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

ABSTRACT

Aims: The aim of the present study is to isolate and characterize root nodulating bacteria from *Cicer arientinum* L cv. GNG 1958 and assess its plant growth promoting properties. The study is based on previous reports that native symbiotic bacteria may be replaced by better performing strains by corss-inoculation to increase productivity and tolarance to bioyic and abiotic stresses

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. arientinum* L. cv. GNG 1958 grown at the agricultural fields' located at the Krishi Vigyan Kendra, Banasthali Vidypapith, 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. arientinum* 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 erdmanii* 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 arientinum* GNG 1958 variety was *Mesorhizobium* sp. which has potent plant growth promoting traits.

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

1. INTRODUCTION

Cicer arientinum 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 [4].

In the present study, isolation of symbiotic bacteria, its biochemical and molecular characterization and assessment of plant growth promoting property through ammonia production, phosphate solubilization 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 Krishi Vigyan Kendra, Banasthali Vidypapith, 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^{o}C. The morphological characteristics of the isolate were noted [5].

2.2 Biochemical Characterization

To check the presence of *Agrobacterium*, alpha ketolactose test was done with the help of Benedict's reagent [4]. Citrate test [6], Indole test [7], Methyl Red-Voges Proskauer 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 (CTCGCTGCCCACTGTCAC).
The reactions were carried out in a PCR tube containing 2.5mM dNTPs, 1XTaq 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 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^{o}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^{o}C. Phosphate solubilization index (PSI) was calculated [13] as follows:
PSI = (colony diameter + halo zone diameter)/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% Na_2CO_3 and incubated for 3 days at 30±2^{o}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 had fast growing characteristics and was 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].

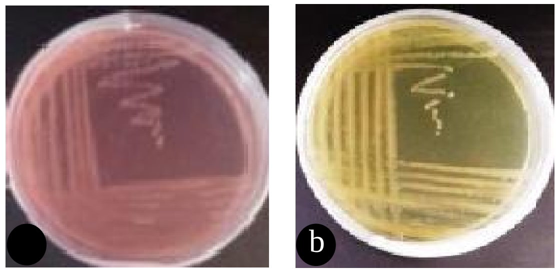

Fig. 1. Bacterial growth on YEMA media supplemented with (a) BTB and (b) Congo red dye

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).

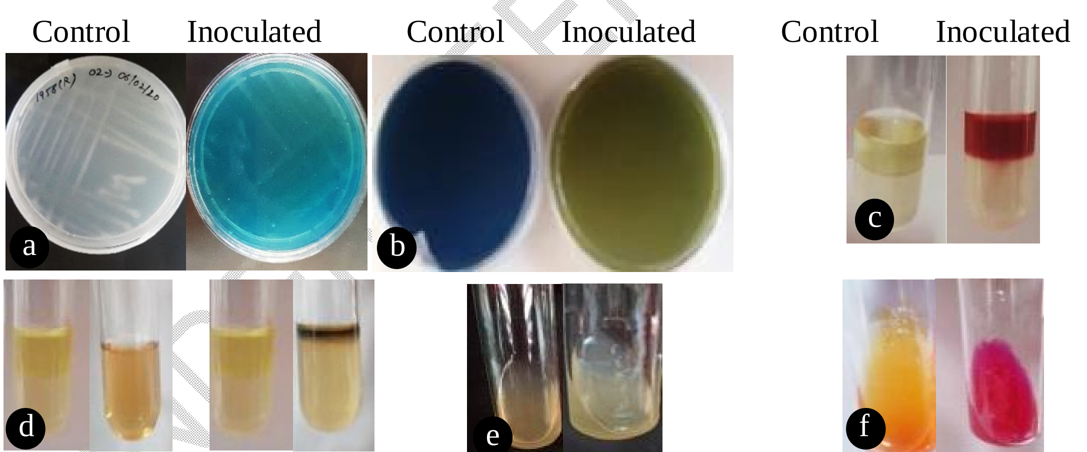

Fig. 2. Biochemical characterization of BVCA-58: (a) alpha-keto test (b) citrate test (c) indole test (d) Methyl Red test and Voges Proskauer test (e) gelatin test (f) urease test

3.3 Molecular Characterization

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 analyzed with NCBI Blast tool which showed 99.89% and 99.62% similarity with *Mesorhizobium jarvisii* ATCC-33669 and *Mesorhizobium erdmanii*

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	Similarity (NCBI BLAST)		NCBI Genebank submission	
	base pair	%	Nearest	Strain name	Accession no.
16f-16r	1165	99.89	*Mesorhizobium jarvisii* ATCC-33669	*Mesorhizobium* sp. BVCA-58	OP646813
63f-1244r	1064	99.62	*Mesorhizobium erdmanii* USDA-3471	*Mesorhizobium* sp. BVCA-58.	OP646810

3.4 Plant growth promoting properties

Ammonia production was observed in BVCA-58by 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, 21].

Isolation of bacterial strains, especially, root nodulating bacteria in legumes with plant growth promoting activities assumes significance as it is reported to provide protection to biotic [22] and abiotic stress [23-25].

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

4. CONCLUSION

Root nodulating bacteria play a significant role in agriculture systems by fixing atmospheric nitrogen. The present study revealed that isolated strain from *Cicer arientinum* GNG 1958 variety was *Mesorhizobium* sp. which has potent plant growth promoting traits. It may be used as a bio-inoculum in a biofertiliser formulation for sustainable agriculture.

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