

Original Research Article

Isolation and Characterization of Plant Growth Promoting Bacteria from Root Nodules of *Cicer arietinum* L.

ABSTRACT

Aims: The aim of the present study is to isolate and characterize root nodulating bacteria from *Cicer arietinum* L. cv. GNG 1958 and assess its plant growth promoting properties.

Study design: Isolation of symbiotic bacteria, its biochemical and molecular characterization and assessment of plant growth promoting property through ammonia production, phosphate solubilization and HCN production.

Place and Duration of Study: Department of Bioscience and Biotechnology, Banasthali Vidyapith, Rajasthan, during 2019-2022.

Methodology:

Root nodulating bacterial strain was isolated from *C. arietinum* L. cv. GNG 1958 grown at the agricultural fields located at the Krishi Vigyan Kendra, Banasthali Vidyapith, Rajasthan. Morphological characteristics of the isolates were noted following Bergey's Manual of Systemic Bacteriology and biochemical tests performed as per standard methods. Molecular characterization of the potential symbiont was performed by PCR of 16S rRNA genes and the PCR product was sequenced. Plant growth promoting property was determined through ammonia production, phosphate solubilization and HCN production.

Results: Root nodulating bacterial strain was isolated from *C. arietinum* L. cv. GNG 1958. Out of eighteen isolates, BVCA-58 grew fast and changed the color of BTB dye in media from green to yellow and deep yellow within 2 days of incubation. White colonies were observed when BVCA-58 was inoculated on YEMA media supplemented with Congo red. BVCA-58 has the ability to produce hydrogen cyanide, ammonia and solubilize phosphate. Molecular study of BVCA-58 using 16f-16r and 63f-1244r primers showed 99.89% and 99.62% of similarity with *Mesorhizobium jarvisii* ATCC-33669 and *Mesorhizobium erdmani* USDA-3471, respectively on performing NCBI Blast. BVCA-58 was submitted to NCBI portal as *Mesorhizobium* sp. strain BVCA-58 with accession nos. OP646813 and OP646810.

Conclusion: The present study revealed that isolated strain from *Cicer arietinum* GNG 1958 variety was *Mesorhizobium* sp. which has potent plant growth promoting traits.

Keywords: *Cicer arietinum*, *Mesorhizobium* sp., root nodulating bacteria, plant growth promoting rhizobacteria

Formatted: Font: Italic

1. INTRODUCTION

Cicerarietinum L. (chickpea, family-Fabaceae) is third most cultivated crop globally with 15 million tons (MT) production just after dry peas (16 MT) and dry beans (27 MT) (FAO, 2020). India contributes 27.53% and 59.67% to total global and Asia pulse production respectively. Chickpea is mainly winter-sown crop and is cultivated under rain fed conditions [1].

Chickpea has the highest protein content approximately 40% of its weight. Besides protein, chickpea also contains considerable amount of nutrients such as magnesium, zinc, iron, phosphorus and calcium [2].

Climate change along with its associate abiotic and biotic stresses, leads to huge pressure on agronomic practices. These adverse changes in environment have raised vulnerabilities to global food causing alternation in soil fertility, high disease index, low yield, high use of synthetic inputs. In such cases legumes consider as ideal crop for sustainable agriculture because of their nutritive value, symbiosis with rhizobia, reducing of chemical fertilizers requirement [3].

"Plant growth promoting rhizobacteria" are a group of bacteria that resides in rhizosphere and root nodules of leguminous crops and enhance the plant productivity through various mechanisms.

Comment [A1]: reference

In the present study, isolation of symbiotic bacteria, its biochemical and molecular characterization and assessment of plant growth promoting property through ammonia production, phosphate solubilisation and HCN production was conducted.

2. MATERIAL AND METHODS

Root nodules of *Cicer arietinum* GNG 1958 were collected from the plants grown at the agricultural fields' located at the KrishiVigyan Kendra, BanasthaliVidyapith, Rajasthan.

2.1 Isolation of root nodulating bacteria

The root nodules were surface sterilized by momentarily dipping in 0.1% sodium hypochlorite followed by rinsing in sterile distilled water 3-4 times. The nodules were then placed on clean dry glass slide and gently crushed with another slide to obtain juicy liquid containing bacteria. Bacterial inoculum was streaked on Yeast Extract Mannitol Agra media supplemented with Congo red dye or Bromothymol blue dye separately and incubated for 1-2 days at 25-30°C. The morphological characteristics of the isolate were noted [4].

2.2 Biochemical Characterization

To check the presence of *Agrobacterium*, alpha ketolactose test was done with the help of Benedict's reagent [5], Citrate test [6], Indole test [7], Methyl Red-VogesProskauer test [8], Gelatine test [9] and Urease test [10] was performed.

2.3 Molecular Characterization-16s rRNA sequencing

Bacterial DNA extraction was carried out with the help of Sigma-Aldrich Cat. no. NA2110 GenElute Bacterial Genomic DNA Kit. PCR for DNA amplification was done using the following primer sets:

Universal 16s rRNA primer: Forward: 16F27 (5'-CCAGAGTTTGATCMTGGCTCAG-3'), Reverse: 16R1492 (5'-TACGGYTACCTTGTTACGACTT-3') and
Rhizobium 16s rRNA specific primer: Forward: 63f (AGGCCTAACACATGCAAGTC), Reverse: 1244r (CTCGCTGCCACTGTAC).

The reactions were carried out in a PCR tube containing 2.5mM dNTPs, 1X Taq buffer, 0.2µM primers, 0.5U/µl Taq polymerase and DNA 125ng/µg. PCR amplification was done on a Verity Thermal Cycler, Applied Biosystems [11]. Gel electrophoresis was performed in 1.5% agarose gel containing ethidium bromide and visualized under the gel documentation system. The PCR product was sent to Xcelris Genomics, Ahmadabad, India and NCMR-NCCS, Pune, India for the sequencing. The partial sequences of 16s rRNA gene of the bacterial isolate was [analysed/analyzed](#) on the NCBI through nucleotide BLAST (<https://blast.ncbi.nlm.nih.gov/>) and EZTaxon (<https://www.ezbiocloud.net/>).

2.4 Characterization of plant growth promoting activities

2.4.1 Ammonia Test:

To determine ammonia production, 10ml peptone broth was inoculated with bacterial strain and incubated for 28±2°C for 3 days [12]. Thereafter, 1ml of Nessler's reagent was added to the bacterial culture; formation of a yellow brown color indicates ammonia production.

2.4.2 Phosphate solubilization:

To determine phosphate solubilization, 0.1µl of one day old bacterial suspension was spot inoculated on Pikovskaya agar media and incubated for 8-10 days at 30±2°C. Phosphate solubilization index (PSI) was calculated [13] as follows:
$$\text{PSI} = (\text{colony diameter} + \text{halo zone diameter}) / \text{colony diameter}$$

2.4.3 HCN Production:

Bacterial isolate was streaked on nutrient agar amended with 4.4g/l glycine [14]. The streaked portion was covered with Whatman filter paper dipped in picric acid solution in 2% Na₂CO₃ and incubated for 3 days at 30±2°C. HCN production is indicated by the change in color of the filter paper from yellow to red brown.

3. RESULTS AND DISCUSSION

3.1 Morphology Characterization:

Screening of all isolates was done on the basis of their colony characteristic. Among 18 bacterial isolates only BVCA-58 [hadwas](#) fast growing and able to change BTB dye color green to yellow and deep yellow after 2 days of incubation. White colonies of BVCA-58 was observed on YEMA media supplemented with Congo red dye (Fig. 1).

BTB is used as pH indicator, the coloration is used to distinguish between slow growers - Bradyrhizobium (alkaline reaction-blue) and fast growers - *Rhizobium* strains (acidic reaction-yellow). For testing the purity of *Rhizobium* cultures, Congo red dye is often added to culture medium [15].

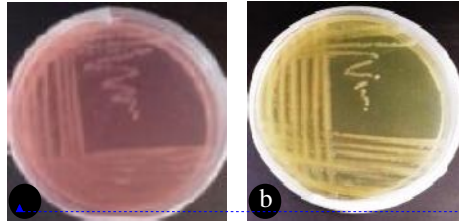


Fig. 1. Bacterial growth on YEMA media supplemented with (a) BTB and (b) Congo red dye

Formatted: Font: 11 pt, Font color: Background 1

3.2 Biochemical Characterization:

No color change of benedict solution was observed with BVCA-58 which confirmed absence of *Agrobacterium* during the alpha-keto lactose test (Fig. 2a). In citrate test, inoculated plate showed blue color which confirms citrate production by BVCA-58 while uninoculated plate remained green in color (Fig. 2b). In indole test, upon addition of Kovac's reagent, red color ring formation occurs indicating indole production (Fig. 2c), BVCA-58 exhibit 2, 3-butadiniol fermentation pathway which is confirmed by positive result for VP while negative result was observed in methyl red test (Fig. 2d). Liquification of media was not observed by isolate which confirmed absence of gelatinase enzyme in gelatine test (Fig. 2e). Urease production was observed by change in color from yellow to pink in BVCA-58 (Fig. 2f).

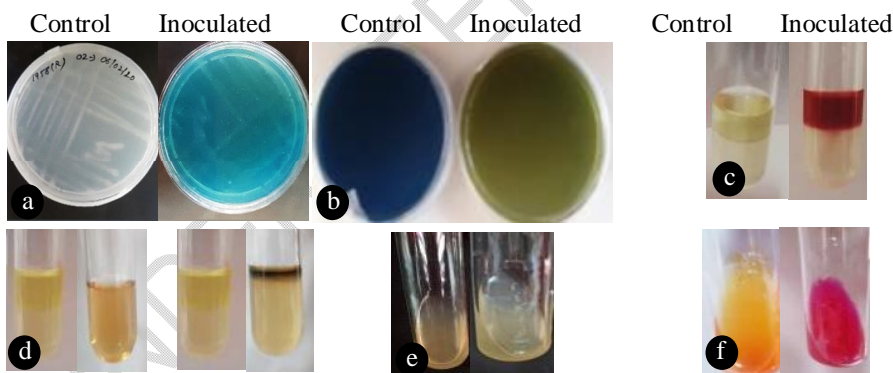


Fig. 2. Biochemical characterization of BVCA-58: (a) alpha-keto test (b) citrate test (c) indole test (d) Methyl Red test and Voges-Proskauer test (e) gelatin test (f) urease test

3.3 Molecular Characterization

A 1165bp and 1064bp amplicon products were obtained with universal primer (16f-16r) and *Rhizobium* specific primer (63f-1244r) respective on PCR. The sequences obtained with 16f-16r and 63f-1244r primers were analysed with NCBI Blast tool which showed 99.89% and 99.62% similarity with *Mesorhizobium jarvisii* ATCC-33669 and

Mesorhizobiumerdmani USDA-3471 respectively. BVCA-58 was submitted at NCBI portal as *Mesorhizobium* sp. strain BVCA-58 with accession no. OP646813 and OP646810 (Table 1).

Mesorhizobium sp. is a native strain for chickpea as reported earlier by several workers authors [16, 17].

Table 1. Identification of BVCA-58 by NCBI BLAST similarity results and subsequent Genebank submission

Primers	Sequence base pair	Similarity (NCBI BLAST)		NCBI Genebank submission	
		%	Nearest	Strain name	Accession no.
16f-16r	1165	99.89	<i>Mesorhizobiumjarvis</i> iiATCC-33669	<i>Mesorhizobium</i> sp. BVCA-58	OP646813
63f-1244r	1064	99.62	<i>Mesorhizobiumerdmani</i> USDA-3471	<i>Mesorhizobium</i> sp. BVCA-58.	OP646810

3.4 Plant growth promoting properties

Ammonia production was observed in BVCA-58 by formation of the light green color (Fig. 3a). It helps in providing nitrogen to plant thereby enhances plant total biomass [18].

HCN production was observed in BVCA-58 as confirmed by change in the color of filter paper from yellow to brown (Fig. 3b).

During phosphate solubilization test using BVCA-58, on the 8th day there was formation of halo zone (15.05 ± 0.05) on Pikovskaya (PVK) agar plates (Fig. 3c). The phosphate solubilization index (PSI) was determined to be 3.15. Phosphate solubilization property of bacteria is considered beneficial as it makes insoluble phosphates available to the plant [19, 20].

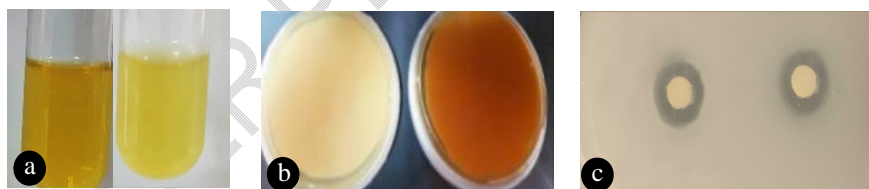


Fig. 3. PGPR characterization by *Mesorhizobium* sp. (a) Ammonia production (b) HCN production (c) Phosphate solubilization

4. CONCLUSION

The present study revealed that isolated strain from *Cicer arietinum* GNG 1958 variety was *Mesorhizobium* sp. which has potent plant growth promoting traits [because.../benefit of that in plants.](#)

Comment [A2]: To enlarge the conclusion

REFERENCES

1. Kaur R, Prasad K. Technological, processing and nutritional aspects of chickpea (*Cicer arietinum*)-A review. Trends Food Sci. Technol. 2021; 109:448-463.

2. Ghadge PN, Vairagar PR, Prasad K. Some physical properties of chickpea split (*Cicer arietinum* L.). *Agric. Eng. Int.* 2008; 10.
3. Dutta A, Trivedi A, Nath CP, Gupta DS, Hazra KK. A comprehensive review on grain legumes as climate-smart crops: challenges and prospects. *Environ. Challenges.* 2022; 100479.
4. Don JB, Noel RK, James TS, George MG. editors. *Bergey's Manual® of Systematic Bacteriology. Volume Two: The Proteobacteria, Part A Introductory Essays.* Springer New York, NY. 2005.
5. Arora DR. *The text book of Microbiology.* New Delhi: CBS.pp.41-48. 2003.
6. Cappuccino JG, Sherman N. *Microbiology:ALaboratory Manual.* New Delhi: Dorling Kindersley. pp. 53-165.2007.
7. Cheesbrough M. *District laboratory practice in tropical countries.* 2nd ed. Cambridge University Press, Cambridge, UK. ISBN-13: 9781139449298.2006.
8. Olutiola PO, Famurewa O, Sonntag HG, *Introduction to General Microbiology: A Practical Approach.* 2nd ed. Bolabay Publications, Ikeja, Nigeria; 2000.
9. Aneja KR. *Experiments in Microbiology and Plant Pathology.* New Age International Pvt. Ltd., New Delhi. 4:632. 2003.
10. Deora GS, Singhal K. Isolation, biochemical characterization and preparation of biofertilizers using *Rhizobium* strains for commercial use. *Biosci.Biotechnol. Res.Commun.* 2010; 3(2):132-136.
11. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. *J.Bacteriol.* 1991; 173:697-703.
12. Chaudhary T, Gera R, Shukla P. Deciphering the potential of *Rhizobium pusense* MB-17a, a plant growth-promoting root endophyte, and functional annotation of the genes involved in the metabolic pathway. *Front. Bioeng.Biotechnol.* 2021; 8:617034.
13. Karpagam T, Nagalakshmi PK. Isolation and characterization of phosphate solubilizing microbes from agricultural soil. *Int. Curr.Microbiol. Appl. Sci.* 2014; 3:601-14.
14. Lorck H. Production of hydrocyanic acid by bacteria. *Plant Physiol.* 2004; 1:142-146.
15. Jida M, Assefa F. Phenotypic and plant growth promoting characteristics of *Rhizobium leguminosarum* bv. *viciae* from lentil growing areas of Ethiopia. 2011; *Afr. J.Microbiol. Res.* 5(24):4133-4142.
16. Laranjo M, Machado J, Young JPW, Oliveira S. High diversity of chickpea *Mesorhizobium* species isolated in a Portuguese agricultural region. *FEMS Microbiol. Ecol.* 2004; 48:101-107.
17. Rivas R, Laranjo M, Mateos PF, Oliveira S, Martinez-Molina E, Velazquez E. Strains of *Mesorhizobium* *morphae* and *Mesorhizobium* *tianshanense* carrying symbiotic genes of common chickpea endosymbiotic species constitute a novel biovar (ciceri) capable of nodulating *Cicer arietinum*. *Letters Appl.Microbiol.* 2007; 44:412-418.
18. Bumunang EW, Babalola OO. Characterization of rhizobacteria from field grown genetically modified (GM) and non-GM maizes. *Braz. Arch. Biol. Technol.* 2014; 57:1-8.
19. Islam M, Deora A, Hashidoko Y, Rahman A, Ito T, Tahara S. Isolation and identification of potential phosphate solubilizing bacteria from the rhizosphere of *Oryza sativa* L. cv. BR29 of Bangladesh. *Z.Naturforsch. C.* 2007; 62(1-2):103-110.
20. Peix A, Rivas-Boyer AA, Mateos PF, Rodriguez-Barrueco C, Martinez-Molina E, Velazquez E,. Growth promotion of chickpea and barley by a phosphate solubilizing strain of *Mesorhizobium mediterraneum* under growth chamber conditions. *Soil Biol.Biochem.* 2001; 33:103-110.