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CHARACTERIZATION OF ROOT-ASSOCIATED RHIZOBACTERIA INCLUDING BACILLUS MESONAE FOR BIOINOCULANT POTENTIAL IN VIGNA UNGUICULATA

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Abstract

Leguminous crops such as *Vigna unguiculata* (cowpea) harbor symbiotic and associative root bacteria that play a vital role in soil fertility and plant growth. This study focuses on isolating and characterizing root-associated bacteria particularly *Rhizobium spp*. and *Bacillus mesonae* with potential for eco-friendly biofertilizer development. Root nodules and rhizospheric soil were collected from cowpea plants in Chinnagottigallu village, Andhra Pradesh, and bacterial isolates were obtained using selective media. Morphological, biochemical, and molecular identification using 16S rRNA gene sequencing was carried out for accurate classification. Func- tional assays assessed indole-3-acetic acid (IAA) production and nitrogen fixation potential. The *Bacillus mesonae* and *Rhizobium* isolates demonstrated traits that support plant growth, improve nutrient uptake, and enhance soil health. These native microbial isolates hold promise as sustainable alternatives to synthetic fertilizers, promoting soil health and crop productivity. Field trials are recommended to evaluate their efficacy across diverse agroecological zones.

Keywords: *Bacillus mesonae*, *Vigna unguiculata*, Rhizobium, plant growth-promoting rhizobacteria (PGPR).

Introduction

Modern agriculture is under increasing pressure due to the excessive use of synthetic agrochemicals, widespread soil degradation, water scarcity, and the accelerating impacts of climate change. These challenges have catalyzed a global movement toward sustain- able farming approaches that strike a balance between productivity and environmental stewardship. A cornerstone of such strategies involves restoring soil health, maintaining biodiversity, and minimizing environmental pollution, all while meeting the growing demand for food. Leguminous crops, particularly *Vigna unguiculata* (cowpea), offer a promising avenue in this regard. These plants naturally enrich soil nitrogen through symbiotic relationships with nitrogen-fixing bacteria such as *Rhizobium spp.,* which inhabit specialized root nodules. By converting atmospheric nitrogen (N) into bioavailable ammonia, these microbes not only sustain legume growth but also improve soil fertility for successive crops. This natural nitrogen supplementation reduces the dependence on chemical fertilizers, thereby cutting costs and mitigating environmental risks.Beyond nitrogen fixation, the rhizosphere of legumes supports a diverse microbial community, including plant growth-promoting rhizobacteria

(PGPR) like *Bacillus spp*. These organisms enhance plant development through mechanisms such as phytohormone production, nutrient mobilization, and stress tolerance. Isolating and characterizing such root-associated microbes provides a gateway to developing effective, eco-friendly biofertilizers tailored for sustainable agricultural systems.*Rhizobium* inoculants are commonly employed as biofertilizers, particularly in the production of legumes, such as cowpeas (*Vigna unguiculata*), where they are essential for increasing yields while preserving ecological equilibrium. Their use is especially beneficial in areas with limited access to chemical fertilisers and in soils with low nitrogen levels.

The gram-positive, rod-shaped, endospore-forming *Bacillus mesonae* bacteria is found in the rhizosphere, the area of soil that is directly impacted by root secretions and related microbial activities. It's a member of the plant growth-promoting rhizobacteria (PGPR) group of helpful bacteria. These microorganisms are significant possibilities for use as biofertilizers because they increase plant growth and productivity through a number of direct and indirect processes.

General Characteristics of *Vigna unguiculata* (Cowpea)

a. Taxonomy

Kingdom: Plantae
Phylum: Magnoliophyta
Class: Magnoliopsida
Order: Fabales
Family: Fabaceae
Genus: Vigna
Species: *Vigna unguiculata*

Commonly known as cowpea, this leguminous plant is widely grown in tropical and subtropical regions for its high-protein seeds and soil-enriching properties.

Materials and Methods

Collection of Root and Soil Samples:

Healthy *Vigna unguiculata* (cowpea) plants with well-developed root nodules were collected from cultivated fields in Chinnagottigallu village, Andhra Pradesh. Rhizospheric soil and root samples were placed in sterile polythene bags, transported to the laboratory under refrigerated conditions, and processed within 24 hours for microbial isolation (Kuykendall et al., 1988).

Isolation of Rhizobium from Root Nodules:

The collected root nodules were first washed under running tap water, then surface-sterilized with 70% ethanol followed by treatment with 0.1% mercuric chloride for 30 seconds. The nodules were thoroughly rinsed with sterile distilled water, crushed in sterile saline, and the suspension was streaked onto Yeast Extract Mannitol Agar (YEMA) for selective isolation of *Rhizobium spp.*

Isolation of Bacillus spp. from Rhizospheric Soil:

Rhizospheric soil samples were serially diluted in sterile water. To enrich for spore-forming *Bacillus sp.*, the soil suspension was heat-treated at 80°C for 10 minutes prior to plating on Nutrient Agar. Colonies with typical Bacillus morphology were selected for further analysis (Claus and Berkeley, 1986).

Preparation of Culture Media:

Yeast Extract Mannitol Agar (YEMA) and Nutrient Agar (NA) were prepared using standard protocols (Pelczar et al., 1993; Vincent, 1970). Isolates were maintained on slants at 4°C for short-term use and preserved at 20°C for long-term storage.

Morphological charecterization

Isolates were characterized by observing colony morphology, Gram staining, and motility.

Biochemical Characterization

Catalase test-

The catalase test was performed by adding a few drops of 3% hydrogen peroxide to a bacterial colony and observing for immediate bubble formation, indicating catalase activity.

IAA production test-

The IAA production test was performed by incubating the bacterial culture in Luria Bertani broth supplemented with L-tryptophan, followed by addition of Salkowski's reagent and observing for pink coloration indicating IAA production.

Biochemical assays such as the catalase and oxidase tests were performed for preliminary identification. IAA production was evaluated in LB broth supplemented with L- tryptophan and confirmed using Salkowski reagent (Gordon and Weber, 1951).

Molecular Identification

PCR Protocol: Polymerase Chain Reaction (PCR) is a process that uses primers to amplify specific cloned or genomic DNA sequences with the help of a very unique enzyme. PCR uses the enzyme DNA polymerase that directs the synthesis of DNA from deoxynucleotide substrates on a single-stranded DNA template. DNA polymerase adds nucleotides to the 3′ end of a custom-designed oligonucleotide when it is annealed to a longer template DNA. Each PCR reaction was initiated with a high-temperature denaturation step to ensure complete strand separation, followed by 25 cycles of amplification, including denaturation, primer annealing, and extension. The final extension step ensured complete synthesis of all amplified products, and the reaction was held at 4 °C until further processing.

Primer Name	Sequence Details	Number of Base
27F	5'-AGAGTTTGATCCTGGCTCAG-3'	20
1492R	5' TACGGTACCTTGTTACGACTT 3'	20

STAGES	TEMPERATURE	TIME
Initial Denaturation	95˚C	2 min
Denaturation	95˚C	30 sec 25 cycles
Annealing	50˚C	30 sec
Extension	72˚C	2 min

Final extension	72˚C	10 min
Hold	4˚C	∞

Phylogenetic Analysis

Amplified sequences were analyzed using BLAST and aligned with MUSCLE. Phylogenetic trees were constructed using PhyML and visualized with TreeDyn.

Results

Collection of Plants

A total of four plant species were sampled, each from a distinct location (Table 1). Groundnut (*Arachis hypogaea*) and Cowpea (*Vigna unguiculata*) were collected from **Chinnagottigallu.** For both species, intact root systems along with rhizospheric soil were carefully excavated using sterile tools to ensure minimal contamination and preserve microbial diversity.Indigo (*Indigofera tinctoria*) plants were sourced from **Chinigepalli**, where only root samples were available due to unavailability of surrounding soil during collection. Similarly, Bluebell vine (*Clitoria ternatea*) roots were obtained from **Lankipallivari Palli**, though soil sampling was not feasible at this sit site.

a.Ground nut

b. Cow pea

c. Indigo

d. Bluebell vine

Fig-1-Different legumenace plants

S.NO	Plant name	Village	Roots	Soil
1.	Ground Nut	Chinnagottigallu	YES	YES
2.	Cow Pea	Chinnagottigallu	YES	YES
3.	Indigo	Chinigepalli	YES	NO
4.	Bluebell vine	Lankipallivari palli	YES	NO

Table1. List of collected plant samples along with site-specific availability of roots and soil

Collection of Roots

Root samples of selected plants were collected from different villages in the Tirupati district. Healthy roots were carefully uprooted using sterile tools to avoid damage and contamination. After removing loosely attached soil, the roots with adhering rhizospheric soil were placed in sterile covers and labeled properly.All samples were transported to the laboratory in ice boxes and processed within 24 hours to ensure microbial viability. These root samples are shown in **Figure 2**, representing collections from various agricultural fields.

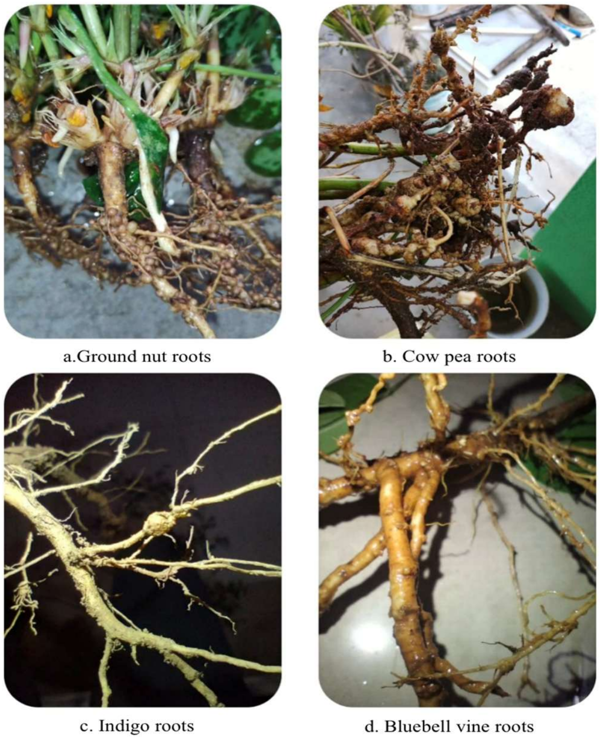
a.Ground nut roots
b. Cow pea roots
c. Indigo roots
d. Bluebell vine roots

Fig 2. Root samples collected from different agriculture fields .

Culturing and Colony Characteristics :

On YEMA, *Rhizobium* isolates produced mucoid, convex, semi-translucent colonies, while *Bacillus* spp. on Nutrient Agar formed creamy white, opaque colonies with smooth margins.

Colony Morphology and Microscopy

- **Rhizobium:** Mucoid, white colonies on YEMA; Gram-negative rods.
- **Bacillus:** Opaque, irregular colonies on Nutrient Agar; Gram-positive rods with endospores.

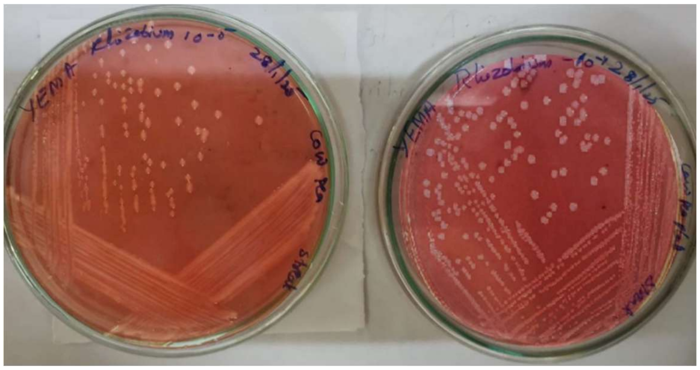

Fig -3 Microbial growth on YEMA media

Microscopy and Biochemical Tests

Rhizobium appeared as Gram-negative rods and tested positive for catalase but did not produce IAA. *Bacillus mesonae* was Gram-positive, rod-shaped with endospores, catalase-positive, and produced IAA (evidenced by pink coloration with Salkowski reagent). In the culturing of bacillus shown positive results of colony morphology.

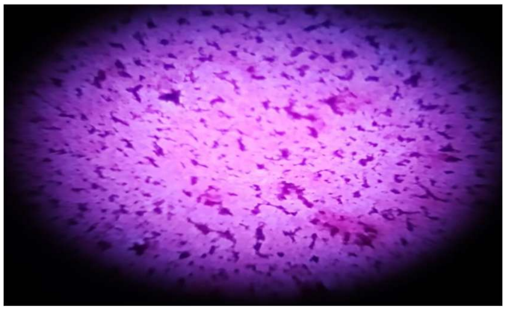

Fig- 4 Gram positive bacillus retaining crystal violet Gram Staining

Gram Staining:(**Microscopic analysis**) The *Bacillus* isolate was found to be **Gram-positive**, appearing purple under the microscope due to retention of the crystal violet stain.

Biochemical Test Results

Both isolates tested positive for catalase. The *Bacillus mesonae* isolate showed IAA production as evidenced by pink coloration after Salkowski's reagent addition. The *Rhizobium* isolate did not produce IAA.

Catalase test- Hydrogen peroxide conveted into water + oxygen indicates the positive catalase

Fig-5 positive for catalase test- *Bacillus*

Catalase test: The Bacillus isolate tested positive for catalase, indicated by immediate bubble formation upon the addition of hydrogen peroxide, confirming the presence of the catalase enzyme.

IAA production test or Indole test before adding Salkowski reagent

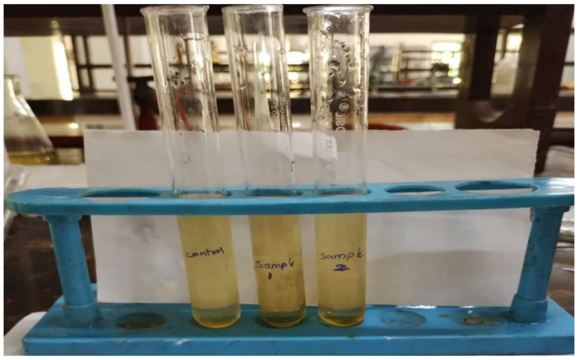

Fig-6 IAA production test

After Salkowski addition-(45 mins)

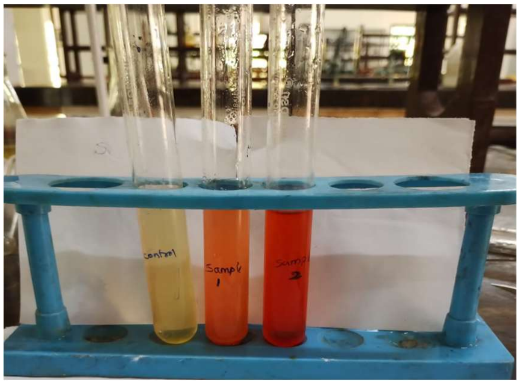

Fig-7 IAA confirmed by Turning pink colour

IAA test: The *Bacillus* isolate tested **positive** for Indole-3-Acetic Acid (IAA) production, as indicated by the development of a pink to reddish color upon the addition of Salkowski's reagent, confirming its potential role in plant growth promotion.

Rhizobium conformed based on the tests include Microscopic analysis and catalase test.

Microscopic analysis

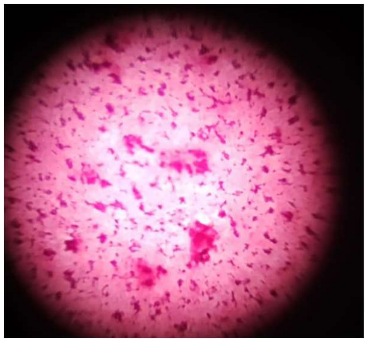

Fig-8 Gram negative *rhizobium* stays pink

Gram Staining:(**Microscopic analysis**)The *Rhizobium* isolate appeared as **Gram-negative**, **rod-shaped** bacteria, typically occurring singly or in pairs, and stained **pink** under the microscope due to safranin counterstaining.

Catalase test- Hydrogen peroxide converted into water + oxygen indicates the positive catalase

Fig-9 Formation of positive for catalase test- *Rhizobium*

catalase test: The isolate showed a **positive catalase reaction**, indicated by brisk bubble formation upon the addition of hydrogen peroxide.

Sequencing

Molecular Identification of Isolates by 16S rRNA Sequencing The selected bacterial isolates were subjected to 16S rRNA gene sequencing for molecular identification. BLAST analysis revealed that the rhizospheric isolate shared 99.3. The *Bacillus mesonae* isolate grouped closely with other known PGPR strains, indicating its ecological relevance and potential functional role in the rhizosphere. Similarly, the *Rhizobium* isolate aligned with strains previously reported in symbiotic associations with legumes, supporting its identity as a nitrogen-fixing symbiont. These m lar findings validated the results from the biochemical tests and further established the identity of the root-associated bacteria as *Bacillus mesonae* and *Rhizobium sp.,* both of which are candidates for development into eco-friendly biofertilizers.

Dr.RAD-
GAGGTCGATGAGTGCTAGTGTTAGAGGGTTTCCAGCCCTTTAGG
T-
GCTCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCC
GCAAGGCTGAAACTCAA

1.2. Molecular Identification

- The 16S rRNA gene sequences revealed: 99.3% identity with *Bacillus mesonae* **Sequencing-**
- **Bacillus spp** *Bacillus mesonae* is confirmed in the 16s RNA sequencing by using next gen sequencing

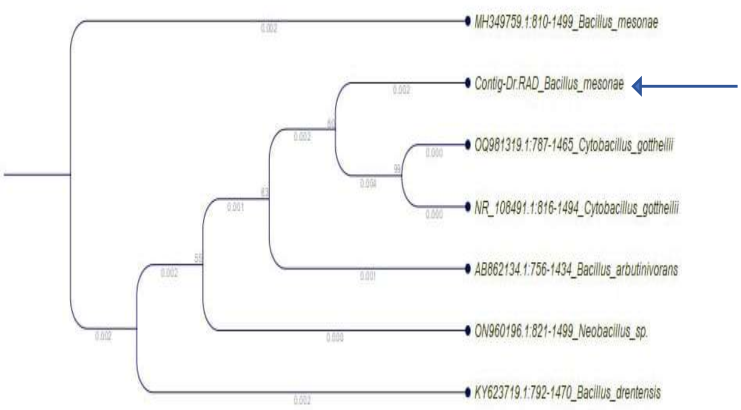

Fig-10 Phylogenetic tree for *Bacillus Mesonae* marked with arrow mark

Future prospective: Developing biofertilizer from beneficiary bacteria.

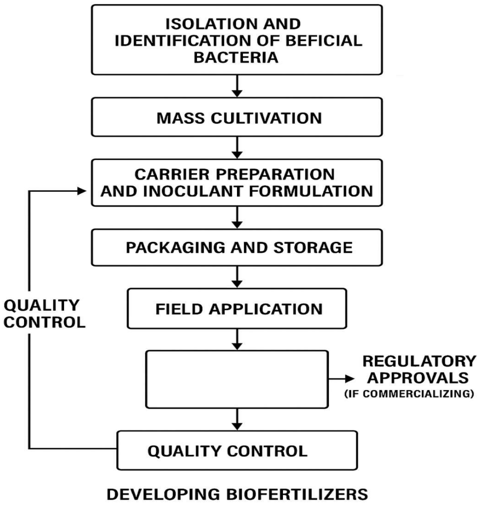

Fig-11 Flow chart for developing bio fertilizers

2. Discussion

The successful isolation and identification of *Bacillus mesonae* and *Rhizobium sp.* from *Vigna unguiculata* highlights their potential as biofertilizer candidates for sustainable agriculture. Both isolates demonstrated key plant growth-promoting traits, such as nitrogen fixation and indole-3-acetic acid (IAA) production, which are known to enhance plant growth, nutrient uptake, and soil health (Glick, 2012). The Rhizobium isolate, retrieved from root nodules, exhibited classical features of symbiotic nitrogen-fixing bacteria and aligns with earlier reports of its association with legumes (Vincent, 1970). In contrast, *Bacillus mesonae*, isolated from the rhizosphere, showed efficient IAA production and spore-forming ability, which suggests robustness in soil environments—traits valuable for field application (Kloepper et al., 2004). Molecular characterization via 16S rRNA sequencing confirmed the identities of both strains, and phylogenetic analysis re- inforced their taxonomic placement. These findings are consistent with previous reports on the ecological relevance of these genera in promoting crop productivity under low-input conditions (Bhattacharyya Jha, 2012). Overall, this study supports the potential use of native microbial strains as bioinoculants. However, field-level validation across agroclimatic zones is necessary before recommending them for commercial biofertilizer formulations. Molecular identification using 16S rRNA gene sequencing confirmed the taxonomic identity of the isolates, showing 99 similarity.

Functional traits of Rhizobium and Bacillus mesonae:

Nitrogen fixation by Rhizobium was demonstrated using a nitrogen free medium, with observable colorimetric changes indicating nitrogenase enzyme activity. This affirms its established symbiotic role in legumes (Zahran, 1999). The mutualistic interaction between *Vigna unguiculata* and *Rhizobium* supports natural nitrogen supplementation in soils. IAA production by both Rhizobium and *Bacillus mesonae* was confirmed using Salkowski's reagent after incubation in L-tryptophan–supplemented media. Pink coloration signified the presence of IAA, a phytohormone known to enhance root elongation and nutrient uptake (Patten Glick, 2002). Furthermore, *Bacillus mesonae* demonstrated salt tolerance by sustaining growth in media containing up to 6Implications for sustainable agriculture: The observed nitrogen fixation, IAA production, and probable phosphate solubilization collectively indicate that *Rhizobium* and *Bacillus mesonae* are strong candidates for use as biofertilizers. In the Indian context—where overuse of synthetic agrochemicals leads to environmental degradation and reduced soil fertility—the use of native plant growth-promoting rhizobacteria (PGPR) like *Rhizobium* and *Bacillus mesonae* offers a sustainable alterna- tive (Bhattacharyya Jha, 2012).

Conclusion

In summary, *Rhizobium* and *Bacillus mesonae* strains were successfully isolated and char- acterized from the rhizosphere and root nodules of *Vigna unguiculata*. The findings demonstrated that these bacterial isolates possess important plant growth-promoting traits, including nitrogen fixation and IAA production. Their potential to enhance soil fertility and promote sustainable agricultural practices was clearly established. However, further validation through field trials and long-term ecological studies is necessary before recommending their widespread application in commercial farming systems.The observed nitrogen fixation, IAA production, and probable phosphate solubilization collectively in- dicate that *Rhizobium* and *Bacillus mesonae* are strong candidates for use as biofertilizers. These functional traits can reduce the dependency on chemical fertilizers, enhance plant nutrient uptake, and improve soil structure and fertility.

Acknowledgments

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References

1. Bhattacharyya PN, Jha DK. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World J Microbiol Biotechnol*. 2012;28(4):1327–1350.

2. Cappuccino JG, Sherman N. *Microbiology: A Laboratory Manual*. 10th ed. Pearson Education; 2013.

3. Chen XH, Vater J, Piel J, et al. Biosynthesis of antibiotics in Bacillus species: genes, regulation, and potential for biofertilizer development. *Appl Microbiol Biotechnol*. 2006;73:13–23. Choudhury ATMA, Kennedy IR. Nitrogen fertilizer losses from rice soils and control of environmental pollution problems. *Commun Soil Sci Plant Anal*. 2005;36(11–12):1625–1639. Chun J, Lee JH, Jung Y, et al. EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int J Syst Evol Microbiol*. 2007;57(10):2259–2261.

4. Dakora FD, Keya SO. Contribution of legume nitrogen fixation to sustainable agriculture in Sub-Saharan Africa. *Soil Biol Biochem*. 1997;29(5–6):809–817. Dereeper A, Guignon V, Blanc G, et al. Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res*. 2008;36(Web Server issue):W465–W469.

5. Dobbelaere S, Vanderleyden J, Okon Y. Plant growth-promoting effects of diazotrophs in the rhizosphere. *Crit Rev Plant Sci*. 2003;22(2):107–149. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res*. 2004;32(5):1792–1797.

6. Glick BR. Plant growth-promoting bacteria: mechanisms and applications. *Scientifica*. 2012;2012:963401.

7. Gordon SA, Weber RP. Colorimetric estimation of indoleacetic acid. *Plant Physiol*. 1951;26(1):192–195.

8. Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST. *Bergey's Manual of Determinative Bacteriology*. 9th ed. Baltimore: Williams & Wilkins; 1994.

9. Jain D, Patel R. Isolation and characterization of Bacillus spp. from rhizosphere soil and their effect on plant growth. *Int J Curr Microbiol App Sci*. 2020;9(6):1701–1710.

10. Kumar A, Kumar A, Devi S, et al. Isolation, screening and characterization of bacteria from rhizospheric soils for different plant growth promotion (PGP) activities: an in vitro study. *Recent Res Sci Technol*. 2014;6(1):210–215.

11. Kuykendall LD, Saxena B, Devine TE, Udell SE. Genetic diversity in *Bradyrhizobium japonicum* serogroup 123 and its relationship to serogroup 122. *Appl Environ Microbiol*. 1988;54(7):1796–1801.

12. Lane DJ. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, eds. *Nucleic Acid Techniques in Bacterial Systematics*. Wiley; 1991:115–175.

13. Lee SA, et al. *Bacillus mesonae* sp. nov., a novel plant growth-promoting rhizobacterium. *Int J Syst Evol Microbiol*. 2017;67(2):422–427.

14. Lugtenberg B, Kamilova F. Plant-growth-promoting rhizobacteria. *Annu Rev Microbiol*. 2009;63:541–556.

15. MilliporeSigma. *Montage PCR Clean-up Kit User Guide*. Merck KGaA, Darmstadt, Germany; 2020.

16. Patten CL, Glick BR. Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. *Appl Environ Microbiol*. 2002;68(8):3795–3801.

17. Peix A, Ramírez-Bahena MH, Velázquez E, Bedmar EJ. Bacterial associations with legumes. *Crit Rev Plant Sci*. 2015;34(1–3):17–42.

18. Ramos B, de Faria MV, Hungria M. Synergism between rhizobia and PGPR: promising interaction for enhanced productivity and soil health. *Microbiol Res*. 2020;238:126529.

19. Sambrook J, Russell DW. *Molecular Cloning: A Laboratory Manual*. 3rd ed. Cold Spring Harbor Laboratory Press; 2001.

20. Singh B, Sharma RA. Cowpea (*Vigna unguiculata*): a valuable legume crop for sustainable agriculture. *Legume Res*. 2020;43(5):641–648. Somasegaran P, Hoben HJ. *Handbook for Rhizobia: Methods in Legume-Rhizobium Technology*. Springer-Verlag; 1994.

21. Talavera G, Castresana J. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Syst Biol*. 2007;56(4):564–577.

22. Vincent JM. *A Manual for the Practical Study of Root-Nodule Bacteria*. IBP Handbook No. 15. Blackwell Scientific Publications; 1970.

23. Vessey JK. Plant growth promoting rhizobacteria as biofertilizers. *Plant Soil*. 2003;255:571–586.

24. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol*. 1991;173(2):697–703.

25. Zahran HH. Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiol Mol Biol Rev*. 1999;63(4):968–989.

26. Zhao K, Penttinen P, Zhang X, et al. *Bacillus* species as potential biocontrol agents against plant pathogens. *Microbiol Res*. 2014;169(5–6):292–298.