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Nitrogen fixation, plant growth and yield enhancements by diazotrophic growth-promoting bacteria in two cultivars of chickpea (*Cicer arietinum* L.)

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ABSTRACT

A total of 11 rhizobia-like-bacteria, isolated from the nodules of chickpea, were characterized for nitrogen fixation potential and growth promoting ability. All the isolates nodulated chickpea, amplified *nifH* gene and fixed nitrogen but, four isolates (ICKM-9, ICKM-15, ICS-31 and ICS-32) were found to fix nitrogen more than 4.0 nmoles of ethylene g^{-1} fresh weight of nodules h^{-1}. Under field conditions, seeds of chickpea varieties ICCV 2 and JG 11, when treated with the bacteria, enhanced the nodule number (up to 46% and 46%), nodule mass (up to 76% and 50%), shoot mass (up to 21% and 42%) and grain yield (up to 27% and 25%), respectively, over the un-inoculated control. At the harvest, organic carbon (up to 7% and 24%), total nitrogen (up to 11% and 19%) and available phosphorous (up to 14% and 29%) were found enhanced, respectively, in the rhizosphere of ICCV-2 and JG-11 treated with bacteria over the un-inoculated control. All the isolates produced plant growth-promoting traits including indole acetic acid, β-1,3-glucanase, hydro cyanic acid (except ICKM-17 and ICS-31) and siderophore (except ICS-31). The 16 S rDNA gene sequences of bacterial isolates of ICKM-1, ICKM-4, ICKM-7, ICKM-9, ICKM-12, ICKM-14, ICKM-15, ICKM-17, ICS-30, ICS-31 and ICS-32 showed maximum identity with *Pantoea dispersa*, *Chryseobacterium indologenes*, *Pseudomonas geniculata*, *Stenotrophomonas pavanii*, *P. geniculata*, *P. geniculata*, *Stenotrophomonas maltophilia*, *Chryseobacterium* sp., *P. geniculata*, *Chryseobacterium indologenes* and *Stenotrophomonas acidaminiphila*, respectively. This study indicates nodule-associated bacteria could be a valuable pool for improving nitrogen fixation and crop yields in chickpea.

1. Introduction

Chickpea (*Cicer arietinum* L.) is the second most important pulse crop grown around the world. It is grown in more than 55 countries on an area of about 14 million hectares during 2014 (FAOSTAT, 2017). India is the largest chickpea producing country with 71% of global chickpea production. Chickpea grain is mainly used as food because of its high protein (12.4—31.5%), carbohydrate (52.4—70.9%), minerals (such as phosphorous, calcium, magnesium, iron and zinc) and β-carotene contents (Awasthi et al., 1991). Global yield of chickpea has been relatively stagnant (0.5 and 1.0 t ha^{-1}) since last five decades (FAOSTAT, 2017) in spite of adopting conventional breeding and molecular approaches and extensively using synthetic fertilizers, pesticides and supplements. Productivity of chickpea may be considerably improved if the adverse effects of abiotic (climate and soil) and biotic (insect pests and pathogens) stresses are reduced. With the ever increasing cost of synthetic pesticides and fertilizers and concern over environmental pollution and/or degradation, there has been a resurgence of interest to develop eco-friendly methods of crop production and protection. The environment-friendly options include the use of plant growth-promoting (PGP) microbes, biocontrol potential microbes, animal wastes, botanicals and crop residues which serve as an alternative to synthetic fertilizers and pesticides (Rupela et al., 2005).

Rhizobacteria that benefit plant growth by producing plant growth regulators, enhancing the nutrient(s) availability, inducing root exudation and controlling phytopathogens are termed as PGP bacteria (Kloepper and Schroth, 1978). PGP bacteria actively colonize plant roots and increase plant growth and yield. Further, indigenous PGP bacteria help in substantially reducing the chemical inputs as they can easily acclimatize to the natural conditions and thus enhance the plant-microbe interactions (Verma et al., 2013). PGP bacteria including species of *Streptomyces*, *Pseudomonas*, *Bacillus*, *Azotobacter*, *Azospirillum*, *Acinetobacter*, *Enterobacter*, *Serratia* and *Brevibacterium* have been reported to enhance plant growth and yield in chickpea (Weller et al., 2002; Singh et al., 2008; Soe et al., 2010; Gopalakrishnan et al., 2015a, 2016; Sreevidya and Gopalakrishnan, 2017). The mechanisms of PGP bacteria promoting plant growth and yield include nitrogen fixation, ability to synthesize molecules such as indole acetic acid, siderophores,

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organic acids and exopolysaccharides and solubilize phosphorus and other nutrients to enhance micronutrient uptake (Ahmad et al., 2008; Gopalakrishnan et al., 2014, 2016). Actively growing PGP bacteria are commonly found in the rhizosphere and rhizoplane as plants release root exudates that contains sugars, growth regulators, amino acids, organic acids, flavonoids, enzymes, fatty acids and vitamins (Uren, 2000). The major objective of this study was to identify diazotrophic PGP bacteria from the nodules of chickpea, which promote plant growth and enhance chickpea yield.

2. Materials and methods

2.1. Root nodule bacteria isolation and preservation

Healthy root nodules, collected from ICCV 2 and JG 11 varieties of chickpea grown at International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, India during December 2014, were surface sterilized with 2.5% sodium hypochlorite for 2 min and washed (5 times) with sterilized distilled water. They were aseptically crushed on yeast extract mannitol agar (YEMA) and incubated at 28 °C for 4 days. At the end of incubation, a single colony representing from each nodule was picked and further purified on YEMA plates. The most prominent isolates were maintained on YEMA slants at 4 °C for further studies.

2.2. Symbiotic tests

To investigate nitrogen fixing ability of rhizobacteria, the pure cultures of isolates were grown to log phase and genomic DNA isolated according to Bazzicalupo and Fani (1995). The genomic DNA was used for the amplification of *nifH* gene using primers: *nifH* for (5′-TAY GGN AAR GGN GGHATY GGY ATC-3′) and *nifH* rev (5′-ATR TTR TTN GCN GCR TAV ABB GCC ATC AT-3′) (Sarita et al., 2007). The PCR reaction mixture (25 µl) contained 2 µl template DNA (0.1–0.14 µg/µl), 0.5 µl Taq DNA polymerase (3 U μl^{-1}), 2 µl of each primer (10 pmol each), 0.5 µl dNTP mixture (10 mM), 10× PCR assay buffer with 25 mM $MgCl_2$ (2.5 µl) and 15.5 µl sterile ultra-pure water. PCR conditions were: denaturation at 95 °C for 4 min; 35 cycles of denaturation at 94 °C for 30 s; annealing at 54 °C for 1 min; extension at 72 °C for 6 min. The PCR product was electrophorised on 2% agarose gel stained with ethidium bromide and photographed using Gel Documentation system (Syngene GBOX).

2.2.1. Acetylene reduction activity

Nitrogenase activity of rhizobacteria was measured by acetylene (C_2H_2) reduction assay as per the protocols of Hardy et al. (1968) with slight modifications. The effect of bacterial culture for their nodulation potential was studied in greenhouse conditions. The experiment was laid with 12 treatments (11 nodule associated bacteria and one water inoculated negative control) in three replications. Chickpea seeds of ICCV 2 (acquired from chickpea breeding, ICRISAT) were surface sterilized with sodium hypochlorite (2.5% for 3 min) followed by ethanol (70% for 3 min) and rinsed with sterile water (5 times). The surface sterilized seeds were transferred into culture of test bacterial isolates grown in YEM broth and kept for an hour. The treated seeds were dibbled in pots (6 seeds/pot but thinned to 3 after one week). Booster doses of bacterial cultures (5 ml per seedling, 10^8 CFU ml^{-1}) were given twice (at 10 and 20 days after sowing) by drenching the soil. At 35 days after sowing (DAS), plants were uprooted and the roots were separated. The roots, along with nodules, were washed gently to remove the soil particles and transferred into a glass bottle (300 ml) and sealed. Thirty millilitres (v/v; 10%) of air was drawn from the glass bottle, with a hypodermic needle and replaced with an equal volume of acetylene gas and incubated at room temperature for 1 h. At the end of incubation, 5 ml of gas drawn from the glass bottle was transferred into a vacutainer and stored at 4 °C until analysed in gas chromatograph (GC). One ml of the above sample was injected into a GC (Agilent 7890B), equipped with a flam ionization detector (FID) to detect ethylene (C_2H_4) and acetylene gas. The results were expressed as nmoles of ethylene gas formed g^{-1} nodule fresh weight h^{-1}. Leaves were used for estimating total chlorophyll content as per the protocols of Hiscox and Israelstam (1979). Other growth parameters including shoot dry weight, root dry weight, nodule number and nodule dry weight were also determined.

2.3. Field inoculation trial

The field trial for two chickpea cultivars (ICCV 2 and JG 11; acquired from chickpea breeding, ICRISAT) was undertaken in 2014–2015 at ICRISAT, Patancheru (17°30' N; 78°16' E; altitude 549 m), Hyderabad, India. Soils at the experimental site are classified as Vertisols with an alkaline pH (7.5 − 8.2) and an OC content of 0.4 − 0.5%. The top 15 cm rhizosphere soil consists of 22 mg kg^{-1} soil of available N, 10 mg kg^{-1} soil of available P and 285 mg kg^{-1} soil of available K. Di-ammonium phosphate (DAP @ 20 kg ha^{-1}) was incorporated in the soil three days before sowing. The trial was conducted in a RCBD design with three replicates and subplot sizes of 4 m × 3 ridges. The selected root nodule bacteria were cultured individually on YEM broth at 28 °C for four days. The seeds of chickpea (ICCV 2 and JG 11) were treated with the root nodule bacterium (individually; containing 10^8 CFU ml^{-1}) for 45 min and sown immediately in rows 30 cm apart at a depth of 4–5 cm to achieve an estimated plant population of at least 25 plants m^{-2}. Plants were inoculated with respective root nodule bacterium at root zone every 15 days till the flowering stage. Control seeds and plots were not treated with root nodule bacteria. No pesticide was sprayed during the cropping period, as no serious insect pest attacks or phytopathogens were observed. Weeding was done 20 days after sowing. The crop was harvested manually on 23 February 2015 at 35 DAS and observations on the number of nodule, nodule weight and shoot weight were recorded. At 60 DAS, observations were made on plant height, shoot weight, leaf weight and leaf number. At crop maturity, pod number, pod weight, seed weight, grain yield and stover yield were recorded. After harvest, rhizosphere soil samples (from top 15 cm of soil profile) were collected from both ICCV 2 and JG 11 plots and analysed for total nitrogen, available phosphorous and organic carbon as per the protocols of Novozamsky et al. (1983), Olsen and Sommers (1982) and Nelson and Sommers (1982), respectively.

2.4. PGP traits of the root nodule bacteria

The root nodule bacteria were characterized for their PGP traits including cellulase, lipase, protease, chitinase, indole acetic acid (IAA), β-1,3-glucanase, siderophore, hydrocyanic acid (HCN) and phosphorous solubilization. The trait for the production of cellulase (Hendricks et al., 1995), lipase (Bhattacharya et al., 2009) and protease (Bhattacharya et al., 2009) was studied as per the standard protocols. Chitinase production was studied by amending agar plates with colloidal chitin and mineral salts according to Hsu and Lockwood (1975). IAA, β-1,3-glucanase and siderophore were estimated as per Patten and Glick (2002), Singh et al. (1999) and Schwyn and Neilands (1987), respectively. One unit of β-1,3-glucanase activity was defined as the amount of enzyme that liberated 1 µmol of glucose $hour^{-1}$ at defined conditions. HCN was qualitatively assessed by the protocol described by Lorck (1948). For HCN production, the following scale was used: 0 = no color change, 1 = light reddish brown, 2 = medium reddish brown and 3 = dark reddish brown. Phosphorous solubilization was tested in National Botanical Research Institute's Phosphate (NBRIP) as per the methods of Nautiyal (1999).

2.5. Molecular identification of the root nodule bacteria

The selected root nodule bacteria were sent to Macrogen Inc. Seoul,

Korea for identification by 16S rDNA analysis. The sequences obtained from Macrogen Inc. were compared with similar sequences in GenBank, using the BLAST program (Altschul et al., 1990), aligned with the Clustal W software (Thompson et al., 1997) and the dendrogram inferred by Neighbor-joining method (Saitou and Nei, 1987). Bootstrap analysis was performed using the MEGA version 4 program to estimate the statistical stability of the branches in cluster with 1000 replications. The sequences were submitted to NCBI and accession numbers obtained.

2.6. Statistical analysis

For field studies, data were analysed by using analysis of variance (ANOVA), by SAS GLM (General Linear Model) procedure (SAS Institute 2002-08, SAS version 9.3) considering isolates and replication as fixed in randomized complete block design. Isolate means were tested for significance and compared using Fisher's protected least significant difference. For greenhouse study, data were analysed statistically by ANOVA and the mean values were compared at 5% level of significance.

3. Results

3.1. Root nodule bacteria isolation and preservation

Totally 11 root nodule bacteria ICKM-1, ICKM-4, ICKM-7, ICKM-9, ICKM-12, ICKM-14, ICKM-15, ICKM-17, ICS-30, ICS-31 and ICS-32 were isolated from the healthy nodules of ICCV 2 and JG 11 chickpea varieties.

3.2. Symbiotic tests of the root nodule bacteria

The nitrogen fixing ability of all the 11 root nodule bacteria was demonstrated by nodulation and acetylene reduction assay (ARA). Under greenhouse conditions, all the 11 root nodule bacteria not only nodulated the chickpea plants (in ICCV 2) but also significantly enhanced the shoot dry weight (up to 33%), root dry weight (up to 64%), total chlorophyll content (up to 27%; except ICKM-1), nodule number (up to 78%) and nodule dry weight (up to 98%) (Table 1). In the ARA, all the 11 root nodule bacteria exhibited nitrogenase activity. The nitrogenase activity ranged from 0.447 (ICKM-1) to 4.920 (ICKM-9) nmoles of ethylene/g fresh weight nodules/h. More than 4 nmoles of ethylene/g fresh weight nodules/h was observed in four root nodule bacteria ICKM-9, ICKM-15, ICS-31 and ICS-32, which is > 97% increase compared to control (Table 1).

Amplification of *nifH* gene segment yielded the product of expected size (400 bp) from DNA template for all the 11 isolates indicating the presence of nitrogen fixing genes in these isolates.

3.3. Field inoculation trial

Under field conditions, in both cultivars (ICCV 2 and JG 11), significant increase in number of agronomic and soil mineral properties were noted in all the 11 root nodule bacteria treated plots over control. At 30 DAS, plots treated with root nodule bacteria, in both cultivars, enhanced the nodule number (up to 46% and 46%), nodule dry weight (up to 76% and 50%) and shoot dry weight (up to 21% and 42%). At 60 DAS, there was rise in plant height (up to 16% and 12%), shoot dry weight (up to 33% and 27%), leaf dry weight (up to 33% and 35%) and leaf area (up to 31% and 16%), respectively, over the un-inoculated control plots (Tables 2 and 3). At grain maturity/harvest stage, the root nodule bacteria treated plots exhibited enhanced pod number (up to 27% and 31%), pod dry weight (up to 28% and 35%), seed weight (up to 30% and 34%), grain yield (up to 27% and 25%) and stover yield (up to 25% and 8%), respectively, over the un-inoculated control plots (Table 4). At grain maturity/harvest, the rhizosphere soil from root nodule bacteria treated plots, enhanced organic carbon (up to 7% and 24%), total N (up to 11% and 19%) and available P (up to 14% and 29%), respectively, over un-inoculated control plots (Table 5).

3.4. PGP traits of the root nodule bacteria

Under *in vitro* conditions, all the root nodule bacteria were found to produce cellulase, lipase, protease, IAA, β-1,3-glucanase, chitinase (except ICKM-1, ICKM-7, ICKM-15, ICS-30 and ICS-31), siderophore (except ICS-31) and HCN (except ICKM-17 and ICS-31) and six isolates solubilized P (except ICKM-1, ICKM-4, ICKM-14, ICKM-17 and ICS-32) (Table 6).

3.5. Molecular identification of the root nodule bacteria

The sequences obtained from Macrogen (1033 bp for ICKM-1, 1070 bp for ICKM-4, 992 bp for ICKM-7, 1482 bp for ICKM-9, 986 bp for ICKM-12, 1298 bp for ICKM-14, 1305 bp for ICKM-15, 1390 bp for ICKM-17, 1000 bp for ICS-30, 901 bp for ICS-31 and 1474 bp for ICS-32) were compared with similar sequences from GenBank, aligned and the dendrogram inferred (Fig. 1). The sequences of 16 S rDNA gene of the root nodule bacteria of ICKM-1, ICKM-4, ICKM-7, ICKM-9, ICKM-

Table 1
Nodulation and nitrogen fixation capabilities of the 11 PGP bacteria on high nodulating chickpea cultivar (ICCV 2) under greenhouse conditions- at 35 days after sowing.

Isolates	Shoot dry weight (g plant^{-1})	Root dry weight (g plant^{-1})	Total chlorophyll (mg l^{-1})	Nodule number (plant^{-1})	Nodule dry weight (mg plant^{-1})	ARA µmol (mg)
ICKM-1	0.887*	0.107	27.69	11.3*	13*	0.447*
ICKM-4	0.830*	0.113	39.61*	16.3*	20*	1.047*
ICKM-7	0.813*	0.150*	33.84*	16.4*	20*	1.397*
ICKM-9	0.950*	0.153*	33.83*	25.5*	30*	4.920*
ICKM-12	0.847*	0.197*	31.33*	22.9*	23*	2.770*
ICKM-14	0.870*	0.177*	37.63*	23.6*	20*	3.087*
ICKM-15	0.933*	0.163*	30.75*	26.6*	40*	4.483*
ICKM-17	1.073*	0.180*	31.93*	25.4*	40*	3.997*
ICS-30	0.917*	0.180*	34.60*	17.8*	20*	3.237*
ICS-31	1.017*	0.220*	30.93*	34.2*	30*	4.600*
ICS-32	0.933*	0.170*	29.89*	27.3*	40*	4.067*
Control	0.723	0.080	28.89	7.7	1	0.097
Mean	0.899	0.158	32.58	21.3	25	2.846
SE ±	0.0089	0.0139	0.049	0.50	1.3	0.0756
LSD (5%)	0.0259	0.0407	0.144	1.46	3.8	0.2218
CV%	2	15	1	4	9	5

*** = Statistically significant at 0.05 compared to control.

Table 2
Effect of the 11 PGP bacteria on nodules and shoot weight on two chickpea cultivars under field conditions- at 35 days after sowing.

	ICCV 2			JG 11		
Isolate	Nodule number (plant^{-1})	Nodule weight (g plant^{-1})	Shoot weight (g plant^{-1})	Nodule number (plant^{-1})	Nodule weight (g plant^{-1})	Shoot weight (g plant^{-1})
ICKM-1	27.3*	22.0*	4.03*	17.0	6.0	4.20*
ICKM-4	17.7	12.7*	4.00*	13.7	6.0	4.7*
ICKM-7	17.7	8.0	4.00*	19.7*	5.3	4.60*
ICKM-9	21.3*	17.0*	4.17*	13.0	10.7*	4.57*
ICKM-12	23.0*	8.0	4.33*	21.3*	6.0	4.27*
ICKM-14	16.3	8.7	3.87*	23.3*	5.3	5.43*
ICKM-15	21.0*	11.3*	4.03*	18.0	6.7	3.67
ICKM-17	18.0	13.3*	4.37*	16.3	6.7	3.93
ICS-30	16.7	10.0	4.07*	14.7	6.0	3.70
ICS-31	24.7*	11.3*	3.77	13.0	7.3	3.90
ICS-32	15.7	6.7	3.93*	12.7	6.0	4.03
Control	14.7	5.3	3.47	12.7	5.3	3.17
Mean	19.5	11.2	4.00	16.3	6.4	4.17
SE ±	2.20	1.79	0.138	2.14	0.93	0.328
LSD (5%)	6.45	5.24	0.406	6.26	2.74	0.963
CV%	20	28	6	23	25	14

SE = Standard error; LSD = least significant differences; CV= coefficients of variation; *= statistically significant at 0.05 compared to control.

Table 3
Effect of the 11 PGP bacteria on agronomic performance on two chickpea cultivars under field conditions- at 60 days after sowing.

	ICCV-2				JG-11			
Isolate	Plant height (cm)	Shoot weight (g plant^{-1})	Leaf weight (g plant^{-1})	Leaf area (m^2 plant^{-1})	Plant height (cm)	Shoot weight (g plant^{-1})	Leaf weight (g plant^{-1})	Leaf area (m^2 plant^{-1})
ICKM-1	47	8.88	13.64*	1285	49	11.98	14.70	1838
ICKM-4	44	7.82	9.79	1302	50	11.34	11.76	1867
ICKM-7	44	7.86	10.13	1500*	52	11.57	11.80	1831
ICKM-9	47	8.08	10.78	1611*	55*	11.75	12.11	2103*
ICKM-12	52*	11.35*	13.50*	1545*	51	15.35*	16.05*	2142*
ICKM-14	51*	11.28*	13.63*	1601*	51	12.48	14.05*	1931
ICKM-15	45	7.88	9.82	1493*	51	12.04	11.14	1844
ICKM-17	53*	8.09	9.47	1481*	50	12.04	11.04	1846
ICS-30	46	7.84	10.10	1331	49	11.86	11.38	1831
ICS-31	46	7.85	9.45	1151	49	11.46	11.34	1838
ICS-32	45	7.95	11.06	1240	52	14.12*	17.16*	2081
Control	44	7.60	9.13	1112	49	11.27	11.11	1798
Mean	47	8.54	10.88	1388	51	12.27	12.080	1913
SE ±	1.9	0.861	0.989	101.8	1.1	0.778	1.173	68.6
LSD (5%)	5.7	2.524	2.812	298.5	3.1	2.294	3.441	201.1
CV%	7	18	15	13	4	11	16	16

SE = Standard error; LSD = least significant differences; CV= coefficients of variation; *= statistically significant at 0.05 compared to control.

12, ICKM-14, ICKM-15, ICKM-17, ICS-30, ICS-31 and ICS-32 were found maximum identity with *Pantoea dispersa*, *Chryseobacterium indologenes*, *Pseudomonas geniculata*, *Stenotrophomonas pavanii*, *P. geniculata*, *P. geniculata*, *Stenotrophomonas maltophilia*, *Chryseobacterium* sp., *P. geniculata*, *Chryseobacterium indologenes* and *Stenotrophomonas acidaminiphila*, respectively. The nucleotide sequences of all the 11 root nodule bacteria were submitted to GenBank and NCBI accession numbers were obtained as follows: ICKM-1:KX583493; ICKM-4:KX583496; ICKM-7:KX583495; ICKM-9:KX583494; ICKM-12:KX583492; ICKM-14:KX611373; ICKM-15:KX611374; ICKM-17:KX611375; ICS-30:KX611376; ICS-31:KY800376 and ICS-32:KX611377.

4. Discussion

In the past, bacteria isolated from nodules were always considered as rhizobia, however, in the recent past, an increasing number of α- β- and γ- Proteobacteria have been reported from nodules of wide range of legumes and reported as nodulating bacteria (Moulin et al., 2001; Vandamme et al., 2002; Valverde et al., 2005; Lin et al., 2008; Saidi et al., 2013). These non-symbiotic nodulating bacteria, isolated from nodules, also establish beneficial interactions with plants (Saidi et al., 2013; Verma et al., 2014; Gopalakrishnan et al., 2015b). However, they have not been studied well as compared to symbiotic bacteria from nodules. In the present investigation, a total of 11 root nodule bacteria were isolated from the root nodules of two chickpea cultivars. In order to test their nitrogen fixing ability, genomic DNA of the isolates was used for amplification of the *nifH* gene. All the 11 bacterial isolates were found to have the nitrogen fixing genes and nodulated the chickpea plants under greenhouse conditions. Further, the nitrogenase activity of the 11 root nodule bacteria were also demonstrated by ARA which is an indirect method to quantify biological nitrogen fixation (BNF) since it measures the conversion of acetylene to ethylene by the nitrogenase enzymes similar to the reduction of N_2 to NH_3 by diazotrophs. Nitrogenase enzymes are important as these are the only family of enzymes known to catalyze this reaction, which is a key step in the process of nitrogen fixation. In the present study, the nitrogenase activity was recorded in the range of 0.447–4.920 nmoles of ethylene g^{-1} fresh nodules h^{-1} (Table 1), with highest activity in ICKM-9 followed

Table 4
Effect of the 11 PGP bacteria on yield performance on two chickpea cultivars under field conditions- at crop maturity.

	ICCV-2					JG-11				
Isolate	Pod number (plant^{-1})	Pod weight (g plant^{-1})	Seed weight (g plant^{-1})	Grain yield (t ha^{-1})	Stover yield (t ha^{-1})	Pod number (plant^{-1})	Pod weight (g plant^{-1})	Seed weight (g plant^{-1})	Grain yield (g) (t ha^{-1})	Stover yield (t ha^{-1})
ICKM-1	58*	18.17*	14.62*	1.979	1.903	77*	22.89*	18.05*	3.108*	2.659
ICKM-4	49	15.81	12.04	2.229*	1.943	84*	25.07*	20.37*	2.910	2.601
ICKM-7	56*	19.89*	14.90*	1.828	1.873	79*	23.83*	19.65*	2.937	2.606
ICKM-9	57*	19.97*	14.37*	1.894	1.864	72*	23.00*	17.84*	3.300*	2.811*
ICKM-12	55*	19.12*	15.19*	2.123*	1.873	76*	23.86*	19.56*	3.316*	2.792*
ICKM-14	62*	20.31*	15.17*	2.119*	2.140*	80*	24.15*	19.65*	3.170*	2.630
ICKM-15	48	15.02	11.64	1.860	1.830	74*	24.02*	18.96*	3.013*	2.688
ICKM-17	57*	19.10*	14.62*	2.138*	2.160*	74*	21.17*	17.33*	3.462*	2.657
ICS-30	56*	18.18*	14.25*	2.191*	2.024*	68	19.78	15.72	3.209*	2.682
ICS-31	53	18.45*	12.71	1.996	1.892	69*	20.87	16.93	3.061*	2.802*
ICS-32	59*	18.84*	13.98*	2.455*	2.366*	71*	20.81	16.84	3.398*	2.758*
Control	46	14.70	10.69	1.803	1.786	58	16.41	13.47	2.602	2.595
Mean	55	18.13	13.68	2.051	1.971	74	22.16	17.86	3.124	2.690
SE ±	2.8	1.153	0.958	0.121	0.099	3.8	1.627	1.274	0.141	0.051
LSD (5%)	8.3	3.381	2.811	0.356	0.292	11.1	4.772	3.737	0.415	0.149
CV%	9	11	12	10	9	9	13	12	8	3

SE = Standard error; LSD = least significant differences; CV = coefficients of variation; * = statistically significant at 0.05 compared to control.

by ICKM-15, ICS-31 and ICS-32, which is more than 97% increase over the un-inoculated control. Kumar and Gera (2014) and Kayasth et al. (2014) reported nitrogen fixing ability of a diastrophic bacterium upon *nifH* gene amplification and acetylene reduction assay. In the present study, all the 11 root nodule bacteria significantly enhanced the shoot dry weight (up to 33%), root dry weight (up to 64%), total chlorophyll contents (up to 27%; except ICKM-1), nodule number (up to 78%) and nodule dry weight (up to 98%) over the un-inoculated control (Table 1).

In field trial, all the 11 root nodule bacteria enhanced PGP and yield traits including nodule number, nodule weight, shoot weight, pod number, pod weight, seed weight, grain yield and stover yield in ICCV 2 and JG 11 varieties. Of the 11 root nodule bacteria, 7 (ICKM-1, ICKM-7, ICKM-12, ICKM-14, ICKM-15, ICKM-17 and ICS-30), 9 (except ICKM-7 and ICKM-14) and 6 (ICKM-12, ICKM-14, ICKM-17, ICS-30, ICS-31 and ICS-32) were found to enhance more than 10% nodule number, nodule weight and grain yield, respectively. The presence of PGP bacteria in the rhizosphere is known to enhance root and shoot growth, root hair development, nitrogen fixation, grain yield, stover yield, plant hormone regulation, solubilization of minerals and the suppression of pathogens in crops including pea, soybean and chickpea (Tokala et al., 2002; Lucas et al., 2009; Minorsky, 2008; Richardson et al., 2009; Soe et al., 2010; Gopalakrishnan et al., 2015a, 2016). However, reports on diazotrophic root nodule bacteria are very limited.

All the root nodule bacteria were found to produce cellulase, lipase, protease, IAA, β-1,3-glucanase, chitinase (except ICKM-1, ICKM-7, ICKM-15, ICS-30 and ICS-31), siderophore (except ICS-31) and HCN (except ICKM-17 and ICS-31) (Table 6). PGP bacteria are widely reported to stimulate plant growth by synthesizing growth hormones which lead to increased nutrient uptake and growth or induce systemic plant resistance against plant pathogens (Lippman et al., 1995; Liu et al., 1995). PGP bacteria such as *Pseudomonas aeruginosa* was reported to produce a maximum IAA of 45.53 μg ml^{-1} followed by *Mesorhizobium* sp. (34.15 μg ml^{-1}), *Trichoderma harzianum* (25.89 μg ml^{-1}) and *Azotobacter chroococcum* (19.24 μg ml^{-1}) (Verma et al., 2014) whereas in the present study, 6 isolates produced 4—8 times more IAA compared to those reported above. These six isolates, ICKM-1, ICKM-9, ICKM-12, ICKM-14, ICS-30 and ICS-32, were found to produce more than 90 μg mg^{-1} of IAA and of which, ICKM-14 produced the maximum (339 μg mg^{-1}) IAA followed by ICS-32 (165 μg mg^{-1}), ICKM-9

Table 5
Effect of the 11 PGP bacteria on soil nutrient traits on two chickpea cultivars under field conditions- at crop maturity.

	ICCV-2			JG-11		
Isolate	Organic carbon (%)	Total N (ppm)	Available P (ppm)	Organic carbon %	Total N (ppm)	Available P (ppm)
ICKM-1	0.47	727*	4.85	0.42	795*	5.76*
ICKM-4	0.48	713*	4.96	0.52*	780*	5.13*
ICKM-7	0.48	709*	4.83	0.44	670	4.42
ICKM-9	0.48	674	4.99	0.56*	677	5.84*
ICKM-12	0.47	675	5.53*	0.46	654	4.23
ICKM-14	0.49*	664	4.83	0.43	656	4.27
ICKM-15	0.48	663	4.98	0.44	662	4.66
ICKM-17	0.50*	668	5.13	0.47	686	4.73
ICS-30	0.47	678	4.75	0.52*	655	4.28
ICS-31	0.49*	723*	4.83	0.44	675	4.55
ICS-32	0.51*	738*	5.30*	0.44	666	4.36
Control	0.47	655	4.75	0.42	642	4.16
Mean	0.48	690	4.98	0.46	685	4.70
SE ±	0.008	17.2	0.134	0.019	24.2	0.277
LSD (5%)	0.023	53.6	0.416	0.060	74.5	0.863
CV%	2	4	4	6	5	8

SE = Standard error; LSD = least significant differences; CV = coefficients of variation; * = Statistically significant at 0.05 compared to control.

Table 6
Enzymatic activities and metabolite production by the 11 PGP bacterial isolates from the nodules of chickpea.

Isolate	At 72 h, production of								
	Cellulase (mm)	Lipase (mm)	Protease (mm)	PS (mm)	Chitinase (mm)	IAA (μg/ml)	β-1, 3-glucanase units[@]	Sid. units	HCN[#]
ICKM-1	27	22	21	0	0	111	2.54	45	3
ICKM-4	27	25	29	0	16	9	2.03	67	1
ICKM-7	24	25	21	8	0	10	1.19	65	1
ICKM-9	26	20	26	10	18	145	1.63	46	1
ICKM-12	25	20	25	6	12	93	1.64	48	2
ICKM-14	25	20	22	0	13	339	1.75	30	1
ICKM-15	25	25	27	10	0	10	1.73	64	3
ICKM-17	26	20	25	0	20	8	2.69	72	0
ICS-30	27	30	29	10	0	115	1.33	34	1
ICS-31	24	25	25	10	0	27	1.07	0	0
ICS-32	18	25	10	0	16	165	1.37	44	3
Mean	25	23	24	5	9	94	1.72	47	2
SE ±	0.2***	0.5***	0.2***	0.1***	0.2***	14.8***	0.075***	1.6***	0
LSD (5%)	0.7	1.5	0.5	0.3	0.6	43.7	0.222	4.7	0
CV%	2	4	1	4	4	27	8	6	0

PS = Phosphate solubilization; Sid. = Siderophore.
Note: the values are zone of inhibition in mm; SE = standard error; LSD = least significant differences; CV = coefficients of variation; *** = statistically significant at 0.001; @ = One unit of β-1,3-glucanase activity was defined as the amount of enzyme that liberated 1 μmol of glucose hour-1 at defined conditions; # HCN production: 0 = Negative growth; 1 = Light growth; 2 = Moderate growth; 3 = Good growth.

(145 μg mg^{-1}), ICS-30 (115 μg mg^{-1}) and ICS-1 (111 μg mg^{-1}). IAA producing bacteria are reported to stimulate seed germination, root formation and plant growth and thereby provides the host plant greater access to water and nutrients (Ahemad and Kibret, 2014).

Siderophores are reported as solubilizing agents for iron under conditions of iron limitation (Indiragandhi et al., 2008). HCN is reported to play a role in disease suppression in tobacco, where HCN producing *Pseudomonas fluorescens* suppressed black root rot disease

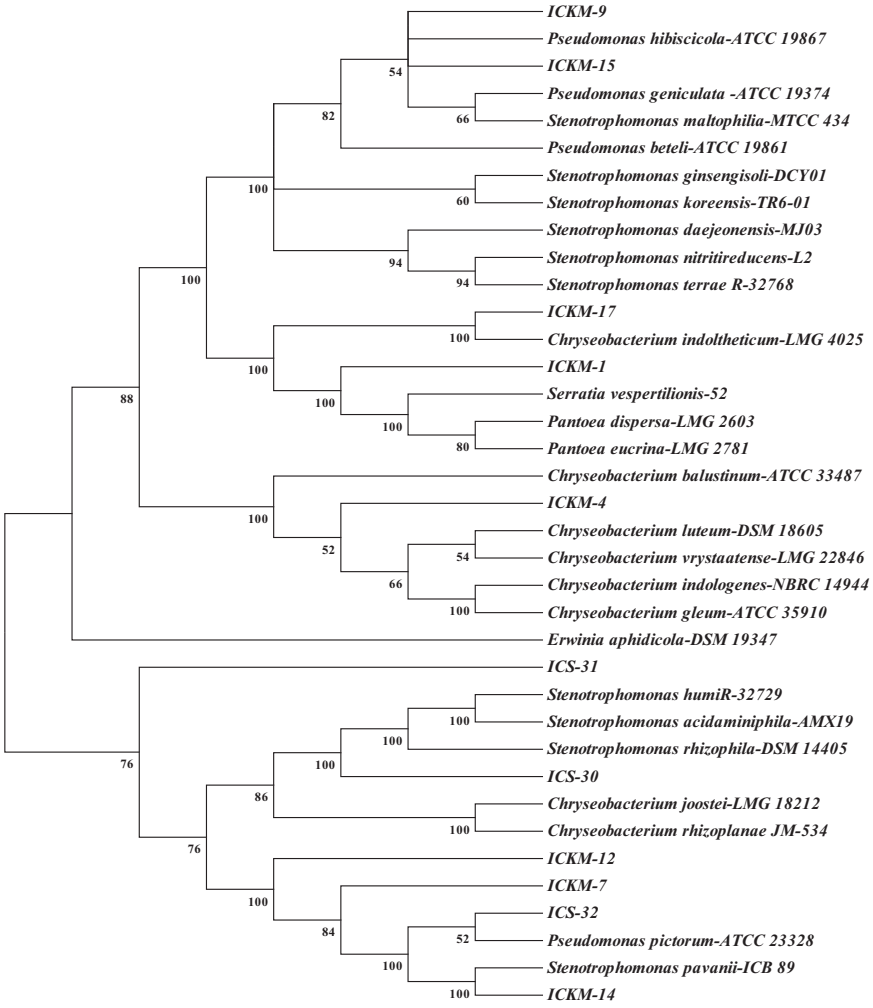

Fig. 1. Phylogenetic relationship between the eleven PGP potential bacteria and representative species based on full length 16S rDNA sequences constructed using the neighbor-joining method.

(Haas et al., 1991). Protease and cellulase-producing bacteria are reported to play an important role in the nutrient mineralization and PGP (Lima et al., 1998). β-1,3-glucanase-producing bacteria are reported to leak the cell wall contents and collapse *Fusarium oxysporum* (Singh et al., 1999). In the present study, 6/11 root nodule bacteria also solubilized phosphorous (except ICKM-1, ICKM-4, ICKM-14, ICKM-17 and ICS-32). Phosphorous (P) is one of the three macronutrients essential for plant growth. It is abundant in many agricultural soils but it is unavailable to plants due to low level of soluble phosphate. Phosphate-solubilizing bacteria such as *Burkholderia cepacia* are reported to be effective in releasing P from total soil P through mineralization and solubilization (Zhao et al., 2014). In the present study, the mechanisms by which the isolates promote plant growth are not fully understood. However, their multiple modes of PGP traits may be responsible for growth promotion activities.

In the present study, all the 11 root nodule bacteria were identified up to species level by 16S rDNA analysis. Of the 11 isolates, 4 were found to be *Pseudomonas geniculata* (ICKM-7, ICKM-12, ICKM-14 and ICS-30), 2 of *Chryseobacterium indologenes* (ICKM-4 and ICS-31), 1 of *Chryseobacterium* spp. (ICKM-17), 3 of *Stenotrophomonas* but different species (ICKM-9, ICKM-15 and ICS-32) and one of *Pantoea dispersa* (ICKM-1).

Plant-microbe interaction involves colonization by a variety of PGP microbes, in and around the roots, in associative or symbiotic relations within the plant. The usefulness of this interaction largely depends on the type of PGP microbes involved, plant defence system environment and soil nutrient status. The 11 diazotrophic root nodule bacteria demonstrated in this study, for their nitrogen fixation, PGP and yield enhancement traits, could be co-inoculated with *Mesorhizobium* sp. of chickpea. PGP bacteria co-inoculated with *Mesorhizobium* sp. was reported to significantly enhance nodulation, nitrogen fixation, P and Fe acquisition, plant growth and yield traits in chickpea under both pot as well as field conditions (Valverde et al., 2006; Verma et al., 2013). Synergistic consortia of *Mesorhizobium* sp. and PGP bacteria with various metabolic traits such as nitrogen fixation, phosphorous mobilization, synthesis of growth hormones and secondary metabolites can be better than single inoculations. The application of such PGP bacteria as inoculants for bio-fertilization, biocontrol and biofortification would be an alternative to reduce the use of synthetic fertilizers and pesticides which effect environmental contamination and/or pollution. The diazotrophic strains used in this study will ultimately help in the development of biofertilizers for use in semi-arid soils to increase the availability of nutrients for both growth and yield of chickpea.

Conflict of interest

All the authors declare that they have no financial/commercial conflicts of interest.

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