded that *Bradyrhizobium* was the most abundant genus of the detected genera and *Chryseobacterium* strains might help cowpea plants to cope with salt stress in semi-arid regions. A recent attempt by Muindi *et al*. (2021) who isolated rhizobia and non rhizobial endophytes associated with cowpea root nodules, the 16S rRNA gene sequencing classified the isolates to the nodulating types to the genera *Rhizobium, Paraburkholderia* and non-rhizobial endophytes to genera *Enterobacter, Strenotrophomonas and Pseudomonas.*

Endophytic bacteria enhance plant growth by aiding in nutrient acquisition through processes like nitrogen fixation, phosphate solubilization, and iron chelation. They also contribute to plant health by their antifungal or antibacterial properties, outcompeting pathogens for nutrients by siderophore production, priming the plant's systemic resistance and production of phytohormones. Among the plant growth promotion traits, phosphate solubilization and ammonia production are the most crucial plants. Phosphorus, stands as the second crucial macronutrient essential for plant development, even though the soil is abundant in phosphorus, the majority of the forms exists in an insoluble state, with only a minimal fraction (~0.1%) accessible to plants (Zou *et al*., 1992). The solubilization of insoluble phosphates by rhizospheric bacteria enhances the nutrient availability to plants (Rodriguez *et al.*, 2006). Similarly, ammonia production plays a role in enhancing plant growth through the accumulation of N and subsequently increasing biomass production (Marques *et al*., 2010). Therefore, this study was carried out to determine these two crucial plant growth promotion traits of eight non nodulating nodule associated bacterial isolates obtained from cowpea and garden pea root nodules.

MATERIAL AND METHODS

Collection of root nodule samples

Intact root samples of cowpea along with their nodules were collected from Prakasam, Bapatla districts of Andhra Pradesh and ICAR-IIHR, Bengaluru, experimental fields. Garden pea root nodules were collected from ICAR-IIHR experimental fields in Bengaluru. The samples were collected in separate ziplock cover pouches, labelled and transported to the laboratory and stored at 4^0C until further processing.

Surface sterilization of root nodules

In the laboratory, the roots were thoroughly washed under running tap water to remove the adhering soil particles and the nodules were detached along with a portion of the roots without damaging them. The intact and undamaged nodules were then immersed in 70% ethanol for 10 seconds and rinsed with sterile water. They were surface sterilized by soaking them in a 0.1% acidified mercuric chloride for 2 minutes, followed by washing with 5 changes of sterile water using sterile forceps. One ml of the last wash water was plated on nutrient agar and incubated at 28^0C for 24 hrs to confirm the surface sterility of the nodules. The surface sterilized nodules were then

crushed with a blunt-ended sterile glass rod in a large drop of sterile water in a test tube.

Isolation of nodule associated bacteria and rapid screening of their plant growth promotion traits

The individual aliquots were serially diluted up to 10^{-7} dilution and plated by the spread plate method on Nutrient Agar and Yeast mannitol Agar w / Congo Red and incubated at 30^0C for 24-48 h. Well-differentiated colonies that appeared on the plates were purified and stored in the respective media under refrigerated conditions for further study. A total of one hundred and fifty isolates were obtained by this process of which 110 isolates were from cowpea and 40 were from garden pea. Based on the colony uniqueness one hundred and two isolates were shortlisted for rapid screening of their ammonia production and phosphate solubilization abilities. The qualitative screening of bacterial isolates for ammonia production was carried out as outlined by (Cappuccino and Sherman, 1992) and qualitative for phosphate solubilization was done as per the protocol of Mehta and Nautiyal (2001). Based on the preliminary results the isolates CPH64, CPK42, CPM11, CPH62 originating from cowpea nodules and GP44, GP71, GP84 and GP102 from garden pea nodules were selected for further study.

Identification of the bacterial isolates

The genomic DNA was isolated from the individual isolates and the 16S rRNA gene was amplified by using the universal primers 27 F and 1492 R. The PCR products of the 16S rRNA gene of the eight efficient bacterial isolates obtained through amplification with specific primers were subjected to Sanger sequencing using the same upstream and downstream primers used for the amplification of 16S rRNA gene at Eurofins Genomics, Bengaluru. The 16S rRNA sequences of different bacterial isolates were BLAST (Basic Local Alignment Search Tool) against publicly available 16S rRNA sequences of bacterial isolates available in the NCBI Genbank Nucleotide Database (*www.ncbi.nlm.nih.gov*) and Ez taxon. Based on the maximum identity score sequences were selected and aligned using the multiple alignment software program Clustal W (*http://clustalw.genome.jp/*).

Quantitative estimation of phosphate solubilization (Murphy and Riley 1962)

The quantitative estimation of phosphate solubilization was conducted using NBRIP (National Botanical Research Institute Phosphate) broth, as described by (Mehta and Nautiyal, 2001). The broth contained 0.5% tri calcium phosphate (TCP) as the phosphate source, but did not include the bromophenol blue indicator. After incubating for 7 days, they were centrifuged at 10000 rpm for 5 minutes and the supernatant was used for estimation of phosphates. To determine the soluble phosphorus content in the medium, 0.5 ml of the supernatant was collected and mixed with 1-2 drops of p-nitrophenol (0.25%) as an indicator. To this 5N HCl was added dropwise to neutralize the colour. The resulting solution was diluted with 40 ml of double distilled water, followed by the addition of 8 ml of ammonium paramolybdate-ascorbic acid reagent. This solution was incubated at room temperature for 20 minutes. The final volume of the solution was adjusted to 50 ml with double distilled water. The absorbance of the solution was measured at 880 nm using a UV-visible spectrophotometer (Thermo Scientific, Biomate 3S, China). A standard curve was prepared using a stock solution of 2 ppm potassium dihydrogen phosphate (KH_2PO_4) in double distilled water, and the curve was plotted using various concentrations ranging from 0.1 to 2.0 µg ml^{-1} of phosphate. All the estimations were performed in triplicates.

Qualitative estimation of ammonia production (Cappuccino and Sherman, 1992)

The bacterial isolates were grown in Nutrient broth for a period of 48 hours. Once the cultures reached the active growth phase, 100 µl of individual culture were added to 10 ml of peptone water. These tubes were incubated for 72 hours at 28^0 C. After the 72-hour incubation period, 0.5 ml of Nessler's reagent was added, and the development of colour was observed. The presence of a brownish yellow colour was considered a positive indication of ammonia production. An uninoculated control was included for comparison purposes.

RESULTS AND DISCUSSION

Taxonomic identity of the isolates

Based on the similarity of the sequences with publically available sequences the isolates originating from cow pea root nodules were identified as Enterobacter *sp.* CPH64., *Enterobacter* sp CPK42.,

Chryseobacterium sp. CPM11, *Stenotrophomonas* sp. CPH62 originating from cowpea nodules, while the isolates from garden pea were identified as and *Enterobacter* sp. GP44, *Enterobacter sp.* GP71, *Enterobacter sp.* GP84, *Bacillus* sp. GP102.

Quantitative estimation of Phosphate solubilization

The quantitative estimation of phosphate solubilization revealed that amongst the cowpea isolates *Enterobacter sp.* CPH64 (2.38 µg ml^{-1}) solubilized the phosphate highest followed by *Enterobacter* sp. CPK42 (1.86 µg ml^{-1}) and *Stenotrophomonas* sp. CPH62 (1.82 µg ml^{-1}) whereas isolate *Chryseobacterium* sp. CPM11 solubilized 1.75 µg ml^{-1} of phosphate (Table 1) (Figure 1). Amongst the garden pea isolates *Bacillus* sp. GP102 (2.17 µg ml^{-1}) solubilized highest phosphate followed by *Enterobacter* sp. GP44 (1.44 µg ml^{-1}), *Enterobacter sp.* GP84 (1.24 µg ml^{-1}) and *Enterobacter sp.* GP71 (1.18 µg ml^{-1}) (Table 2) (Figure 2). Phosphorus is an important plant nutrient that plays a multifarious role in plant growth and development. The availability of this elemental from the phosphate reserved in the soil is enhanced by the activity of rhizospheric microbes (Song *et al*., 2008). Similar results on phosphate solubilisation by nodule associated bacteria have been reported in the past by Pandya *et al*. (2015) who isolated 26 non rhizobial endophytes from the nodules of *Vigna radiata* and reported that only 11 endophytes solubilized phosphate ranged from 37.4 µg mL^{-1} to 134.48 µg mL^{-1}. Yadav *et al.,*(2010) tested five elite nodule bacteria and reported that all the four isolates were potential phosphate solubilizers. Sharma and Choudhary (2013) isolated ten endophytic root-nodule bacterial strains from cultivated legume, mothbean (*Vigna aconitifolia* L.) and reported that all isolates were able to solubilize tri calcium Phosphate. Though the magnitude of the soluble phosphorus released by the isolates in the present study are lower than the previous reports, the ability of the nodule associated non nodulating bacterial isolates has been reinforced once again.

CONCLUSION

The findings of the present study have once again reiterated the presence of non nodulating bacterial genera possessing plant growth promotion traits such as phosphate solubilisation and ammonia production within legume root nodules. The role of these isolates in promoting the nodulation and growth of their respective host plants in association with rhizobia is a researchable issue.

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TABLE 1. PHOSPHATE SOLUBILIZATION BY ELITE NON NODULATING BACTERIA ISOLATED FROM COWPEA

Isolate	Phosphate solubilization (µg ml^{-1})
Enterobacter sp. CPH64	2.38
Enterobacter sp. CPK42	1.86
Chryseobacterium sp. CPM11	1.75
Stenotrophomonas sp. CPH62	1.82
S.E(m). ±	**0.01**
C.D.	**0.06**
C.V (%)	**1.54**

TABLE 2. PHOSPHATE SOLUBILIZATION BY ELITE NON NODULATING BACTERIA ISOLATED FROM GARDEN PEA

Isolate	Phosphate solubilization (µg ml^{-1})
Enterobacter sp. GP44	1.44
Enterobacter sp. GP71	1.18
Enterobacter sp. GP84	1.24
Bacillus sp. GP102	2.17
S.E(m). ±	**0.001**
C.D.	**0.15**
C.V (%)	**0.004**

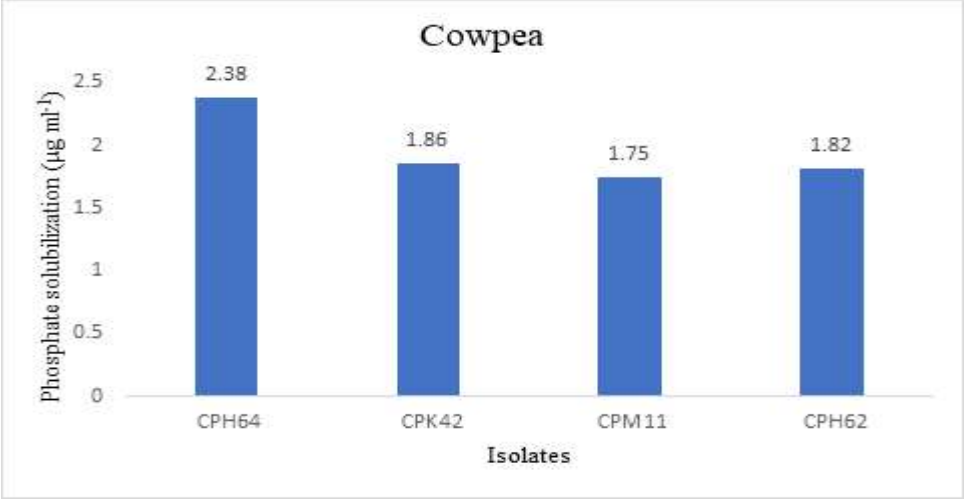

Fig. 1. Phosphate solubilization by the cowpea nodule associated bacterial isolates

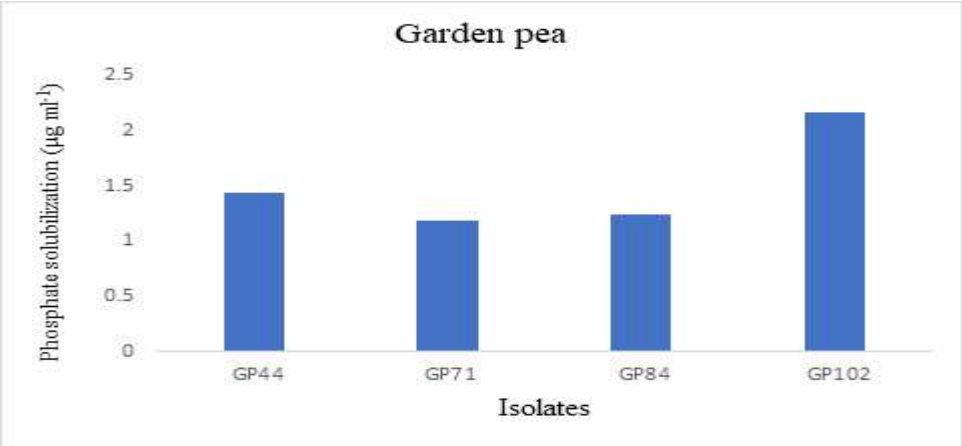

Fig 2. Phosphate solubilization by the garden pea nodule associated bacterial isolates

TABLE 3. AMMONIA PRODUCTION BY THE ELITE NODULE BACTERIA ISOLATED FROM COWPEA

Isolate	Ammonia production
Enterobacter sp. CPH64	++
Enterobacter sp. CPK42	+++
Chryseobacterium sp. CPM11	++
Stenotrophomonas sp. CPH62	+

"+++" High production "++" Moderate production "+" Low production

TABLE 4. AMMONIA PRODUCTION BY THE ELITE NODULE BACTERIA ISOLATED FROM GARDEN PEA

Isolate	Ammonia production
Enterobacter sp. GP44	+
Enterobacter sp. GP71	+
Enterobacter sp. GP84	+
Bacillus sp. GP102	+

"+" Low production

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