Heliyon 7 (2021) e08321

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

# Characterization of rhizobia isolated from leguminous plants and their impact on the growth of ICCV 2 variety of chickpea (*Cicer arietinum* L.)

Mohammad Imran Mir [a], B. Kiran Kumar [a], Subramaniam Gopalakrishnan [b,*], Srinivas Vadlamudi [b], Bee Hameeda [c,**]

[a] Department of Botany, UCS, Osmania University, Hyderabad, 500007, Telangana, India
[b] International Crops Research Institute for the Semi-Arid Tropics, Patancheru, Hyderabad, 502319, Telangana, India
[c] Department of Microbiology, UCS, Osmania University, Hyderabad, 500007, Telangana, India

ARTICLE INFO

*Keywords:*
Root-stem nodules
*Rhizobium* spp.
PGP traits
16S rRNA
Chickpea
SEM analysis

ABSTRACT

Six rhizobia-like-bacterial strains in total, secluded from the root and stem nodules of various leguminous plants were characterized for growth promoting ability on ICCV 2 variety of chickpea. Bacterial strains showed production of IAA, $NH_3$, siderophore, HCN, ACC deaminase, hydrolytic enzyme production such as chitinase, amylase, protease, lipase, β-1, 3-glucanase and solubilization of nutrients such as phosphate, zinc and potassium. However the performance of PGP traits characterized *in-vitro* varied among the six bacterial strains. The sequences of 16S rRNA gene of bacterial strains IHSR, IHRG, IHAA, IHGN-3, IHCP-1 and IHCP-2 showed maximum identity with *Rhizobium* sp., *Rhizobium tropici*, *Rhizobium multihospitium*, *Mesorhizobium* sp., *Burkholderia cepacia* and *Rhizobium pusense*. In plate culture conditions the bacterial strains changed the colour of media (NFB) from green to blue and showed amplification of *nifH* gene by PCR, and also enhanced nodule formation in chickpea under greenhouse conditions, which explains their nitrogen fixing ability. Scanning electron microscopy studies of chickpea roots showed colonization by all the six bacterial strains in solo and by consortium (IHRG + IHGN-3). Under greenhouse conditions, chickpea plants inoculated with different strains showed improvement in plant height, number of branches, total chlorophyll, nodule number, nodule weight, shoot weight, root weight, root volume and root surface area at 30 and 45 days after sowing (DAS) over the uninoculated control plants. It was also observed at the crop maturity stage all the bacterial strains inoculated separately enhanced pod number, seed number and total NPK compared to uninoculated control plants. This study suggests that bacteria associated with root and stem nodules can be a promising resource to enhance nodulation, PGP and crop yields in chickpea.

## 1. Introduction

Chickpea (*Cicer arietinum* L.) is globally the third most important food legume after common bean and soybean (Wolde-Meskel et al., 2018). Approximately 64% of the total chickpea production is from India, covering 8.25 million hectare of land that play a key role in human diet, sustainable and eco-friendly agriculture (Yadav and Verma, 2014). Among vegetarian people, protein energy malnutrition is greatly reduced by this legume as it contains abundant carbohydrates, proteins, minerals and β-carotene (Jukanti et al., 2012). Chickpea display low glycemic index thus useful in lowering the menace of cardiovascular diseases, colon and breast cancer, obesity and diabetes (Foster-Powell et al., 2002; Aisa et al., 2019). In addition to its significant role in human diet, chickpea can fix atmospheric nitrogen in symbiosis with rhizobia called symbiotic nitrogen fixation that improves fixed nitrogen content in environment is another attractive characteristic that separates it when compared to the cereal crops. This fixation is carried in specialized organs called nodules in the form that is directly assimilated i.e., ammonia at an approximate rate of 140 kg/ha/year thereby improve fertility of soil and cereal crops productivity during crop rotation systems (Flowers et al., 2010). Even after manoeuvring ample amounts of synthetic fertilizers and pesticides, conventional breeding and molecular approaches, global chickpea yield/production stands still for the last 50 years i.e., 0.5–1 ton/ha (Gopalakrishnan et al., 2018). According to Akhtar and Siddiqui (2009), during the last decade, the production of chickpea in India is declined due to meagre native soil rhizobial inhabitants or

* Corresponding author.
** Corresponding author.
*E-mail addresses:* s.gopalakrishnan@cgiar.org (S. Gopalakrishnan), drhami2009@gmail.com (B. Hameeda).

https://doi.org/10.1016/j.heliyon.2021.e08321
Received 15 July 2021; Received in revised form 14 September 2021; Accepted 1 November 2021

ineffective biological nitrogen fixation (BNF). Increase in chickpea production encounters considerable limitations in terms of inadequate native soil rhizobial populations, harsh climate, poor soil, inadequate fertilizer, pathogens etc. (Akhtar and Siddiqui, 2009; Gopalakrishnan et al., 2018). With the ever rising cost of chemically synthesized pesticides and fertilizers as well as fears about environmental pollution, there has been a resurgence of interest in finding environmentally sustainable crop production and conservation methods (Jannouraa et al., 2013; Hamid et al., 2021). Furthermore due to excessive use of these plant protection chemicals, rhizosphere microflora gets diminished in a negative way by leaning from associative favourable microbes to detrimental ones. A more sustainable alternative is to use plant growth promoting rhizobacteria (PGPR). PGPR based inoculants are widely being accepted globally as an alternative for chemical fertilizers in view of agricultural sustainability (Ahmad and Zaib, 2020; Rasool et al., 2021).

Rhizobacteria enhance plant growth by production of plant growth hormones, improve the uptake of nutrient, induce root exudation and suppress phytopathogens are termed as plant growth-promoting (PGP) bacteria (Dutta and Podile, 2010). Rhizobia by their ability to convert nitrogen into ammonia, which can be used by the plants, also belong to PGPR. PGP bacteria colonize roots efficiently, enhance plants growth and yield. PGP rhizobacteria either directly or indirectly affect plants positively by increasing availability of nutrients (Verma et al., 2013; Imen et al., 2015; Khalid et al., 2020), regulate nitrogenase activity, synthesize plant growth regulators like phytohormones (Backer et al., 2018; Gopalakrishnan et al., 2018), siderophores (Angus et al., 2013; Datta and Chakrabartty, 2014), hydrocyanic acid (HCN), 1-aminocyclopropane-1--carboxylic acid (ACC) deaminase activity (Tariq et al., 2014; Subramanian et al., 2015; Igiehon et al., 2019) and inhibit phytopathogens.

For many decades, *Rhizobium* sp. were thought to be the only $N_2$ fixers found in legume nodules. However, a range of α- β- and γ-Proteobacteria primarily genera like *Pantoea*, *Burkholderia*, *Serratia*, *Pseudomonas*, *Bacillus* and *Enterobacter* have recently been found from legume nodules (Saidi et al., 2013; Martinez-Hidalgo and Hirsch, 2017; Gopalakrishnan et al., 2018). Most of these nodulating diazotrophic bacteria have been demonstrated to exhibit PGP qualities and yield enhancement in addition to their $N_2$ fixing abilities (Dobbelaere et al., 2003; Gopalakrishnan et al., 2015). As most chickpea growing soils lack adequate quantities of natural compatible rhizobia, rhizobia must be applied to seeds or soil. Furthermore, it is well recognized that the host (cultivars) also differs in their ability to fix nitrogen, and hence necessitating the identification of compatible rhizobia for specific variety. This study principally demonstrates to isolate and identify efficient diazotrophic PGP bacteria from the root and stem nodules of various leguminous plants and their further evaluation for growth promotion on ICCV 2 variety of chickpea under greenhouse conditions.

## 2. Materials and methods

### 2.1. Sample collection and isolation of bacteria from root and stem nodules

Root nodules of the *Cicer arietinum* (chickpea), *Cajanus cajan* (red gram), and stem nodules of the *Sesbania rostrata* (rostrate sesbania) and *Aeschynomene aspera* (shola pith) were collected from agricultural field sites in different states of India (Table 1). The plants were uprooted and loosely adhered soil was detached by gentle shaking. The collected nodules were washed in running water to remove adhered soil and dust particles. Large sized and healthy nodules were selected for isolation of bacteria. The root and stem nodules were immersed in 95 % alcohol for 5–10 s, followed by 2.5% sodium hypochlorite for 2 min and then rinsed thoroughly with sterilized distilled water (six times) in order to remove the chemicals. Each surface sterilized nodule from different legumes was aseptically crushed with a sterile glass rod in a test tube that contained 1 mL sterile physiological saline (0.8 % NaCl). One loopful of the nodule suspension was streaked on Petridishes that contained YEM (yeast extract mannitol) agar medium and incubated in the dark for 3–4 days at 30 °C (Vincent, 1970; Wei et al., 2009). A single colony representing each nodule was selected at the end of the incubation period and further purified on fresh YEM plates. The purified cultures were maintained on YEM agar slants, stored at 4 °C in refrigerator for further characterization.

**Table 1.** Collection details of plant samples.

| Bacterial isolates | Host plant | Source of isolation | Place of collection |
|---|---|---|---|
| IHRG | Red gram | Root nodules | Adilabad, Telangana, India. |
| IHCP-1, IHCP-2, IHGN-3 | Chickpea | Root nodules | Pathancheru, Telangana, India. |
| IHAA | *A. aspera* | Stem nodules | Manipur, India. |
| IHSR | *S. rostrata* | Stem nodules | Tamil Nadu, India. |

### 2.2. Detection of PGP traits of isolated bacteria under in vitro conditions

Bacteria secluded from root and stem nodules were investigated for their PGP properties such as production of siderophore, indole acetic acid (IAA), ammonia, hydrocyanic acid (HCN), 1-aminocyclopropane-1-carboxylate deaminase (ACCD), mineral solubilization like phosphate (tri-calcium phosphate-TCP), rock phosphate (RP), potassium and zinc (Zn), production of hydrolytic enzymes like cellulase, β-1, 3- glucanase, lipase, protease and chitinase under *in vitro* conditions. Qualitative test for IAA production was carried out by spot inoculating the isolated bacteria on YEM medium supplemented with 5 mM L- tryptophan and after incubating for 48–72 h; the inoculated marks were superimposed with 10 mm-diameter nitrocellulose membrane (NCM) disk that had been pre-saturated with few drops of Salkowski reagent. After few minutes, development of pink colour indicated IAA production (Williams and Signer, 1990). Cultures which were positive in plate culture conditions were tested for quantitative assessment of IAA in broth culture by colorimetry as reported by Gordon and Weber (1951). Siderophore production was assayed qualitatively using Chrome Azurol S (CAS) blue agar as described by the method of Schwyn and Neilands (1987). CAS agar plates were spot inoculated with each of the bacterial strain and development of an orange halo zone around the colonies were recorded as the measurement of siderophore production. HCN was qualitatively estimated in YEMA medium incorporated with glycine (4.4 g/L) by method of Bakker and Schipperes (1987). Development of colour from yellow to brown, moderate brown or strong brown indicates production of hydrocyanic acid. Estimation of ammonia was carried out by addition of Nessler's reagent to bacterial culture in peptone water broth and development of slight yellow to brownish color was considered to be a positive test for ammonia production (Kavamura et al., 2013). The bacterial strains were tested for their ability to solubilize phosphate (TCP) under *in vitro* conditions using NBRIP medium according to the method described by Nautiyal (1999). Quantitative estimation of phosphate solubilization was carried out by ammonium phosphate molybdate blue colour method (Fiske and Subbarow, 1925). TRP (Tris buffered rock phosphate) agar medium amended with methyl red pH indicator and 100 mM glucose as sole carbon source was used to check the ability of bacterial isolates to solubilize rock phosphate. Development of red coloration around the bacterial colonies on TRP agar medium indicated rock phosphate (RP) solubilization (Gyaneshwar et al., 1998). The ability of the isolates to solubilize potassium was tested by spot inoculating bacterial isolates on Aleksandrov medium as per the method of Hu et al. (2006), plates were incubated at 28 ± 2 °C for 3–5 days. The formation of zone of clearance around the spots indicated the potassium solubilization. Zinc solubilization was carried out by spot inoculation of bacteria isolates on tris- salt agar medium supplemented with insoluble zinc compounds (zinc carbonate- $ZnCO_3$) at 1000 mg per liter individually as per method of Goteti et al. (2013). After spot inoculation on tris-salt agar medium, the diameter of the solubilization zones around the colonies were measured (Saravanan et al., 2007). ACC deaminase activity was

tested on Petridishes that contained DF (Dworkin and Foster) salt minimal medium supplemented with 3 mM ACC (as the sole nitrogen source), as per method described by Penrose and Glick (2003). Growth of isolates on ACC supplemented plates was compared to positive ($(NH_4)_2$ $SO_4$ as N-source) and negative controls (DF minimal medium without ACC) after 3–4 days incubation at 28 °C. For the detection of hydrolytic enzymes such as protease, cellulase and lipase; casein agar, carboxy methyl cellulose congo red and tween 80 agar were used according to the methods described by (Kasana et al., 2008; Bhattacharya et al., 2009). Minimal media supplemented with 5% colloidal chitin according to the methods of Hirano and Nagao (1988) was used for the detection of chitinase in bacterial isolates. β − 1, 3-glucanase breaks down the glucan polymer laminarin, liberating glucose molecules. Standardized methodology of Singh et al. (1999) was employed for β- 1, 3-glucanase assay. The amount of glucose released was used to determine the activity of β − 1, 3-glucanase. The amount of enzyme required to liberate 1 μmol of glucose/hour is one unit of its activity. By calculating the amount of glucose released, β-glucanase activity was measured.

### 2.3. Screening of isolates for nitrogen-fixing ability- PCR amplification of nifH genes, growth on NFB medium

For PCR amplification of *nifH* gene, purified cultures of bacteria isolated from root and stem nodules were cultured in YEM broth until log phase and GSure bacterial genomic DNA isolation kit according to manufacturer's instructions (GCC Biotech) was used for extraction of genomic DNA. One hundred nanograms of genomic DNA of the isolated bacteria was used as template in PCR for the amplification of *nifH* gene using primers: polF (5′-TGC GAY CCS AAR GCB GAC TC-3′) and polR (5′-ATS GCC ATC ATY TCR CCG GA -3′) (Poly et al., 2001a). The PCR reaction set up and thermal profiling conditions were performed according to methods described by Poly et al. (2001b).

The $N_2$ fixation ability of the isolated strains was also verified by culturing the bacterial isolates on N-free solid malate medium (NFB medium) that contained bromothymol blue (BTB) as an indicator and incubating the plates at 30 °C for 24–48 h. After the incubation period, the color of the medium changed from pale green to blue, indicating the isolates' ability to fix $N_2$ (Baldani et al., 2014).

### 2.4. Identification of the isolated bacteria by 16S rRNA gene sequence analysis

Pure cultures of bacteria secluded from root and stem nodules were cultured in YEM broth until log phase for molecular identification. GSure bacterial genomic DNA isolation kit according to manufacturer's (GCC Biotech) was used for isolation of genomic DNA. Universal eubacterial primers FGPS6 (5′ GGA GAG TTA GAT CTT GGC TCA G 3′) as forward and FGPS1509 (5′ AAG GAG GGG ATC CAG CCG CA 3′) as reverse according to Normand et al. (1992); Zakhia et al. (2004), was used for amplification of 16S rDNA gene. PCR amplification was carried out in a 50 μL reaction mixture comprising template DNA (2 μL), 5 μL of reaction buffer, 1.5 μL of $MgCl_2$ 50 mM, 4 μL of dNTPs 2.5 mM each, 2 μL of each primer (20 μM), 1 unit of Taq polymerase and 33.2 μL of nucleus free water. PCR amplifications were carried out in a thermal cycler (Eppendorf AG Mastercycler Nexus Series Serial No. 6333BR503557) set to 35 cycles, with an initial denaturation step of 5 min at 94 °C, followed by 30 s at 94 °C for denaturation, 30 s at 55 °C for primer annealing, and primer extension of 7 min at 72 °C, followed by a final extension of 7 min at 72 °C. PCR amplified products were verified by electrophoresis in 1.5% agarose gel in TAE buffer containing 2 drops of 0.5 mg/mL ethidium bromide for 1 h at 80-volt constant. Gel Document system (Bio-RAD, Gel DOC, E Z IMAGER US) was used to capture an image of the resulting gel. All the amplified PCR products were purified and sequenced. The BLAST program was used to compare the sequences obtained from Eurofins Genomics India Pvt. Ltd to those from the NCBI and Ez-Taxon, and the Clustal W software was used to align the sequences, and the MEGA7 software was used to build phylogenetic trees (Alschul et al., 1990; Tamura et al., 2007). The neighbor-joining approach was used to infer the dendrogram. Nucleotide sequences of all the six isolated bacteria were submitted to GenBank and the NCBI GenBank accession numbers were received.

### 2.5. Seed material

In the present investigation, ICCV 2 variety of chickpea seeds were used and were procured from International Crops Research Institute for the Semi-Arid Tropics, Patancheru, Telangana, India.

### 2.6. Colonization ability of isolated bacteria with chickpea roots

Root colonization of chickpea by isolated bacteria was investigated using scanning electron microscopy (SEM) according to methodologies of Gopalakrishnan et al. (2015a). ICCV 2 variety of chickpea seeds were disinfected through successive immersion in 2.5% $NaOCl_2$ solution for a period of 2-min, followed by 70% ethanol for 5-min, and six consecutive washings in sterilized distilled water. Surface disinfected seeds were pre-treated for an hour with each of the six bacterial strains individually (IHSR, IHRG, IHAA, IHGN-3, IHCP-1, IHCP-2) and also with a consortium of (IHRG, IHGN-3) before being seeded in sterilized coarse sand pots. The un-inoculated but sterilized seeds dipped in sterile water only were taken as control. The pots were maintained for a period of 14 days in glasshouse at a temperature of 26 °C, humidity 70%. Chickpea seedlings were removed from the sand pots at the end of the incubation period, and the roots were rinsed in phosphate saline buffer (PSB, pH 7.2). The roots were fixed in 2.5% glutaraldehyde prepared in 0.1 M phosphate buffered saline (pH 7.2) for 24 h at 4 °C, washed twice with PSB, postfixed in 2% osmium tetroxide for 4 h. The fixed roots were then dehydrated in an ascending gradient series of ethanol 30–100% (v/v). The samples were later coated with gold-palladium in an automated sputter coater (JEOL JFC-1600) and examined with a scanning electron microscope as per the standardized protocols at Central Analytical Facility, University College of Technology, Osmania University, Hyderabad, Telangana, India. The presence of bacterial strains on root surfaces was observed and recorded.

### 2.7. Evaluation of plant growth promotion of chickpea under greenhouse conditions

Under glasshouse conditions, plant growth promoting ability of isolated bacteria was evaluated using ICCV 2 variety of chickpea. Chickpea seeds were surface sterilized by dipping in 2.5% sodium hypochlorite ($NaOCl_2$) solution for 2 min and in 70% ethanol for 5 min followed by rinsing six times with sterile distilled water. Surface sterilized seeds were pre-treated with all the six bacterial strains separately (IHSR, IHRG, IHAA, IHGN-3, IHCP-1, IHCP-2) and also with consortium of (IHRG, IHGN-3) for an hour before being sown in pots. The bacterial inoculum was prepared in 100 mL YEM broth taken in 250 mL Erlenmeyer flask and incubated for 72 h. However for consortium of IHRG and IGHN-3, both the cultures were mixed (50/50 v/v). Pot mixture was prepared by mixing black soil and sand in the ratio of 3:2 and placed in 8″ plastic pots. Six seeds were sown in each pot and thinning was done to three after 7 days of seedling emergence. The pots were placed in controlled greenhouse conditions with maximum and minimum temperatures maintained at 28 °C and 22 °C, relative humidity 70%, respectively and were under natural day-light oscillations. A total of eight treatments (6 diazotrophic bacteria in solo, one consortium, and one un-inoculated negative control) were made with six replications. Booster doses of each isolated bacteria ($10^8$ CFU $mL^{-1}$), were applied at 10, 25 and 40 days after sowing by soil drench method. At 30 and 45 days after sowing (DAS), PGP parameters such as plant height, branch number, total chlorophyll (as per the methodology of Hiscox and Israelstam, 1979),

nodule number, nodule dry weight, root and shoot dry weight, root volume and root surface area were measured. At crop maturity stage, pod and seed number were recorded (Gopalakrishnan et al., 2018). At crop harvest stage, chickpea plants obtained were dried in hot air oven at 65 °C for three days, ground to fine powder and digested with nitric acid – hydrogen peroxide for total potassium analysis (Wheal et al., 2011) and selenium-sulfuric acid digestion method was used for total nitrogen and phosphorous analysis (Sahrawat et al., 2002), in plant samples treated with isolated bacteria. By running known standards, potassium analysis in digested samples was explored employing inductively coupled plasma-optical emission spectroscopy (ICP-OES).

### 2.8. Statistical analysis

The data were analyzed statistically by analysis of variance (ANOVA; Genstat 20. version) in a completely randomized design (CRD) for greenhouse to evaluate the efficiency of diazotrophic bacteria. Significance of differences between the treatment means was tested at $P = 0.05$.

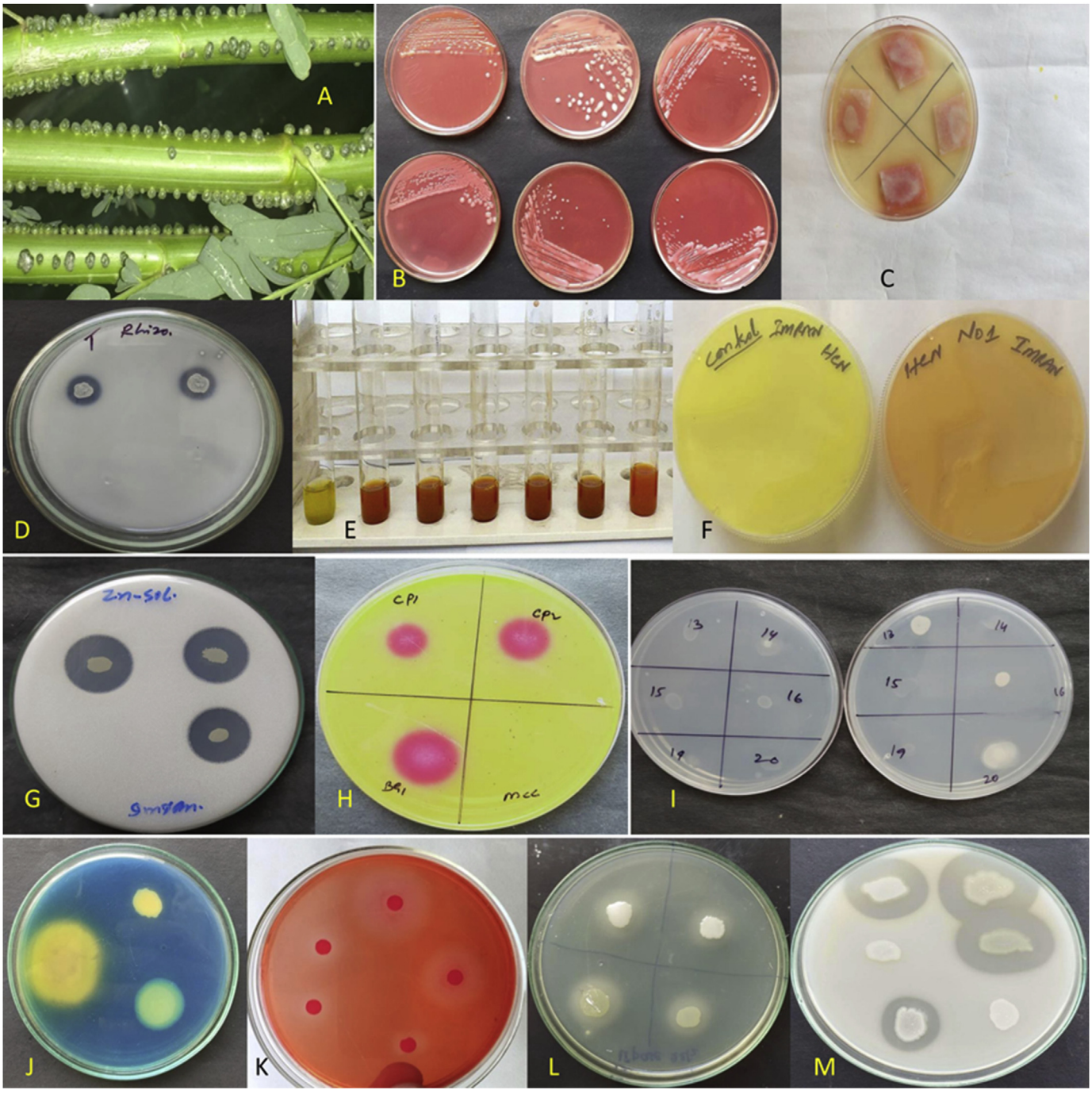


**Figure 1.** *In vitro* Plant growth promoting traits of root and stem nodule bacteria. (A) Photograph showing stem nodules of *Sesbania rostrata*, (B) Purified cultures on YEM agar media, (C) Development of pink colour of nitrocellulose membrane indicated IAA production, (D) Isolates showing halo zones on NBRIP media indicated tri-calcium solubilization, (E) Ammonia production, (F) HCN production, (G) Isolates showing zinc solubilization, (H) Rock phosphate solubilization, (I) Isolates showing ACC deaminase activity on DF salt minimal medium, (J) Orange halo zone on CAS agar plates indicates siderophore production, (K) Cellulase production by the isolates, (L) Lipase production, (M) Protease production.

## 3. Results

### 3.1. Isolation of root and stem nodule bacteria

A total of six bacteria were isolated from the healthy root and stem nodules of different leguminous plants, collected from various sites of India (Table 1). The isolates include, 1 isolate from the root nodules of red gram designated as IHRG, 3 isolates from root nodules of chickpea designated as IHCP-1, IHCP-2 and IHGN-3, 1 isolate from the stem nodules of *A. aspera* designated as IHAA, and 1 isolate from the stem nodules of *S. rostrata* (Figure 1A) designated as IHSR. All the isolates showed well-marked growth on YEM agar medium at pH 7.0 after incubation for 48–72 h at 30 °C (Figure 1B). Microscopic examination revealed that the isolates were Gram negative and rod in shape.

### 3.2. PGP traits of isolated bacteria

The findings of PGP traits of all the six bacterial isolates are mentioned in (Table 2 and Figure 1). Varying levels of PGPR traits were found in the isolated bacteria. All the six isolates produced IAA which was confirmed qualitatively by appearance of pink colour of nitrocellulose membrane (Figure 1C). However quantitative estimation of IAA in broth culture amended with 5 mM L- tryptophan, revealed variation in amount of IAA produced by each isolate. Highest amount of IAA (308 μg/ml) was produced by bacterial isolate IHAA and HGN-3, followed by IHCP-1 (251 μg/ml), IHCP-2 (165 μg/ml), IHRG (158 μg/ml) and IHSR (32 μg/ml). All the bacterial isolates showed phosphate solubilization on NBRIP medium by formation of halo zone around the bacterial colonies. Highest solubilization of phosphate was observed with the supernatant of bacterial isolate IHAA (552 μg/ml), followed by IHSR (356 μg/ml), IHCP-2 (300 μg/ml), IHRG (296 μg/ml), IHCP-1 (292 μg/ml) and IHGN-3 (173 μg/ml) (Figure 1D). The brown coloration of the tubes containing peptone water broth suggested that all of the isolates had positive results for ammonia production. Further out of six bacterial isolates, the isolates IHSR, IHRG, IHAA, IHGN-3 and IHCP-2 showed high ammonia production activity, while the isolate IHCP-1 showed moderate activity (Figure 1E). Among the six bacterial isolates, five isolates were found to produce HCN, which was verified by the appearance of a brown color on the filter paper. Further among five bacterial isolates, IHRG and IHCP-2 showed highest hydrocyanic acid production as they exhibited deep brown colour. Bacterial isolates IHSR, IHAA and IHCP-1 showed moderate HCN production as they exhibited medium brown colour. The isolate IHGN-3 showed negative results with HCN production (Figure 1F). Among the six bacterial isolates, five isolates (except IHSR) showed zinc solubilization on tris mineral salt medium by development of halo zone around the bacterial colonies. Highest zone of solubilization was shown by the bacterial isolate IHCP-1 (32 mm) followed by IHAA (19.3 mm) and IHCP-2 (19 mm) (Figure 1G). Only two isolates (IHCP-1 and IHAA) showed potassium solubilization on Aleksandrov agar medium by formation of halo zones around the bacterial conies. Further of two bacterial isolates, the highest zone of solubilization was shown by the isolate IHCP-1 (11.3 mm) followed by IHAA (11.2 mm). On TRP agar, four of the six bacterial isolates (IHRG, IHAA, IHGN-3, and IHCP-1) had red coloration around the colonies, confirming rock phosphate solubilization. Further out of the four isolates, the isolate IHCP-1 showed maximum zone of red coloration (29 mm), followed by IHAA (19 mm) and (15 mm) by IHRG (Figure 1H). All the six bacterial isolates showed growth on DF minimal medium supplemented with ammonium sulphate and only five isolates (IHRG, IHCP-1, IHCP-2, IHGN-3 and IHAA) showed growth on DF minimal medium supplemented with ACC, indicated that bacterial isolates exhibit ACC deaminase activity (Figure 1I). The ability to produce siderophore was detected in all of the bacterial isolates, as evidenced by the development of orange halos around the colonies on CAS agar plates (Figure 1J). Highest production of siderophore was shown by isolate IHRG (67.7% units) followed by IHAA (62.1% units), IHCP-1 (61.7% units), IHGN-3 (56.5% units), IHCP-2 (33.2% units) and IHSR (30.2% units). All the six bacterial isolates produced β-1, 3-glucanase, isolate IHGN-3 had produced higher level of β-1, 3-glucanase (2.78% units), followed by IHSR (2.41% units), IHCP-1 (2.33% units), IHRG (1.93% units), IHAA (1.89% units) and IHCP-2 (1.75% units). On carboxymethyl cellulose congo red, tween 80 agar and casein agar medium, all six bacterial isolates produced cellulase, lipase and protease respectively, by forming a halo zone around the bacterial colonies (Figure 1K, L, M). Out of six bacterial isolates, three isolates (IHRG, IHGN-3 and IHCP-2) showed zone of hydrolysis when grown on chitin minimal medium indicating chitinase production. Further out of three isolates, the isolates IHRG and IHGN-3 showed highest zone of hydrolysis (19 mm) followed by IHCP-2 (18 mm).

### 3.3. Nitrogen-fixing ability of the isolated bacteria

All the six bacterial isolates were examined qualitatively for nitrogen fixation ability in N-free solid malate medium (NFB medium) containing bromothymol blue as an indicator. All the bacterial isolates were able to

**Table 2.** PGP traits of bacteria isolated from root and stem nodules.

| Isolate | Cell (mm) | Lip (mm) | Prot (mm) | Chit (mm) | Amy (mm) | IAA (μg/ml) | β-1, 3-glucanase (% units) | Sid (% units) | HCN | PS (μg/ml) | KS (mm) | ZS (mm) | RPS (mm) | ACCD |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| IHSR | 20 | 19 | 16 | 0 | 0 | 32 | 2.41 | 30.2 | 2 | 356 | 0.0 | 0.0 | 0.0 | - |
| IHRG | 7 | 8 | 16 | 19 | 0 | 158 | 1.93 | 67.7 | 3 | 296 | 0.0 | 9.7 | 15.0 | + |
| IHAA | 31 | 23 | 20 | 0 | 0 | 308 | 1.89 | 62.1 | 2 | 552 | 11.2 | 19.3 | 18.9 | + |
| IHGN-3 | 27 | 24 | 30 | 19 | 20 | 308 | 2.78 | 56.5 | 0 | 173 | 0.0 | 9.3 | 14.7 | + |
| IHCP-1 | 29 | 22 | 25 | 0 | 17 | 251 | 2.33 | 61.7 | 2 | 292 | 11.3 | 32.0 | 29.2 | + |
| IHCP-2 | 21 | 23 | 24 | 18 | 20 | 165 | 1.75 | 33.2 | 3 | 300 | 0.0 | 18.7 | 0.0 | + |
| Mean | 23 | 20 | 22 | 9 | 9 | 204 | 2.18 | 51.9 | 2 | 328 | 3.7 | 14.8 | 13.0 | |
| SE± | 0.8*** | 0.7*** | 0.4*** | 0.3*** | 0.5*** | 2.0*** | 0.011*** | 0.35*** | 0.02*** | 3.9*** | 0.31*** | 0.59*** | 0.43*** | |
| LSD (5%) | 2.5 | 2.2 | 1.2 | 0.9 | 1.7 | 6.3 | 0.034 | 1.10 | 0.07 | 12.4 | 0.96 | 1.85 | 1.36 | |
| CV% | 6 | 6 | 3 | 5 | 10 | 2 | 1 | 1 | 2 | 2 | 14 | 7 | 6 | |

Cell- Cellulase production; Lip- Lipase production; Prot- Protease production; Chit- Chitinase production; Amy- Amylase production; IAA- Indole acetic acid production; Sid- Siderophore production; HCN- Hydrocyanic acid production; PS- Phosphate solubilization; KS- Potassium solubilization; ZS- Zinc solubilization; RP- Rock phosphate solubilization; ACCD- 1-aminocyclopropane-1-carboxylate deaminase; SE- Standard error; LSD- least significant differences; CV- coefficients of variation; *** = statistically significant at 0.001; '+', Activity is present; '-', Activity is absent.

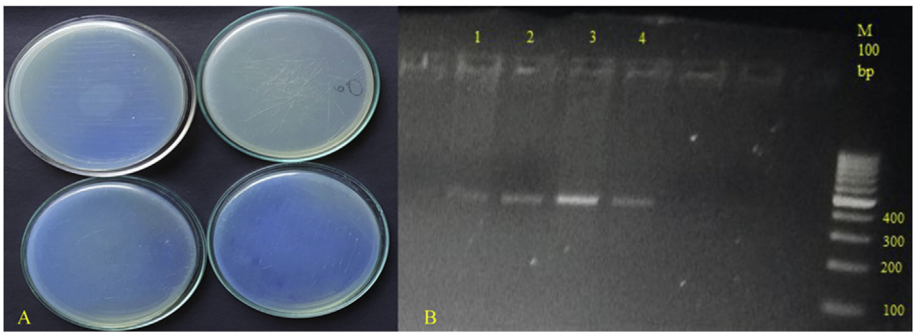


**Figure 2.** (A) Change in colour of NFB media due to pH increase. (B) Amplification of *nifH* gene. Lane 1, IHSR; Lane 2, IHGN-3; Lane 3, IHCP-1; Lane 4, IHCP-2.

change the colour of the medium from green to blue, suggested that the bacterial isolates can fix atmospheric nitrogen (Figure 2A). Further PCR amplification of *nifH* gene was carried out for determining nitrogen fixation potential of the isolated bacteria. Results revealed that four isolates (IHSR, IHGN-3, IHCP-1 and IHCP-2) amplified *nifH* gene and the product of predicted size (360–400 bp) were obtained indicated the presence of nitrogen fixing genes in these isolated bacteria (Figure 2B).

### 3.4. Molecular characterization of the isolated bacteria based on 16S rRNA gene sequence analysis

The sequences obtained from Eurofins Genomics India Pvt. Ltd, were compared to other similar sequences from GenBank, aligned and the dendrogram constructed. The sequences of 16S rRNA gene of the root and stem nodule bacteria of IHSR, IHRG, IHAA, IHGN-3, IHCP-1 and IHCP-2 showed maximum identity with *Rhizobium* sp., *Rhizobium tropici*, *Rhizobium multihospitium*, *Mesorhizobium* sp., *Burkholderia cepacia* and *Rhizobium pusense*, respectively. All six bacterial strains nucleotide sequences were submitted to NCBI GenBank and accession numbers were received (Figure 3, Table 3).

### 3.5. Colonization studies of chickpea roots by SEM analysis

In order to establish in the rhizosphere and boost plant development and yield, an efficient PGPR should bind to the root surface. The ability of bacterial isolates, IHSR, IHRG, IHAA, IHGN-3, IHCP-1, IHCP-2 and consortium (IHGN-3+IHRG) to colonize root tissues was investigated in 14 days old seedlings of chickpea by scanning electron microscopy (SEM). The obtained SEM images are represented in Figure 4. From the images it is clearly visible that all the bacterial isolates strictly adhered to the root surfaces of the inoculated seedling while they are not present in the uninoculated control seedlings.

### 3.6. Plant growth promotion in greenhouse conditions

Chickpea plants (ICCV 2) treated with the six selected bacterial strains in solo (IHSR, IHRG, IHAA, IHGN-3, IHCP-1, IHCP-2) and consortium (IHGN-3+IHRG) under greenhouse conditions significantly enhanced both plant growth parameters and yield traits. Results revealed that chickpea plants treated with the above bacterial strains increased plant height (up to 18%, 18%, 22%, 23%, 16%, 20%, and 27% by consortium of IHGN-3+IHRG), number of branches (up to 42%, 38%, 45%, 53%, 30%, 42%, and 53% by consortium), total chlorophyll content (up to 33%, 20%, 43%, 43%, 6%, 41% and 55% by consortium), nodule number (up to 44%, 44%, 48%, 50%, 35%, 44% and 58% by consortium), nodule dry weight (up to 62%, 55%, 68%, 83%, 55%, 65% and 91% by consortium), shoot dry weight (up to 29%, 27%, 35%, 45%, 27%, 29% and 56% by consortium), root dry weight (up to 50%, 50%, 61%, 65%, 36%, 50% and 82% by consortium), root volume (up to 48%, 44%, 49%, 50%, 36%, 48% and 55% by consortium), and root surface area (up to 46%, 45%, 54%, 56%, 45%, 51% and 57% by consortium) respectively at 30 DAS over the control plants (Table 4, Figure 5) while at 45 DAS, chickpea plants treated with the above bacterial strains increased plant height (up to 14%, 13%, 18%, 19%, 12%, 14% and 19% by consortium), number of branches (up to 33%, 33%, 43%, 43%, 20%, 33% and 43% by consortium), nodule number (up to 38%, 36%, 45%, 45%, 22%, 42% and 49% by consortium), nodule dry weight (up to 47%, 41%, 55%, 62%,

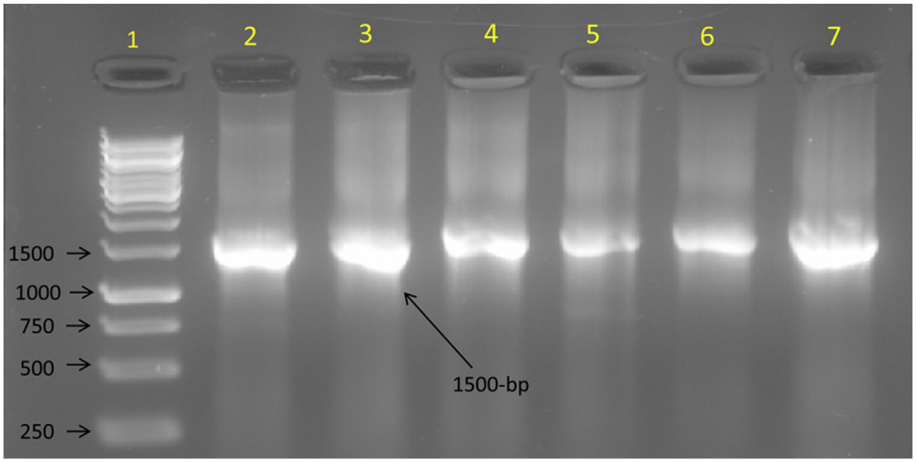


**Figure 3.** Amplification of 16S rDNA gene with amplicon size of 1500-bp. Lane 1, Thermo Scientific GeneRuler 1 kb ladder; Lane 2, IHSR; Lane 3, IHRG; Lane 4, IHAA; Lane 5, IHGN-3; Lane 6, IHCP-1; Lane 7, IHCP-2.

**Table 3.** Identification of isolates based on 16S rRNA gene sequence analysis.

| Isolate | Cell morphology | 16S rRNA Sequence Length | Hit strain | Similarity (%) | GenBank accession number [EMBL] |
|---|---|---|---|---|---|
| IHSR | Gram -ve rods | 1352 bp | *Rhizobium* sp. | 100% | MW478312 |
| IHRG | Gram -ve rods | 1280 bp | *Rhizobium tropici* | 100% | MW478313 |
| IHAA | Gram -ve rods | 1336 bp | *Rhizobium multihospitium* | 99% | MW478301 |
| IHGN-3 | Gram -ve rods | 1449 bp | *Mesorhizobium* sp. | 99% | MW478300 |
| IHCP-1 | Gram -ve rods | 1167 bp | *Burkholderia cepacia* | 99% | MW485490 |
| IHCP-2 | Gram -ve rods | 1232 bp | *Rhizobium pusense* | 99% | MW478346 |

26%, 52% and 66% by consortium), root dry weight (up to 40%, 39%, 44%, 52%, 39%, 41% and 56% by consortium) and shoot dry weight (up to 24%, 23%, 32%, 41%, 21%, 24% and 42% by consortium) over the control plants (Table 5). At crop maturity stage, chickpea plants inoculated with six bacterial strains in solo and consortium, increased pod number (up to 25% by IHSR, IHRG, 40% by IHAA, IHGN-3, IHCP-2 and consortium, 0% by IHCP-1), shoot weight (up to 32% by IHSR, IHRG, 33% by IHAA, 38% by IHGN-3, 31% by IHCP-1, 32% by IHCP-2 and 41% by consortium of IHGN-3 and IHRG) and seed number (up to 50% by IHSR, IHAA, IHGN-3, IHCP-2, 33% by IHRG, IHCP-1 and 60% by consortium of IHGN-3 and IHRG) over control plants (Table 6). Chickpea plants inoculated with isolated bacteria had higher total N (up to 12% except IHCP-1), total P (up to 15% except IHSR and IHCP-1) and total K (up to 15%) respectively than un-inoculated control plants at crop harvest stage (Table 7).

## 4. Discussion

In present study, a total of six rhizobia like bacteria were secluded from the root and stem nodules of *C. arietinum*, *C. cajan*, *S. rostrata* and *A. aspera* and were designated as IHSR, IHRG, IHAA, IHGN-3, IHCP-1 and IHCP-2. The isolates showed well marked growth on YEM agar medium and microscopic investigation revealed that the isolates were Gram –ve and rod in shape. All the six bacteria were identified up to species level by 16S rRNA analysis. The sequences of 16S rRNA genes of IHSR, IHRG, IHAA, IHGN-3, IHCP-1 and IHCP-2 showed highest similarity with *Rhizobium* sp., *Rhizobium tropici*, *Rhizobium multihospitium*, *Mesorhizobium* sp., *Burkholderia cepacia* and *Rhizobium pusense*, respectively (Table 3). Rhizobia were considered to be the only nitrogen fixing inhabitant of legume nodules for many decades. Recently various researchers have reported number of α, β and γ proteobacteria from nodules of wide range of legumes, such as *Pantoea*, *Burkholderia*, *Serratia*, *Pseudomonas*, *Bacillus* and *Enterobacter* (Saidi et al., 2013; Gopalakrishnan et al., 2018). Besides their nitrogen fixing capabilities, some of these non-symbiotic nodulating diazotrophic bacteria have also been shown to have PGP capabilities and yield enhancement (Martinez-Hidalgo and Hirsch, 2017). One such non-symbiotic nodulating diazotrophic bacteria designated as IHCP-1 (*Burkholderia cepacia*) was isolated from the root nodules of chickpea in our study. The results are in line with the prior findings of Benjelloun et al. (2019); Gopalakrishnan et al. (2018), reported the isolation of *Burkholderia* sp. from the nodules of chickpea.

Majority of legumes form symbiotic relationship with diazotrophic bacteria (alpha and beta rhizobia) and carryout biological nitrogen fixation, by root and stem nodules, and hence legumes gain significant ecological advantage (Figueiredo et al., 2013). These beneficial microbes provide nitrogen, phytohormones such as indole acetic acid, produce exopolysaccharides, siderophores, mineral solubilizations and antagonistic activity against various phytopathogenic fungi (Gopalakrishnan et al., 2015b). In current study, bacterial strains isolated from root and stem nodules showed production of IAA, $NH_3$, siderophore, HCN, ACC deaminase, hydrolytic enzyme production such as chitinase, amylase, protease, lipase, β-1, 3-glucanase and solubilization of nutrients such as phosphate, zinc and potassium. However the performance of PGP traits characterized *in-vitro* varied among the six bacterial strains. Phytohormones are plant growth-regulators, which influence the growth of plants. Majority of *Rhizobium* species are known to synthesize IAA (Ahemad and Kibret, 2014) since, IAA is engaged in a number of activities, including cell division, differentiation, and the development of vascular bundles, all of which are necessary for nodule formation. Exogenous application of IAA producing *Rhizobium* sp. have been found to increase root length/-biomass, shoot growth and seedling germination in chickpea (Yadav and Verma, 2014; Gopalakrishnan et al., 2018). In the current investigation all six bacteria isolated from root and stem nodules were found to produce IAA in the range of 32–.308 μg/ml (Table 2), which is higher than the prior reports of IAA production by various species of *Rhizobium* (Singha et al., 2018; Gopalakrishnan et al., 2018; Khalid et al., 2020).

PGP bacteria also enhance plant growth by scavenging useable iron ($Fe^{3+}$), through the production of siderophores, which are high affinity, low molecular weight iron chelating ligands. Siderophore producing PGP bacteria play vital role in the bio-control of variety of soil-borne plant diseases caused by various pathogens. Since siderophores sequester the rhizosphere's limited supply of iron, they reduce pathogens access to it, ultimately suppressing their development (Rasool et al., 2021). In present investigation all six bacteria were capable to produce siderophore. Highest production of siderophore was shown by isolate IHRG (67.7% units) followed by IHAA (62.1% units), IHCP-1 (61.7% units), IHGN-3 (56.5% units), IHCP-2 (33.2% units) and IHSR (30.2% units). Production of siderophore by *Mesorhizobium* sp., *Rhizobium tropici*, *Rhizobium multihospitium*, *Rhizobium pusense* and *Burkholderia* sp., is in line with the previous finding of various researchers (Solanki et al., 2017; Gopalakrishnan et al., 2018; Igiehon et al., 2019; Tagele et al., 2019; Menéndez et al., 2020). In the present investigation isolated bacteria were able to produce various hydrolytic enzymes such as cellulase, lipase, β- 1, 3-glucanase, protease and chitinase (except IHSR, IHAA and IHCP-1). PGP bacteria that produce one or more of these hydrolytic enzymes have been reported to have bio-control potential against a range of phytopathogenic fungi and bacteria (Rasool et al., 2021). Another important property of PGP bacteria is the production of $NH_3$ which is an inorganic versatile compound, which helps in bio-control mechanism against various phyopathogens. In present investigation all the isolated bacteria produced ammonia. The accumulation of ammonia in the soil by PGP bacteria has been observed to limit the growth of certain pathogenic fungi and inhibit the germination of spores (Kumari et al., 2018). Endogenous levels of ethylene in plants are greatly raised under various stressful situations, which have a negative impact on overall plant growth and development. Many *Rhizobium* sp. are able to synthesis ACC deaminase that splits ACC to α-ketobutyrate and ammonia. This reduces the ethylene levels in plants which in turn promotes plant growth in addition to defence against numerous biotic and abiotic stresses. In this study, all the isolated bacteria produced ACC deaminase (except IHSR). Production of ACC deaminase by *Rhizobium tropici*, *Rhizobium pusense*, *Mesorhizobium* sp., and *Burkholderia* sp., is also reported by many researchers (Igiehon et al., 2019; Gopalakrishnan et al., 2018; Tagele et al., 2019; Shahid et al., 2021).

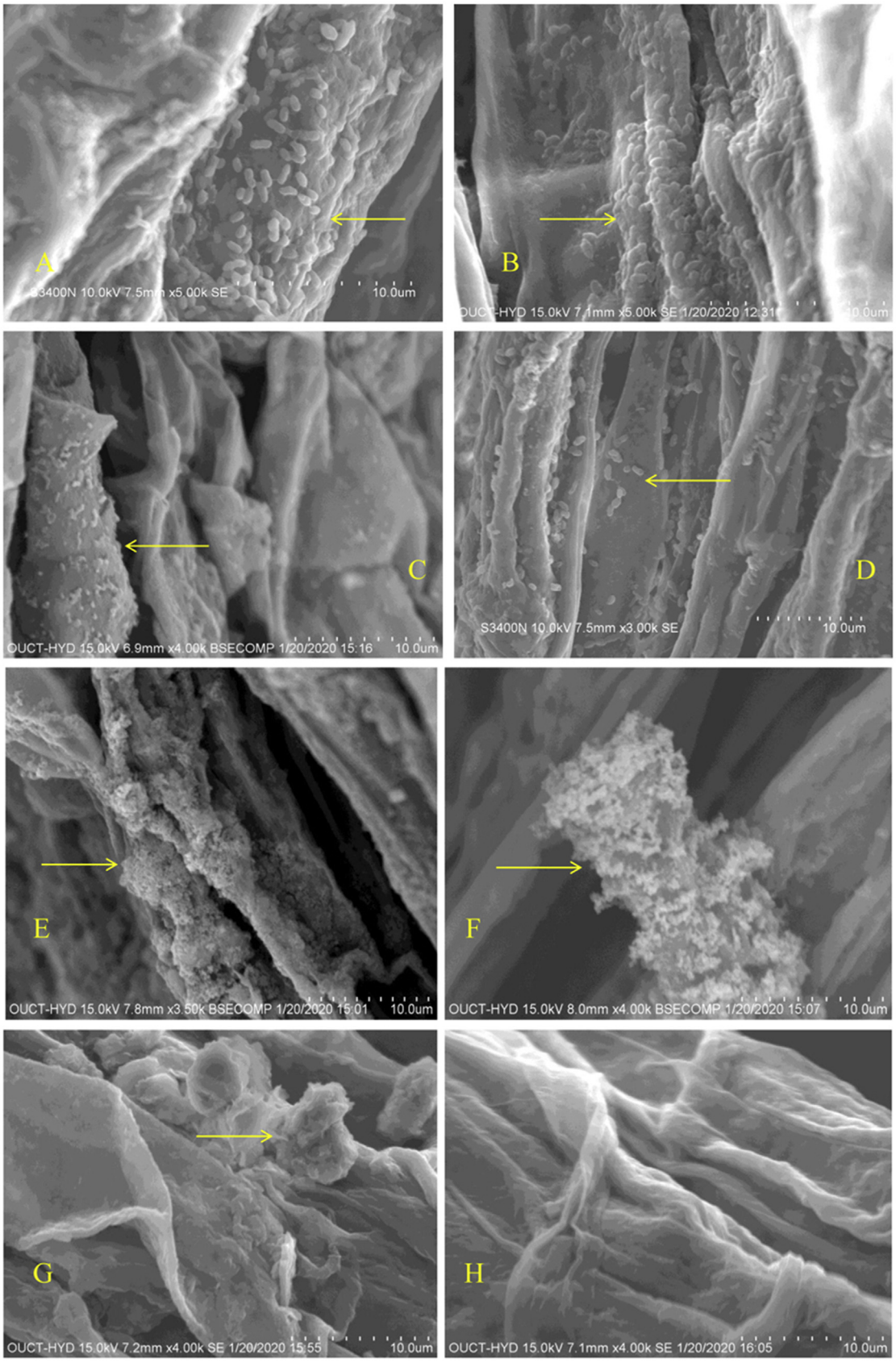


**Figure 4.** Scanning electron microscopy images of chickpea roots treated with isolated bacteria. (A) Chickpea roots colonized by bacterial strain IHSR, (B) IHRG, (C) IHAA, (D) IHGN-3, (E) IHCP-1, (F) IHCP-2, (G) consortium of IHGN-3+IHRG, (H) Chickpea root surface without any bacterial treatment (control).

**Table 4.** Effect of isolated bacteria and a co-inoculation of *Rhizobium tropici* with *Mesorhizobium* sp., on the growth of chickpea under greenhouse conditions after 30 days of treatment.

| Treatment | Plant height (mean) | No of Branches/ plant | Total chlorophyll (mg $l^{-1}$) | No. of nodules/ plant | Nodule dry weight (mg/plant) | Shoot dry weight (gm/plant) | Root dry weight (gm/plant) | Root volume ($cm^3$/plant) | Root surface area ($cm^2$/plant) |
|---|---|---|---|---|---|---|---|---|---|
| IHSR | 38.3 | 5.7 | 33.2 | 27 | 24 | 0.91 | 0.14 | 2.08 | 122 |
| IHRG | 38.3 | 5.3 | 28.0 | 27 | 20 | 0.89 | 0.14 | 1.94 | 121 |
| IHAA | 39.9 | 6.0 | 39.1 | 29 | 28 | 1.00 | 0.18 | 2.14 | 144 |
| IHGN-3 | 40.6 | 7.0 | 39.4 | 30 | 53 | 1.18 | 0.20 | 2.19 | 149 |
| IHCP-1 | 37.4 | 4.7 | 23.7 | 23 | 20 | 0.89 | 0.11 | 1.70 | 119 |
| IHCP-2 | 39.2 | 5.7 | 38.1 | 27 | 26 | 0.91 | 0.14 | 2.10 | 135 |
| IHRG + IHGN-3 | 42.6 | 7.0 | 49.3 | 36 | 99 | 1.47 | 0.38 | 2.40 | 155 |
| Untreated | 31.3 | 3.3 | 22.3 | 15 | 9 | 0.65 | 0.07 | 1.09 | 66 |
| Mean | 38.5 | 5.6 | 34.1 | 27 | 35 | 0.99 | 0.17 | 1.95 | 126 |
| SE± | 1.76* | 0.42*** | 0.36*** | 2.9** | 12.1** | 0.086*** | 0.024*** | 0.243* | 13.5* |
| LSD (5%) | 5.33 | 1.26 | 1.09 | 8.9 | 36.8 | 0.259 | 0.073 | 0.737 | 41.1 |
| CV% | 8 | 13 | 2 | 19 | 60 | 15 | 24 | 22 | 19 |

SE = Standard error; LSD = least significant differences; CV = coefficients of variation; * = statistically significant at 0.05, ** = statistically significant at 0.01, *** = statistically significant at 0.001.

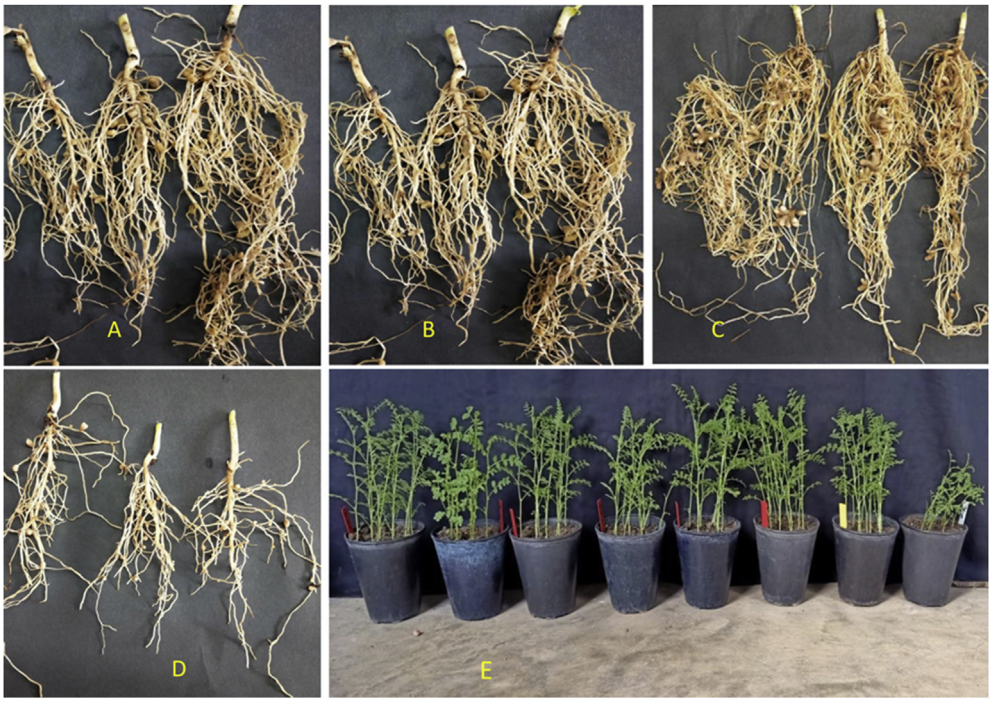


**Figure 5.** (A) Enhancement of nodules in chickpea plants treated with *Mesorhizobium* sp. strain IHGN-3 at 30 DAS, (B) Enhancement of nodules in chickpea plants treated with *Rhizobium multihospitium* strain IHAA at 30 DAS, (C) Enhancement of nodules in chickpea plants treated with consortium of *Rhizobium tropici* strain IHRG + *Mesorhizobium* sp. strain IHGN-3 at 30 days, (D) Un-inoculated chickpea plants, (E) 15 days old chickpea plants treated with root and stem nodule bacteria.

After nitrogen, phosphorus is the most limited nutrient for plant growth. It is plentiful in many agricultural soils but it is inert to plants due to low level of soluble phosphate. The potential of rhizobacteria to solubilize insoluble phosphates has attracted the attentions of agricultural microbiologists as it can improve plant development and yield by enhancing the availability of phosphorus to the plant. One of the alternative approaches in sustainable agriculture to fulfill the phosphate needs of plants is the use of phosphate solubilizing PGPR as bio-inoculants. In present investigation, all the six isolated bacteria solubilize tri-calcium phosphate in the range of 173–552 μg/ml (Table 2). The results are in line with the prior findings of Verma et al. (2013), Zhao et al. (2014); Imen et al. (2015); Singha et al. (2018); Gopalakrishnan et al. (2018); Tagele et al. (2019); Khalid et al. (2020), reported various species of *Rhizobium*, *Mesorhizobium*, *Burkholderia* are known to solubilize phosphate. Similarly, Sridevi and Mallaiah (2009), reported phosphate solubilization by *Rhizobium* sp. isolated from the stem nodules of *Sesbania sesban*. In present investigation, the isolated bacteria were able to solubilize rock phosphate under *in vitro* conditions. The results are in line with the findings of Halder et al. (1990) reported rock phosphate solubilization by various *Rhizobium* sp. The isolated bacteria were able to solubilize an insoluble zinc source ($ZnCO_3$) in tris- salt agar medium. Highest zinc solubilization was shown by the isolate IHCP-1(32 mm). *Burkholderia* sp. and *Rhizobium* sp. have the potential to solubilize insoluble forms of zinc (Khanghahi et al., 2018; Tagele et al., 2019).

**Table 5.** Effect of isolated bacteria and a co-inoculation of *Rhizobium tropici* with *Mesorhizobium* sp., on the growth of chickpea under greenhouse conditions after 45 days of treatment.

| Treatment | Plant height (cm) | No of branches/ plant | No. of nodules/ plant | Nodule dry weight (mg/plant) | Shoot dry weight (gm/plant) | Root dry weight (gm/plant) |
|---|---|---|---|---|---|---|
| *Rhizobium* sp., IHSR | 40.8 | 6 | 29 | 43 | 1.433 | 0.273 |
| *Rhizobium tropici* IHRG | 40.6 | 6 | 28 | 39 | 1.422 | 0.267 |
| *Rhizobium multihospitium* IHAA | 42.7 | 7 | 33 | 51 | 1.597 | 0.290 |
| *Mesorhizobium* sp., IHGN-3 | 43.2 | 7 | 33 | 60 | 1.846 | 0.340 |
| *Burkholderia cepacia* IHCP-1 | 39.8 | 5 | 23 | 31 | 1.378 | 0.267 |
| *Rhizobium pusense* IHCP-2 | 41.1 | 6 | 31 | 48 | 1.433 | 0.274 |
| *Rhizobium tropici* + *Mesorhizobium* sp. (IHRG + IHGN-3) | 43.5 | 7 | 35 | 68 | 1.867 | 0.372 |
| Untreated | 35.2 | 4 | 18 | 23 | 1.089 | 0.163 |
| Mean | 40.9 | 6 | 29 | 46 | 1.508 | 0.281 |
| SE± | 1.36* | 0.34*** | 1.3*** | 4.4*** | 0.078*** | 0.031** |
| LSD (5%) | 4.14 | 1.02 | 3.9 | 13.2 | 0.238 | 0.094 |
| CV% | 6 | 10 | 8 | 17 | 9 | 19 |

SE = Standard error; LSD = least significant differences; CV = coefficients of variation; * = statistically significant at 0.05, ** = statistically significant at 0.01, *** = statistically significant at 0.001.

**Table 6.** Effect of the isolated bacteria in solo and one consortium on chickpea (ICCV 2) under glasshouse conditions at crop maturity.

| Treatment | No of pods/ plant | Shoot weight (gm/plant) | Seed number/ plant |
|---|---|---|---|
| *Rhizobium* sp., IHSR | 4 | 2.56 | 4 |
| *Rhizobium tropici* IHRG | 4 | 2.56 | 3 |
| *Rhizobium multihospitium* IHAA | 5 | 2.61 | 4 |
| *Mesorhizobium* sp., IHGN-3 | 5 | 2.83 | 4 |
| *Burkholderia cepacia* IHCP-1 | 3 | 2.53 | 3 |
| *Rhizobium pusense* IHCP-2 | 5 | 2.58 | 4 |
| *Rhizobium tropici* + *Mesorhizobium* sp. (IHRG + IHGN-3) | 5 | 2.97 | 5 |
| Untreated | 3 | 1.75 | 2 |
| Mean | 4 | 2.55 | 4 |
| SE± | 0.3*** | 0.083*** | 0.3*** |
| LSD (5%) | 0.9 | 0.238 | 0.8 |
| CV% | 18 | 8 | 19 |

SE = Standard error; LSD = least significant differences; CV = coefficients of variation; *** = statistically significant at 0.001.

All the six bacterial strains were able to change the colour of the NFB medium from green to blue, that suggested their nitrogen fixing ability (Figure 2A). This is due to the increase in pH attributed to the formation of ammonia and nitrates from the atmospheric $N_2$ fixation. Nitrogen fixation is carried out by nitrogenase enzyme whose multiple subunits are encoded by the genes *nifH, nifD* and *nifK*. Of the three, *nifH* (encoding the nitrogenase reductase subunit) is the most widely sequenced marker gene used to identify nitrogen-fixing bacteria and archaea in various environments. Out of 6 bacterial isolates, four isolates (*Rhizobium* sp., strain IHSR, *Mesorhizobium* sp., strain IHGN-3, *Burkholderia cepacia* strain IHCP-1 and *Rhizobium pusense* strain IHCP-2) showed amplification with *nifH* gene (Figure 2B). The results are in accordance with the previous studies of Poly et al. (2001a); Chen et al. (2005); Gopalakrishnan et al. (2018); Khalid et al. (2020) reported the presence of *nifH* genes in various species of *Rhizobium* and *Burkholderia* isolated from nodules of different leguminous plants.

Scanning electron microscopy was employed to study the colonization ability of isolated bacterial strains with chickpea roots. Results revealed that all the bacterial strains in solo (IHSR, IHRG, IHAA, IHGN-3, IHCP-1, IHCP-2) and consortium (IHRG + IHGN-3) showed significant level of colonization (Figure 4). Bacterial colonization to the root surfaces is an essential, early and mandatory feature of plant-microbe interaction in the rhizosphere for plant growth and development as well provides protection to the plants against various soil pathogens and abiotic stresses (Pagnani et al., 2018). The adherence of the bacteria to plant surfaces starts with attraction by seedling root exudates including various phenolic substances, amino acids, sugars and organic acids (Begonia and Kremer, 1994). The capability of rhizobacteria to migrate chemotactically to substances emitted by seedling roots of chickpea may lead to increased bacterial colonization of roots. Observation at the root morphology including nodule number, nodule weight, root weight and other agronomical traits along with the SEM micrograph clearly indicate that the PGP effects of the isolated bacteria had been caused by successful colonization of the inoculated chickpea roots.

In present investigation, seed inoculation of chickpea with *Rhizobium* sp., *Rhizobium tropici*, *Rhizobium multihospitium*, *Mesorhizobium* sp., *Burkholderia cepacia*, *Rhizobium pusense* and combination of *Rhizobium tropici* with *Mesorhizobium* sp., resulted significant increase in plant height, number of branches, shoot dry weight, total chlorophyll content, root dry weight, root volume and root surface area at 30 and 45 days after sowing over the un-inoculated control plants (Table 4, Table 5). Several authors

**Table 7.** Effect of the isolated bacteria in solo and one consortium on nutrient traits of chickpea (ICCV 2) under glasshouse conditions at crop maturity.

| Treatment | Total N% | Total P% | Total K% |
|---|---|---|---|
| *Rhizobium* sp., IHSR | 2.65 | 0.28 | 2.39 |
| *Rhizobium tropici* IHRG | 2.63 | 0.29 | 2.31 |
| *Rhizobium multihospitium* IHAA | 2.76 | 0.32 | 2.56 |
| *Mesorhizobium* sp., IHGN-3 | 2.85 | 0.33 | 2.60 |
| *Burkholderia cepacia* IHCP-1 | 2.50 | 0.27 | 2.31 |
| *Rhizobium pusense* IHCP-2 | 2.74 | 0.31 | 2.54 |
| *Rhizobium tropici* + *Mesorhizobium* sp. (IHRG + IHGN-3) | 2.89 | 0.33 | 2.67 |
| Untreated | 2.54 | 0.28 | 2.26 |
| Mean | 2.70 | 0.30 | 2.46 |
| SE± | 0.059* | 0.011* | 0.046** |
| LSD (5%) | 0.197 | 0.038 | 0.154 |
| CV% | 3 | 5 | 3 |

SE = Standard error; LSD = least significant differences; CV = coefficients of variation * = statistically significant at 0.05, ** = statistically significant at 0.01.

**Table 8.** Comparison of symbiotic parameters (nodule number and nodule dry weight) by various species of *Rhizobium*, *Mesorhizobium* and the consortia with the present results.

| Microbial inoculant | No of nodules/ plant | Nodule dry weight (mg plant$^{-1}$) | Reference | Present study | | |
|---|---|---|---|---|---|---|
| | | | | Microbial inoculant | No of nodules/ plant | Nodule dry weight (mg plant$^{-1}$) |
| *Rhizobium pusense* | 25 | 27 | Gopalakrishnan et al. (2018) | *Rhizobium* sp. strain IHSR | 27 | 24 |
| *Paraburkholderia kururiensis* | 34 | 37 | | | | |
| *Stenotrophomonas maltophilia* | 38 | 31 | | | | |
| *Mesorhizobium* sp. | 35 | 86 | Verma et al. (2013) | *Rhizobium tropici* strain IHRG | 27 | 20 |
| *Mesorhizobium* sp. + *A. chroococcum* | 41 | 103 | | | | |
| *Mesorhizobium* sp. + *B. megaterium* | 46 | 94 | | | | |
| *Mesorhizobium* sp. + *P. aeruginosa* | 62 | 132 | | | | |
| *Rhizobium* sp. | 135 | 157 | Rudresh et al. (2005) | *Rhizobium multihospitium* strain IHAA | 29 | 28 |
| *Bacillus megaterium* sub sp. *phospaticum* + *T. harzianum* + *Rhizobium* sp. | 144 | 178 | | | | |
| *R. leguminosarum* | 35 | 86 | Yadav and Verma (2014) | *Mesorhizobium* sp.IHGN-3 | 30 | 53 |
| *R. leguminosarum* + *A. chroococcum* | 41 | 103 | | | | |
| *R. leguminosarum* + *B. megaterium* | 46 | 94 | | | | |
| *R. leguminosarum* + *P. aeruginosa* | 62 | 132 | | | | |
| *Rhizobium* | 19 | 67 | Tagore et al. (2014) | *Burkholderia cepacia* strain IHCP-1 | 23 | 20 |
| PSB | 10 | 55 | | | | |
| *Rhizobium* + PSB | 21 | 95 | | | | |
| *Mesorhizobium* strain Ca181 | 49 | 1312 | Sindhu et al. (2002) | *Rhizobium pusense* strain IHCP-2 | 27 | 26 |
| Ca181 + *Pseudomonas* strains MRS13 | 71 | 1612 | | | | |
| Ca181 + *Pseudomonas* strains CRS55b | 56 | 1482 | | | | |
| Ca181 + *Pseudomonas* strains CRS68 | 58 | 1420 | | | | |
| | | | | *Rhizobium tropici* strain IHRG + *Mesorhizobium* sp. strain IHGN-3 | 36 | 99 |

have reported the beneficial effects of plant growth promoting *Rhizobium* sp., *Mesorhizobium* sp., on chickpea (Rudresh et al., 2005; Akhtar and Siddiqui, 2008; Elkoca et al., 2008; Singh et al., 2011; Yadav and Verma, 2014).

Comparisons of symbiotic parameters (nodule number, nodule weight) by various species of *Rhizobium*, *Mesorhizobium* and the consortia with the present results are represented in Table 8. Gopalakrishnan et al. (2018), reported the isolation of *Rhizobium pusense*, *Paraburkholderia kururiensis* and *Stenotrophomonas maltophilia* from the root nodules of chickpea and found enhancement of nodule number and nodule dry weight, when the seeds of ICCV 2 variety of chickpea were treated by these bacteria mentioned above under greenhouse conditions. Verma et al. (2013) reported seed inoculation of chickpea with *Mesorhizobium* sp., and co-inoculation of *Mesorhizobium* sp. with PGPR bacteria such as *A. chroococcum*, *B. megaterium* and *P. aeruginosa* significantly increased the nodule number (41%, 46% and 62%), and nodule dry weight over control under greenhouse conditions at 70 DAS. Rudresh et al. (2005), reported treatment of chickpea seeds with *Rhizobium* sp., and co-inoculation of *Rhizobium* sp., with phosphate solubilizing *Bacillus megaterium* sub sp. *phospaticum* and biocontrol fungus *Trichoderma* sp., significantly increased the nodule number and nodule dry weight over control under greenhouse conditions at 45 days after sowing. Similarly, Yadav and Verma (2014), studied the effect of indigenous PGPR and *R. leguminosarum* (*Cicer* sp.) on growth of chickpea under greenhouse conditions. Seed treatment of chickpea with *R. leguminosarum* strain alone and co-inoculation with *A. chroococcum*, *B. megaterium* and *P. aeruginosa* significantly increased the nodule number and nodule dry weight over control under greenhouse conditions at 70 DAS. Tagore et al. (2014) studied the effect of *Rhizobium* and phosphate solubilizing bacterial (PSB) inoculants on symbiotic traits in chickpea. Co-inoculation of *Rhizobium* and PSB recorded significantly higher nodule number and dry weight than *Rhizobium* and PSB alone at 35 days after sowing under field conditions. In our studies all the isolated bacteria were found to enhance the nodule formation in the chickpea plants under greenhouse conditions at 30 and 45 DAS. The highest number of nodules and nodule biomass was observed in the chickpea plants treated with *Rhizobium tropici* strain IHRG in combination with *Mesorhizobium* sp. strain IHGN-3 followed by *Mesorhizobium* sp. strain IHGN-3, *Rhizobium multihospitium* strain IHAA, *Rhizobium pusense* strain IHCP-2, *Rhizobium* sp. strain IHSR, *Rhizobium tropici* strain IHRG and *Burkholderia cepacia* strain IHCP-1 (Table 8).

It has been observed that at crop maturity stage, chickpea plants treated with isolated bacteria separately enhanced pod number, seed number and total NPK, compared to the control plants (Table 6, Table 7). These findings are consistent with prior studies carried out by many researchers (Akhtar and Siddiqui, 2009; Gopalakrishnan et al., 2017, 2018). Increase in the concentration of N in shoots of chickpea plants treated with diazotrophic bacteria over un-inoculated control was reported by Wani et al. (2007). Similarly Akhtar and Siddiqui (2008); Verma et al. (2013), reported significant increase in the uptake of N, P and K by chickpea plants treated with *Mesorhizobium* sp. *Pseudomonas* sp. and *Rhizobium* sp. over un-inoculated control. Increased uptake of NPK in chickpea treated with the different rhizobial isolates in this study may be specifically due to their nitrogen fixing ability, phosphate and potassium solubilization. It may also be noted that all the six bacterial isolates possessed different PGP traits which may be responsible for enhanced plant growth, yield and uptake of nutrients.

## 5. Conclusion

During last decade, the production of chickpea in India is declined due to meagre native soil rhizobial inhabitants or ineffective BNF. Increase in chickpea production encounters considerable limitations in terms of inadequate native soil rhizobial populations, harsh climate, poor soil, inadequate fertilizers, pathogens etc. Hence development of environmentally friendly crop production process with inoculation of nitrogen fixing plant growth promoting bacteria will lead to sustainability in agriculture. Present study concludes that bacteria associated with root and stem nodules enhanced chickpea plant height, number of branches,

total chlorophyll, nodule number, nodule weight, shoot weight, root weight, pod number, seed number and NPK uptake when compared to uninoculated control plants. This study also explains that co-inoculation of *Mesorhizobium* sp. strain IHGN-3 with *Rhizobium tropici* strain IHRG can be a potential bioinoculant for chickpea. In future the isolates could be subjected to field trails to evaluate their stability under different environmental conditions.

## Declarations

### *Author contribution statement*

Mohammad Imran Mir: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

B. Kiran Kumar: Analyzed and interpreted the data; Wrote the paper.

Srinivas Vadlamudi: Analyzed and interpreted the data; Wrote the paper.

Bee Hameeda: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Gopalakrishnan S: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

### *Funding statement*

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

### *Data availability statement*

Data will be made available on request.

### *Declaration of interests statement*

The authors declare no conflict of interest.

### *Additional information*

No additional information is available for this paper.

## References

Ahemad, M., Kibret, M., 2014. Mechanisms and applications of plant growth-promoting rhizobacteria: current perspective. J. King Saud. Univ. Sci. 26, 1–20.

Ahmad, I., Zaib, S., 2020. Mighty microbes: plant growth promoting microbes in soil health and sustainable agriculture. In: Soil Health. Springer, Cham, pp. 243–264.

Aisa, H.A., Gao, Y., Yili, A., Ma, Q., Cheng, Z., 2019. Beneficial role of chickpea (*Cicer arietinum* L.) functional factors in the intervention of metabolic syndrome and diabetes mellitus. In: Bioactive Food as Dietary Interventions for Diabete. Academic Press, pp. 615–627.

Akhtar, M.S., Siddiqui, Z.A., 2008. Biocontrol of a root-rot disease complex of chickpea by *Glomus intraradices, Rhizobium* sp. and *Pseudomonas straita*. Crop Protect. 27, 410–417.

Akhtar, M., Siddiqui, Z., 2009. Effects of phosphate solubilizing microorganisms and *Rhizobium* sp. on the growth, nodulation, yield and root-rot disease complex of chickpea under field condition. Afr. J. Biotechnol. 8, 3489–3496.

Alschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Angus, A.A., Lee, A., Lum, M.R., Shehayeb, M., Hessabi, R., Fujishige, N.A., Yerrapragada, S., Kano, S., Song, N., Yang, P., Estrada de los Santos, P., de Faria, S.M., Dakora, F.D., Weinstock, G., Hirsch, A.M., 2013. Nodulation and effective nitrogen fixation of *Macroptilium atropurpureum* (siratro) by *Burkholderia tuberum*, a nodulating and plant growth promoting beta-proteobacterium, are influenced by environmental factors. Plant Soil 369, 543–562.

Backer, R., Rokem, J.S., Ilangumaran, G., Lamont, J., Praslickova, D., Ricci, E., Smith, D.L., 2018. Plant growth-promoting rhizobacteria: context, mechanisms of action, and roadmap to commercialization of biostimulants for sustainable agriculture. Front. Plant Sci. 9, 1473.

Bakker, A.W., Schipperes, B., 1987. Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomoas* sp. mediated plant growth stimulation. Soil Biol. Biochem. 19, 451–457.

Baldani, J.I., Reis, V.M., Videira, S.S., Boddey, L.H., Baldani, V.L.D., 2014. The art of isolating nitrogen-fixing bacteria from non-leguminous plants using N-free semi-solid media: a practical guide for microbiologists. Plant Soil 384, 413–431.

Begonia, M.F.T., Kremer, R.J., 1994. Chemotaxis of deleterious rhizobacteria to velvet leaf (*Abutilon theophrasti* Medik.) seeds and seedlings. FEMS Microbiol. Ecol. 15, 227–236.

Benjelloun, I., Thami Alami, I., Douira, A., Udupa, S.M., 2019. Phenotypic and genotypic diversity among symbiotic and non-symbiotic bacteria present in chickpea nodules in Morocco. Front. Microbiol. 10, 1885.

Bhattacharya, A., Chandra, S., Barik, S., 2009. Lipase and protease producing microbes from the environment of sugar beet field. Ind. J. Agric. Biochem. 22, 26–30.

Chen, W.M., De Faria, S.M., Straliotto, R., Pitard, R.M., Simoes-Araujo, J.L., Chou, J.H., James, E.K., 2005. Proof that *Burkholderia* strains form effective symbioses with legumes: a study of novel Mimosa-nodulating strains from South America. Appl. Environ. Microbiol. 71, 7461–7471.

Datta, B., Chakrabartty, P.K., 2014. Siderophore biosynthesis genes of *Rhizobium* sp. isolated from *Cicer arietinum* L. 3 Biotech 4, 391–401.

Dobbelaere, S., Vanderleyden, J., Okon, Y., 2003. Plant growth-promoting effects of diazotrophs in the rhizosphere. Crit. Rev. Plant Sci. 22, 107–149.

Dutta, S., Podile, A.R., 2010. Plant growth promoting rhizobacteria (PGPR): the bugs to debug the root zone. Crit. Rev. Microbiol. 36, 232–244.

Elkoca, E., Kantar, F., Sahin, F., 2008. Influence of nitrogen fixing and phosphorus solubilizing bacteria on the nodulation, plant growth, and yield of chickpea. J. Plant Nutr. 31, 157–171.

Figueiredo, M.D.V.B., Santo Mergulhão, A.C.D.E., Sobral, J.K., Junior, M.D.A.L., de Araújo, A.S.F., 2013. Biological nitrogen fixation: importance, associated diversity, and estimates. In: Plant Microbe Symbiosis: Fundamentals and Advances. Springer, New Delhi, pp. 267–289.

Fiske, C.H., Subbarow, Y., 1925. The colorimetric determination of phosphorus. J. Biol. Chem. 66, 375–400.

Flowers, T.J., Gaur, P.M., Gowda, C.L., Krishnamurthy, L., Samineni, S., Siddique, K.H., Turner, N.C., Vincent, V., Varshney, R.K., Colmer, T.D., 2010. Salt sensitivity in chickpea. Plant Cell Environ. 33, 490–509.

Foster-Powell, K., Holt, S.H.A., Brand-Miller, J.C., 2002. International table of glycemic index and glycemic load values. Am. J. Clin. Nutr. 76, 5–56.

Gopalakrishnan, S., Srinivas, V., Prakash, B., Sathya, A., Vijayabharathi, R., 2015. Plant growth-promoting traits of *Pseudomonas geniculata* isolated from chickpea nodules. 3 Biotech 5, 653–661.

Gopalakrishnan, S., Srinivas, V., Alekhya, G., Prakash, B., 2015a. Effect of plant growth promoting *Streptomyces* sp. on growth promotion and grain yield in chickpea (*Cicer arietinum* L.). 3 Biotech. 5, 799–806.

Gopalakrishnan, S., Sathya, A., Vijayabharathi, R., Varshney, R.K., Gowda, C.L., Krishnamurthy, L., 2015b. Plant growth promoting rhizobia: challenges and opportunities. 3 Biotech 5, 355–377.

Gopalakrishnan, S., Srinivas, V., Samineni, S., 2017. Nitrogen fixation, plant growth and yield enhancements by diazotrophic growth-promoting bacteria in two cultivars of chickpea (*Cicer arietinum* L.). Biocatal. Agric. Biotechnol. 11, 116–123.

Gopalakrishnan, S., Srinivas, V., Vemula, A., Samineni, S., Rathore, A., 2018. Influence of diazotrophic bacteria on nodulation, nitrogen fixation, growth promotion and yield traits in five cultivars of chickpea. Biocatal. Agric. Biotech. 15, 35–42.

Gordon, S.A., Weber, R.P., 1951. Colorimetric estimation of indole acetic acid. Plant Physiol. 26, 192–195.

Goteti, P.K., Leo, D.A.E., Desai, S., Mir Hassan Ahmed, S., 2013. Prospective zinc solubilising bacteria for enhanced nutrient uptake and growth promotion in maize (*Zea mays* L.). Internet J. Microbiol. 2013, 1–7.

Gyaneshwar, P., Naresh Kumar, G., Parekh, L.J., 1998. Effect of buffering on the phosphate-solubilizing ability of microorganisms. World J. Microbiol. Biotechnol. 14, 669–673.

Halder, A.K., Mishra, A.K., Bhattacharyya, P., Chakrabartty, P.K., 1990. Solubilization of rock phosphate by *rhizobium* and *Bradyrhizobium*. J. Gen. Appl. Microbiol. 36, 81–92.

Hamid, B., Zaman, M., Farooq, S., Fatima, S., Sayyed, R.Z., Baba, Z.A., Suriani, N.L., 2021. Bacterial plant biostimulants: a sustainable way towards improving growth, productivity, and health of crops. Sustainability 13, 2856.

Hirano, S., Nagao, N., 1988. An improved method for the preparation of colloidal chitin by using methanesulfonic acid. Agric. Biol. Chem. 52, 2111–2112.

Hiscox, J.D., Israelstam, G.F.A., 1979. A method for the extraction of chlorophyll from leaf tissue without maceration. Can. J. Bot. 57, 1332–1334.

Hu, X., Chen, J., Guo, J., 2006. Two phosphate and potassium solubilizing bacteria isolated from Tianmu Mountain, Zhejiang, China. World J. Microbiol. Biotechnol. 22, 983–990.

Igiehon, N.O., Babalola, O.O., Aremu, B.R., 2019. Genomic insights into plant growth promoting rhizobia capable of enhancing soybean germination under drought stress. BMC Microbiol. 19, 1–22.

Imen, H., Neila, A., Adnane, B., Manel, B., Mabrouk, Y., Saidi, M., Bouaziz, S., 2015. Inoculation with phosphate solubilizing *Mesorhizobium* strains improves the performance of chickpea (*Cicer arietinum* L.) under phosphorus deficiency. J. Plant Nutr. 38, 1656–1671.

Jannouraa, R., Bruns, C., Joergensena, R.G., 2013. Organic fertilizer effects on pea yield, nutrient uptake, microbial root colonization and soil microbial biomass indices in organic farming systems. Eur. J. Agron. 49, 32–41.

Jukanti, A.K., Gaur, P.M., Gowda, C.L.L., Chibbar, R.N., 2012. Nutritional quality and health benefits of chickpea (*Cicer arietinum* L.): a review. Br. J. Nutr. 108, S11–S26.

Kasana, R.C., Salwan, R., Dhar, H., Dutt, S., Gulati, A., 2008. A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. Curr. Microbiol. 57, 503–507.

Kavamura, V.N., Santos, S.N., da Silva, J.L., Parma, M.M., Ávila, L.A., Visconti, A., de Melo, I.S., 2013. Screening of Brazilian cacti rhizobacteria for plant growth promotion under drought. Microbiol. Res. 168, 183–191.

Khalid, R., Zhang, X.X., Hayat, R., Ahmed, M., 2020. Molecular characteristics of rhizobia isolated from *Arachis hypogaea* grown under stress environment. Sustainability 12, 6259.

Khanghahi, M.Y., Ricciuti, P., Allegretta, I., Terzano, R., Crecchio, C., 2018. Solubilization of insoluble zinc compounds by zinc solubilizing bacteria (ZSB) and optimization of their growth conditions. Environ. Sci. Pollut. Res. 25, 25862–25868.

Kumari, P., Meena, M., Upadhyay, R.S., 2018. Characterization of plant growth promoting rhizobacteria (PGPR) isolated from the rhizosphere of *Vigna radiata* (mung bean). Biocatal. Agric. Biotechnol. 16, 155–162.

Martinez-Hidalgo, P., Hirsch, A.M., 2017. The nodule microbiome: $N_2$-fixing rhizobia do not live alone. Phytobiomes J. 1, 70–82.

Menéndez, E., Pérez-Yépez, J., Hernández, M., Rodríguez-Pérez, A., Velázquez, E., León-Barrios, M., 2020. Plant growth promotion abilities of phylogenetically diverse *Mesorhizobium* strains: effect in the root colonization and development of tomato seedlings. Microorganisms 8, 412.

Nautiyal, C.S., 1999. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol. Lett. 170, 265–270.

Normand, P., Cournoyer, B., Simonet, P., Nazaret, S., 1992. Analysis of a ribosomal RNA operon in the actinomycete *Frankia*. Gene 111, 119–124.

Pagnani, G., Pellegrini, M., Galieni, A., D'Egidio, S., Matteucci, F., Ricci, A., Del Gallo, M., 2018. Plant growth-promoting rhizobacteria (PGPR) in *Cannabis sativa* 'Finola'cultivation: an alternative fertilization strategy to improve plant growth and quality characteristics. Ind. Crop. Prod. 123, 75–83.

Penrose, D.M., Glick, B.R., 2003. Methods for isolating and characterizing ACC deaminase- containing plant growth-promoting rhizobacteria. Physiol. Plantarum 118, 10–15.

Poly, F., Monrozier, L.J., Bally, R., 2001a. Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in soil. Res. Microbiol. 152, 95–103.

Poly, F., Ranjard, L., Nazaret, S., Gourbière, F., Monrozier, L.J., 2001b. Comparison of *nifH* gene pools in soils and soil microenvironments with contrasting properties. Appl. Environ. Microbiol. 67, 2255–2262.

Rasool, A., Mir, M.I., Zulfajri, M., Hanafiah, M.M., Unnisa, S.A., Mahboob, M., 2021. Plant growth promoting and antifungal asset of indigenous rhizobacteria secluded from saffron (*Crocus sativus* L.) rhizosphere. Microb. Pathog. 150, 104734.

Rudresh, D.L., Shivaprakash, M.K., Prasad, R.D., 2005. Effect of combined application of *Rhizobium*, phosphate solubilizing bacterium and *Trichoderma* sp. on growth, nutrient uptake and yield of chickpea (*Cicer aritenium* L.). Appl. Soil Ecol. 28, 139–146.

Sahrawat, K.L., Kumar, G.R., Murthy, K.V.S., 2002. Sulfuric acid–selenium digestion for multi-element analysis in a single plant digests. Commun. Soil Sci. Plant Anal. 33, 3757–3765.

Saidi, S., Chebil, S., Gtari, M., Mhamdi, R., 2013. Characterization of root-nodule bacteria isolated from *Vicia faba* and selection of plant growth-promoting traits. World J. Microbiol. Biotechnol. 29, 1099–1106.

Saravanan, V.S., Kalaiarasan, P., Madhaiyan, M., Thangaraju, M., 2007. Solubilization of insoluble zinc compounds by *Gluconacetobacter diazotrophicus* and the detrimental action of zinc ion ($Zn^{2+}$) and zinc chelates on root knot nematode *Meloidogyne incognita*. Lett. Appl. Microbiol. 44, 235–241.

Schwyn, B., Neilands, J.B., 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. 160, 47–56.

Shahid, M., Khan, M.S., Syed, A., Marraiki, N., Elgorban, A.M., 2021. *Mesorhizobium ciceri* as biological tool for improving physiological, biochemical and antioxidant state of *Cicer aritienum* (L.) under fungicide stress. Sci. Rep. 11, 1–18.

Sindhu, S.S., Suneja, S., Goel, A.K., Parmar, N., Dadarwal, K.R., 2002. Plant growth promoting effects of *Pseudomonas* sp. on co-inoculation with *Mesorhizobium* sp. Cicer strain under sterile and "wilt sick" soil conditions. Appl. Soil Ecol. 19, 57–64.

Singh, P.P., Shin, Y.C., Park, C.S., Chung, Y.R., 1999. Biological control of *Fusarium* wilts of cucumber by chitinolytic bacteria. Phytopathology 89, 92–99.

Singh, G., Sekhon, H.S., Sharma, P., 2011. Effect of irrigation and biofertilizer on water use, nodulation, growth and yield of chickpea (*Cicer arietinum* L.). Arch. Agron Soil Sci. 57, 715–726.

Singha, B., Mazumder, P.B., Pandey, P., 2018. Characterization of plant growth promoting Rhizobia from root nodule of two legume species cultivated in Assam, India. Proc. Natl. Acad. Sci. India B Biol. Sci. 88, 1007–1016.

Solanki, M.K., Wang, Z., Wang, F.Y., Li, C.N., Lan, T.J., Singh, R.K., Li, Y.R., 2017. Intercropping in sugarcane cultivation influenced the soil properties and enhanced the diversity of vital diazotrophic bacteria. Sugar Tech. 19, 136–147.

Sridevi, M., Mallaiah, K.V., 2009. Phosphate solubilization by *Rhizobium* strains. Indian J. Microbiol. 49, 98–102.

Subramanian, P., Kim, K., Krishnamoorthy, R., Sundaram, S., Sa, T., 2015. Endophytic bacteria improve nodule function and plant nitrogen in soybean on co-inoculation with *Bradyrhizobium japonicum* MN110. Plant Growth Regul. 76, 327–332.

Tagele, S.B., Kim, S.W., Lee, H.G., Lee, Y.S., 2019. Potential of novel sequence type of *Burkholderia Cenocepacia* for biological control of root rot of maize (*Zea mays* L.) caused by *Fusarium temperatum*. Int. J. Mol. Sci. 20, 1005.

Tagore, G.S., Namdeo, S.L., Sharma, S.K., Kumar, N., 2014. Effect of *Rhizobium* and phosphate solubilizing bacterial inoculants on symbiotic traits, nodule leghemoglobin, and yield of chickpea genotypes. Int. J. Agron. 581627, 1–8.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.

Tariq, M., Hameed, S., Yasmeen, T., Zahid, M., Zafar, M., 2014. Molecular characterization and identification of plant growth promoting endophytic bacteria isolated from the root nodules of pea (*Pisum sativum* L.). World J. Microbiol. Biotechnol. 30, 719–725.

Verma, J.P., Yadav, J., Tiwaric, K.N., Kumarb, A., 2013. Effect of indigenous *Mesorhizobium* sp. and plant growth promoting rhizobacteria on yields and nutrients uptake of chickpea (*Cicer arietinum* L.) under sustainable agriculture. Ecol. Eng. 51, 282–286.

Vincent, J.M., 1970. A Manual for the Practical Study of Root -nodule Bacteria, vol. 348. Blackwell Scientific Publication, Oxford.

Wani, P.A., Khan, M.S., Zaidi, A., 2007. Synergistic effect of the inoculation with nitrogen-fixing and phosphate-solubilizing rhizobacteria on performance of field-grown chickpea. J. Plant Nutr. Soil Sci. 170, 283–287.

Wei, G., Fan, L., Zhu, W., Fu, Y., Yu, J., Tang, M., 2009. Isolation and characterization of the heavy metal resistant bacteria CCNWRS33-2 isolated from root nodule of *Lespedeza cuneata* in gold mine tailings in China. J. Hazard Mater. 162, 50–56.

Wheal, M.S., Fowles, T.O., Palmer, L.T., 2011. A cost-effective acid digestion method using closed polypropylene tubes for inductively coupled plasma optical emission spectrometry (ICP-OES) analysis of plant essential elements. Anal. Methods 3, 2854–2863.

Williams, M.N.V., Signer, E.R., 1990. Metabolism of tryptophan and tryptophan analogs by *Rhizobium meliloti*. Plant Physiol. 92, 1009–1013.

Wolde-Meskel, E., van Heerwaarden, J., Abdulkadir, B., Kassa, S., Aliyi, I., Degefu, T., Giller, K.E., 2018. Additive yield response of chickpea (*Cicer arietinum* L.) to rhizobium inoculation and phosphorus fertilizer across smallholder farms in Ethiopia. Agric. Ecosyst. Environ. 261, 144–152.

Yadav, J., Verma, J.P., 2014. Effect of seed inoculation with indigenous *Rhizobium* and plant growth promoting rhizobacteria on nutrients uptake and yields of chickpea (*Cicer arietinum* L.). Eur. J. Soil Biol. 63, 70–77.

Zakhia, F., Jeder, H., Domergue, O., Willems, A., Cleyet-Marel, J.C., Gillis, M., De Lajudie, P., 2004. Characterisation of wild legume nodulating bacteria (LNB) in the infra-arid zone of Tunisia. Syst. Appl. Microbiol. 27, 380–395.

Zhao, K., Penttinen, P., Zhang, X., Ao, Z., Liu, M., Yu, X., Chen, Q., 2014. Maize rhizosphere in Sichuan, China, hosts plant growth-promoting *Burkholderia cepacia* with phosphate solubilizing and antifungal activities. Microbiol. Res. 169, 76–82.