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Influence of diazotrophic bacteria on nodulation, nitrogen fixation, growth promotion and yield traits in five cultivars of chickpea

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ABSTRACT

Three bacteria, IC-59, IC-76A and IC-2002, isolated from the nodules of chickpea, were characterized for nodulation, nitrogen fixation, plant growth-promoting (PGP) and yield traits in five cultivars of chickpea such as BG256, RSG888, Subhra, K850 and ICCV2. All the bacteria produced cellulase, protease, β-1,3-glucanase, indole acetic acid, siderophore, hydro cyanic acid and 1-aminocyclopropane-1-carboxylate deaminase while none produced lipase and chitinase. The 16 S rDNA gene sequences of IC-59, IC-76A and IC-2002 were found to match closely with *Rhizobium pusense*, *Paraburkholderia kururiensis* and *Stenotrophomonas maltophilia*, respectively. The three bacteria nodulated all the cultivars of chickpea well, amplified *nifH* gene and fixed nitrogen. Under greenhouse conditions at 30 and 45 days after sowing, treatment of five cultivars of chickpea with bacterial cultures IC-59, IC-76A and IC-2002, enhanced the nodule number (up to 45%, 38% and 43%), nodule weight (up to 31%, 15% and 39%), shoot weight (11%, 16% and 14%) and root weight (37%, 48% and 62%), respectively, over the un-inoculated control. At crop maturity, IC-59, IC-76A and IC-2002 were found to enhance the shoot weight (16%, 40% and 26%), pod number (37%, 69% and 81%), pod weight (17%, 45% and 49%), seed number (21%, 31% and 39%) and seed weight (14%, 56% and 65%), respectively, over the un-inoculated control. Among the five cultivars, Subhra was found to enhance most of the PGP traits when treated with the three diazotrophic bacteria. It is concluded that the three diazotrophic bacteria could be potentially exploited for improving nodulation, nitrogen fixation, PGP and yields of chickpea.

1. Introduction

Chickpea (*Cicer arietinum* L.), the second most important grain legume crop after bean (*Phaseolus vulgaris* L.), is grown in more than 55 countries (FAOSTAT, 2017), of which India is the largest producer. Chickpea play important roles on farm health, in human diets and for the sustainability of agriculture. Many of the poorest countries in the world derive 10–20% of their total dietary protein from chickpea and/or other grain legumes (Akibode and Maredia, 2011). Chickpea grain consists of high protein (12.4—31.5%), carbohydrates (52.4–70.9%), minerals (including iron, zinc, phosphorous, calcium and magnesium) and β-carotene (Awasthi et al., 1991). Chickpea has significant quantity of all the essential amino acids (except sulphur-containing amino acids) and un-saturated fatty acids such as linoleic acid, oleic acid, β-sitosterol, campesterol and stigmasterol (Jukanti et al., 2012). Chickpea exhibit low glycemic index and thus reducing the risk of obesity and diabetes (Foster-Powell et al., 2002), colon and breast cancer (Thompson et al., 2008) and cardiovascular diseases (Kabagambe et al., 2005). Global yields of chickpea has been stagnant (0.5 and 1.0 t ha^{-1}) for the last 50 years in spite of adopting conventional breeding and molecular approaches and extensively using synthetic fertilizers and pesticides (FAOSTAT, 2017). Symbiotic nitrogen fixation (SNF) is a trait that distinguishes chickpea from cereal crops. The ability of chickpea to fix nitrogen in their root nodules benefits not only the chickpea itself but also the subsequent crops, the finances of smallholder farmers and the agricultural system. Through gradual release of nitrogen from decaying root biomass, chickpea can improve overall nitrogen balance in farming systems as compared to chemical nitrogen-only strategies (Nyiraneza and Snapp, 2007). The lack of sufficient numbers of natural compatible rhizobia in most of the chickpea-grown soils imposes a need for rhizobia application to seeds. Further, it is widely known that the host (cultivars) also vary in their potential for nitrogen fixation. Hence, to exploit the advantages of SNF, there is an urgent need to identify compatible rhizobia for specific cultivars.

For several decades, rhizobia were thought to be the only N_2 fixing inhabitants of legume nodules. However, recently a number of α- β- and

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γ-Proteobacteria have been reported from nodules of legumes (Valverde et al., 2006; Saidi et al., 2013; Martinez-Hidalgo and Hirsch, 2017). Some of these nodulating diazotrophic bacteria were also shown to possess abilities of plant growth-promotion (PGP) and yield improvement in addition to their N_2 fixing abilities (Saidi et al., 2013; Verma et al., 2014; Gopalakrishnan et al., 2015, 2017). The mechanisms of these PGP diazotrophic bacteria promoting plant growth and yield were shown to include N_2 fixation, ability to synthesize siderophores, indole acetic acid (IAA) and organic acids that solubilize phosphorus and other nutrients to enhance nutrient uptake (Ahmad et al., 2008; Gopalakrishnan et al., 2017). However, they have not been studied that well in comparison to symbiotic bacteria from nodules, i.e. rhizobia. Therefore, the present investigation was aimed to identify diazotrophic PGP bacteria from the nodules of chickpea, which promote plant growth and yield of chickpea.

2. Materials and methods

2.1. Chickpea cultivars

A total of five chickpea cultivars such as BG256 (desi), RSG888 (desi), Subhra (kabuli), K850 (desi) and ICCV2 (kabuli) were used in this study. The cultivars were acquired from chickpea breeding unit, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India. The selected cultivars varied in maturity duration including extra early duration (ICCV2; 80 90 days), medium duration (BG256, K850 and Subhra; 110–120 days) and late duration (RSG888; 120—130 days) types.

2.2. Diazotrophic bacteria

A total of three diazotrophic bacteria, designated as IC-59, IC-76A and IC-2002, acquired from microbial gene bank at ICRISAT, Patancheru, India, were used in this study. These bacteria were originally isolated from the nodules of chickpea by ICRISAT from the alluvial soils of Haryana, India.

2.3. In vitro PGP traits of the diazotrophic bacteria

The selected three diazotrophic bacteria were characterized for their PGP traits including cellulase, lipase, protease, chitinase, β-1,3-glucanase, indole acetic acid (IAA), siderophore, hydrocyanic acid (HCN) and 1-aminocyclopropane-1-carboxylate (ACC) deaminase. The traits for the production of cellulase, lipase and protease was studied as per the protocols in cellulose congo red agar, tween 80 agar and casein agar, respectively (Bhattacharya et al., 2009; Hendricks et al., 1995). Chitinase production was done in minimal media with 5% colloidal chitin as per the methods of Hirano and Nagao (1988). β − 1,3-glucanase production was done as per the methodology of Singh et al. (1999) in tryptic soy broth (supplemented with 1% colloidal chitin), where one unit of it was defined as the amount of enzyme that liberated 1 μmol of glucose hour^{-1} at defined conditions. IAA and siderophore were estimated as per the protocols in yeast extract mannitol broth supplemented with L-tryptophan (1 μg ml^{-1}) and King's B broth, respectively (Patten and Glick, 2002; Schwyn and Neilands, 1987). HCN was qualitatively estimated in yeast extract mannitol agar amended with glycine (4.4 g L^{-1}) by sulfocyanate method (Lorck, 1948). The following scale was used for HCN production: 0 = no color change, 1 = light reddish brown, 2 = medium reddish brown and 3 = dark reddish brown. ACC deaminase activity was tested as per Penrose and Glick (2003) using ACC as the sole nitrogen source. The presence of colonies in the plate was considered that the colony is capable of producing ACC deaminase.

2.4. Nodulation and N_2 fixation traits of the diazotrophic bacteria

2.4.1. Symbiotic tests

The N_2 fixing ability of the diazotrophic bacteria was done by symbiotic tests. For this, the pure cultures of the three diazotrophic bacteria were grown to log phase and genomic DNA isolated as per the methods of Bazzicalupo and Fani (1995). The genomic DNA of the diazotrophic bacteria 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 and conditions were followed as per the protocols mentioned in Gopalakrishnan et al. (2017).

2.4.2. Acetylene reduction assay (ARA)

The nitrogenase activity of the three diazotrophic bacteria was quantified by acetylene (C_2H_2) reduction assay as per the methods of Hardy et al. (1968) with slight modifications under greenhouse conditions (Gopalakrishnan et al., 2017). In brief, the experiment was laid with 4 treatments (3 diazotrophic bacteria and one water inoculated negative control) in three replications. Chickpea seeds of BG256, RSG888, Subhra, K850 and ICCV2 were surface sterilized and transferred into culture of test diazotrophic bacterial isolates (IC-59, IC-76A and IC-2002) for an hour. The treated seeds were dibbled in pots (6 seeds/pot but thinned to 3 after one week). Booster doses of diazotrophic bacteria (5 ml per seedling, 10^8 CFU ml^{-1}) were applied twice (at 7 and 14 days after sowing [DAS]) by drenching the soil. At 35 DAS, ARA was done as per the protocols of Gopalakrishnan et al. (2017). ARA was done in a gas chromatograph (GC; Agilent 7890B), equipped with a flame ionization detector (FID) to detect ethylene (C_2H_4) and C_2H_2 gas. The results were expressed as nmoles of C_2H_4 gas formed g^{-1} nodule fresh weight h^{-1}. At 35 DAS, leaves of chickpea were also estimated for total chlorophyll content as per the methods of Hiscox and Israelstam (1979). Other plant growth traits including shoot weight, root weight, nodule number and nodule weight were also recorded.

2.5. In vivo PGP traits of the diazotrophic bacteria

The three diazotrophic bacteria (IC-59, IC-76A and IC-2002) were evaluated for their PGP potential in greenhouse on five cultivars of chickpea (BG256, RSG888, Subhra, K850 and ICCV2). Plants were grown in controlled greenhouse conditions. The day and night temperatures and relative humidity (RH %) were on average 28/22 °C and 70/90%, respectively, and were under natural day-light oscillations. The greenhouse trial was conducted in a completely randomized design (CRD). A total of four treatments (three diazotrophic bacteria and one un-inoculated control) were made with six replications for each cultivar of chickpea. Pot mixture (black soil and sand at 3:2) was prepared by mixing and placed in 8″ plastic pots. Chickpea seeds (all five cultivars) were surface sterilized with sodium hypochlorite (2.5% for 5 min) and rinsed thoroughly with sterilized water. The sterilized seeds were transferred into the three diazotrophic bacteria culture broth (10^8 CFU ml^{-1}; grown in yeast extract mannitol broth separately) and incubated for 1 h. At the end of incubation, the seeds were sown in the pots (three seeds/pot but thinned to one after one week). Booster doses of the three diazotrophic bacteria (5 ml per pot, 10^8 CFU ml^{-1}) were applied at 15, 30, 45 and 60 DAS by soil drench method. Plants were irrigated once in every three days with sterilized deionized water (30 ml). PGP traits including plant height, nodule number, nodule weight, shoot weight and root weight were determined at 30 and 45 DAS. At crop maturity, shoot weight, pod number, pod weight, seed number and seed weight were recorded.

2.6. Molecular identification of the diazotrophic bacteria

For molecular identification of the three diazotrophic bacteria, pure cultures of them were grown in yeast extract mannitol broth until log

phase. Genomic DNA was isolated as per the methods of Bazzicalupo and Fani (1995). 16 S rDNA gene was amplified using universal primer 1492 R (5′-TAC GGY TAC CTT GTT ACG ACT T-3′) and 27 F (5′- AGA GTT TGA TCM TGG CTC AG-3′) according to Pandey et al. (2005). The PCR products were sequenced at Macrogen Inc. Seoul, Korea. The sequences obtained from Macrogen Inc. were compared with those from the NCBI and Ez-Taxon using the BLAST program, aligned using the Clustal W software and phylogenetic trees inferred using the MEGA version 4 program (Alschul et al., 1990; Thompson et al., 1997; Tamura et al., 2007). The dendrogram was inferred by neighbor-joining method (Saitou and Nei, 1987). The nucleotide sequences of the three diazotrophic bacteria were submitted to GenBank and the NCBI GenBank accession numbers were obtained.

2.7. Statistical analysis

For the greenhouse studies, analysis of variance was performed using SAS GLM (General Linear Model) procedure (SAS Institute Inc, 2017) considering bacterial culture and chickpea cultivars as fixed effects. Least Square means were calculated for culture, cultivars and culture*cultivars and pairwise comparisons were tested at 5% level of significance using Least Significant Difference.

3. Results

3.1. In vitro PGP traits of the diazotrophic bacteria

Under in vitro conditions, all the three diazotrophic bacteria produced cellulase, protease, β-1,3-glucanase, IAA, siderophore, HCN and ACC deaminase whereas none of them produced lipase and chitinase. Among the three diazotrophic bacteria, IC-76A and IC-2002 produced significantly higher levels of cellulase, β-1,3-glucanase, IAA, siderophore and HCN when compared to IC-59 (Table 1).

3.2. Nodulation and N_2 fixation traits of the diazotrophic bacteria

Amplification of *nifH* gene segment resulted in the product of expected size (about 400 bp) from DNA template for all the three bacteria indicating the presence of N_2 fixing genes in these diazotrophs. The N_2 fixing ability of all the three diazotrophic bacteria was demonstrated by nodulation and ARA. All the three diazotrophic bacteria not only significantly nodulated the chickpea plants in all the five cultivars (BG256, RSG888, Subhra, K850 and ICCV2) but also enhanced the shoot weight (up to 15%, 41%, 19%, 31% and 8%, respectively), root weight (up to 27%, 24%, 5%, 42% and 34%, respectively), nodule number (up to 45%, 54%, 35%, 51% and 55%, respectively), nodule weight (up to 62%, 52%, 54%, 34% and 43%, respectively) and total chlorophyll content (up to 25%, 17%, 14%, 17% and 29%, respectively) under greenhouse conditions over the un-inoculated control (Table 2). Among the three diazotrophic bacteria, IC-76A and IC-2002 produced more nodules both by number and weight than IC-59. This was true in 4 out of the 5 chickpea cultivars studied (except BG256). By ARA, all the three diazotrophic bacteria exhibited good nitrogenase activity. The nitrogenase activity ranged from 1.78 to 8.83, 3.39 to 7.31 and 2.75 to 4.67 nmoles of C_2H_4/g fresh weight nodules/h for IC-59, IC-76A and IC-2002, respectively. Among the five chickpea cultivars tested, BG256 and K850 were found to exhibit more nitrogenase activity compared to RSG888, Subhra and ICCV2 (Table 2).

3.3. In vivo PGP traits of the diazotrophic bacteria

Under greenhouse conditions, in all the five cultivars, significant increase in both agronomic and yield traits were noted in all the three diazotrophic bacteria (IC-59, IC-76A and IC-2002) treated plants over the un-inoculated control. At 30 DAS, in BG256, RSG888, Subhra, K850 and ICCV2 cultivars, all the three diazotrophic bacteria significantly enhanced the nodule number (up to 41%, 43%, 38%, 45% and 16%, respectively), nodule weight (up to 39%, 14%, 4%, 6% and 31%, respectively), shoot weight (up to 14%, 11%, 9%, 12% and 16%, respectively) and root weight (up to 58%, 42%, 24%, 21% and 62%, respectively) while at 45 DAS, plant height (up to 6%, 8%, 26%, 9% and 8%, respectively), nodule number (up to 29%, 8%, 21%, 41% and 34%, respectively), nodule weight (up to 16%, 35%, 8%, 22% and 8%, respectively), shoot weight (up to 7%, 36%, 12%, 28% and 18%, respectively) and root weight (up to 22%, 44%, 40%, 27% and 43%, respectively) over the un-inoculated control (Tables 3–6). At crop maturity, the diazotrophic bacteria treated pots exhibited enhanced shoot weight (up to 29%, 40%, 14%, 39% and 14%, respectively), pod number (up to 52%, 19%, 12%, 63% and 81%, respectively), pod weight (up to 36%, 14%, 24%, 24% and 49%, respectively), seed number (up to 31%, 35%, 19%, 39% and 33%, respectively) and seed weight (up to 37%, 16%, 22%, 23%, 23% and 65%, respectively) over the un-inoculated control pots (Tables 7 and 8). Among the three diazotrophic bacteria, IC-76A and IC-2002 were found better than IC-59 based on shoot weight, root weight, nodule weight, nodule number, pod number, pod weight, seed number and seed weight. This was true for all the 5 chickpea cultivars studied (Tables 3–8).

3.4. Molecular identification of the diazotrophic bacteria

The sequences obtained from Macrogen (621 bp for IC-59, 1583 bp for IC-76A, and 943 bp for IC-2002) 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 IC-59, IC-76A and IC-2002 were found to match maximum with *Rhizobium pusense*, *Paraburkholderia kururiensis* and *Stenotrophomonas maltophilia*, respectively (Fig. 1). The nucleotide sequences of all the three diazotrophic bacteria were submitted to GenBank and NCBI accession numbers were obtained as follows: IC-59: MF372582; IC-76A: MF373465 and IC-2002: MF372584.

Table 1
In vitro PGP traits of the three diazotrophic bacteria.

Isolate	Cellulase (mm)	Lipase (mm)	Protease (mm)	Chitinase (mm)	β -1,3-glucanase (% units)	IAA (μg/ml)	Sidrophore (% units)	HCN	ACC deaminase
IC-59	18.3	0	29.7	0	0.37	21.9	30.6	1	+
IC-76A	23.3	0	24	0	2.53	31.9	44.7	2	+
IC-2002	21.7	0	28	0	2.49	39.8	34.2	3	+
Mean	21.1	0	27.2	0	1.79	31.2	36.5	2	
SE ±	0.77*	0	0.19***	0	0.065***	1.49**	2.52*	0	
LSD (5%)	3.02	0	0.8	0	0.257	5.85	9.9	0	
CV%	6	0	1	0	6	8	12	0	

IAA = Indole acetic acid; HCN = Hydrocyanic acid; ACC deaminase = 1-aminocyclopropane-1-carboxylate (ACC) deaminase; SE = Standard error; LSD = least significant differences; CV = coefficients of variation; * = statistically significant at 0.5, ** = statistically significant at 0.1, *** = statistically significant at 0.001.

Table 2
Nodulation and N_2 fixation traits of the three diazotrophic bacteria on five cultivars of chickpea.

	Shoot weight (mg plant^{-1})					Root weight (mg plant^{-1})					Nodule number (plant^{-1})				
Isolate	BG256	RSG888	Subhra	K850	ICCV2	BG256	RSG888	Subhra	K850	ICCV2	BG256	RSG888	Subhra	K850	ICCV2
IC-59	492	391*	375	223	390	151*	101	209	168	134	44*	29*	13	46*	25*
IC-76A	510	287	434*	273	363	110	136*	207	267*	160*	33*	37*	19*	39*	34*
IC-2002	449	462*	359	338*	373	107	107	181	128	98	40*	33 *	20*	51*	38*
Control	432	273	352	234	360	110	104	199	155	105	24	17	13	25	17
Mean	471	353	380	267	372	120	112	199	180	124	35	29	16	40	28
SE ±	28.9	17.9	13.4	18.7	13.4	7.2	5.5	5.2	23.2	11.1	2.3	2.9	1.5	4.2	1.9
LSD (5%)	100	61.9	46.5	64.7	46.2	25	19.2	18.1	80.4	38.5	8	9.9	5.2	14.7	6.6
CV%	11	9	6	12	6	11	9	5	22	16	11	17	16	18	12
	Nodule weight (mg plant^{-1})					**Total chlorophyll (mg 1^{-1})**					**ARA (μ mol/mg)**				
Isolate	BG256	RSG888	Subhra	K850	ICCV2	BG256	RSG888	Subhra	K850	ICCV2	BG256	RSG888	Subhra	K850	ICCV2
IC-59	47*	34*	15	53*	27*	21*	21*	21*	21	18	8.83*	1.78*	3.55*	5.69*	2.43*
IC-76A	31*	42*	28*	56*	37*	18	21*	18	19	21*	4.70*	3.39*	4.84*	7.31*	4.27*
IC-2002	35*	36*	22*	48*	31*	24*	24*	22*	24*	24*	4.67*	2.76*	3.06*	4.66*	2.92*
Control	18	20	13	37	21	18	18	19	20	17	1.3	0.83	1.3	2.76	1.58
Mean	33	33	20	49	29	20	21	20	21	20	4.88	2.19	3.19	5.11	2.8
SE ±	2.7	2.7	1.9	1	1.7	0.5	0.2	0.6	0.7	0.9	0.45	0.17	0.26	0.31	0.16
LSD (5%)	9.2	9.2	6.7	3.5	5.9	1.7	0.7	2.1	2.6	3	2.02	0.77	1.17	1.4	0.74
CV%	14	14	17	13	10	4	2	5	6	8	13	11	11	9	8

* = Statistically significant at 0.5 compared to control; ARA = acetylene reduction assay; SE = Standard error; LSD = least significant differences; CV = coefficients of variation.

4. Discussion

Rhizobia were thought to be the only N_2 fixing bacteria of legumes for decades. However, in the recent past, non-symbiotic nodulating bacteria, including diazotrophic bacteria, were reported to have beneficial interactions with legumes (Saidi et al., 2013; Verma et al., 2014). Many of these non-rhizobial diazotrophic bacteria also induce N_2 fixation in the nodules of legume roots (Martinez-Hidalgo and Hirsch, 2017), though, they have not been characterized as that of the rhizobial bacteria. In the present study, three such diazotrophic bacteria (IC-59, IC-76A and IC-2002), isolated previously from the nodules of chickpea, were characterized for their nodulation, N_2 fixation, PGP and yield related traits.

Beneficial bacteria were reported to exhibit indirect mechanisms of PGP activities including ability to produce hormones and enzymes and exhibit antagonistic activities against plant pathogens. The present study had revealed that all the three diazotrophic bacteria produced cellulase, protease, β-1,3-glucanase, IAA, siderophore, HCN and ACC deaminase while these did not produce lipase and chitinase (Table 1). Among the three diazotrophic bacteria studied, IC-76A and IC-2002 had produced significantly higher levels of β-1,3-glucanase, IAA, siderophore and HCN when compared to IC-59 while other traits showed no difference. Between the two promising isolates, IC-76A had produced higher levels of β-1,3-glucanase (2.53% units) and siderophore (44.7% units) whereas IC-2002 had produced higher levels of IAA (39.8 μg ml^{-1}) and HCN (dark reddish brown in color). Cellulase and protease-producing microbes play an important role in the degradation of organic wastes, mineralization of nutrients and promotion of plant

Table 3
Effect of the three diazotrophic bacteria on plant growth-promoting traits on five cultivars of chickpea under greenhouse conditions, at 30 days after sowing.

	Plant height (cm)					Nodule number (plant^{-1})					Nodule weight (mg plant^{-1})				
Isolate	BG256	RSG888	Subhra	K850	ICCV2	BG256	RSG888	Subhra	K850	ICCV2	BG256	RSG888	Subhra	K850	ICCV2
IC-59	37.2*	27.3	34.3**	26.3**	31.0	75*	55.0	43*	43	39.0	690**	580	800	430	330**
IC-76A	33.8	27.7	35.2**	27.3**	30.2	87**	69**	52**	49*	41.0	640*	610	740	480	470*
IC-2002	31.2**	29.7**	32.0	22.3*	31.3	97**	63**	43*	63**	43*	800**	730**	770	510	540**
Control	35.3	26.2	31.5	24.2	30.0	69.0	50.0	37.0	43.0	37.0	570	570	770	480	410
Mean	34.4	27.7	33.3	25	30.6	82.0	59.0	44.0	50.0	40.0	675	623	770	475	438
SE ±	0.69	0.4	0.67	0.6	0.41	2.7	4.7	1.3	0.6	0.6	18.5	49.8	20.3	11.9	15.7
LSD (5%)	2.38	1.38	2.3	2.06	1.4	9.3	16.3	4.5	2.1	2.2	64.1	172.3	70.4	41.5	54.6
CV%	4	3	4	4	2	6	13	5	2	3	5	14	5	4	6
	Shoot weight (g plant^{-1})					**Root weight (g plant^{-1})**				
Isolate	BG256	RSG888	Subhra	K850	ICCV2	BG256	RSG888	Subhra	K850	ICCV2
IC-59	0.84*	0.64	0.96	0.67*	0.88**	0.53**	0.54	0.80*	0.53	0.48**
IC-76A	0.85**	0.70*	0.99**	0.68**	0.92**	0.61**	0.71**	0.84**	0.62**	0.52**
IC-2002	0.89**	0.71**	0.93	0.65	0.84*	0.67**	0.61**	0.91**	0.62**	0.57**
Control	0.78	0.65	0.91	0.61	0.79	0.43	0.5	0.73	0.51	0.35
Mean	0.84	0.67	0.95	0.65	0.86	0.6	0.62	0.85	0.59	0.52
SE ±	0.019	0.027	0.012	0.014	0.012	0.032	0.018	0.028	0.013	0.017
LSD (5%)	0.065	0.092	0.043	0.047	0.041	0.11	0.063	0.092	0.043	0.057
CV%	4	7	2	4	2	10	5	6	4	6

* = Statistically significant at 0.5; ** = statistically significant at 0.01; SE = Standard error; LSD = least significant differences; CV = coefficients of variation.

Table 4
Analysis of Variance of mean sum of squares for the three diazotrophic bacteria on plant growth-promoting traits on five cultivars of chickpea under greenhouse conditions, at 30 days after sowing.

Source	DF	Plant height	Nodule number	Nodule weight	Shoot weight	Root weight
Culture	3	14.33**	396.71**	109.02**	0.017**	0.09**
Cultivars	4	179.19**	3369.99**	2282.62**	0.19**	0.19**
Culture*Cultivars	12	9.26**	167.14**	136.46**	0.002*	0.004**
Error	40	0.90	10.99	12.64	0.0009	0.001

growth (Lima et al., 1998). β-1,3-glucanase plays an important role in control of plant pathogens. The cell wall of plant pathogens, for e.g. *Fusarium oxysporum* f. sp. *ciceri* (FOC; causes wilt in chickpea), is composed of β-1,3-glucan and lysis of this by β-1,3-glucanase-producing bacteria leads to leakage of cell contents and collapse of the FOC (Singh et al., 1999). Phytohormones are plant growth-regulators, which influence the growth of plants. For instance, auxins (such as IAA) producing bacteria are reported to stimulate seed germination, root formation and root prolificacy thereby providing the host plant greater access to soil nutrients and water (Ahemad and Kibret, 2014). Siderophores forms stable complexes with heavy metals such as uranium (U), neptunium (Np), aluminum (Al), copper (Cu), cadmium (Cd), gallium (Ga), zinc (Zn) and lead (Pb) and increases the soluble metal concentrations (Rajkumar et al., 2010). Thus, this process helps to alleviate the heavy metal stresses in soils. Siderophores also act as solubilizing agents for iron from minerals under conditions of iron limitation (Indiragandhi et al., 2008). HCN production by bacteria such as *Pseudomonas fluorescens* is reported to play a role in suppression of black root rot disease in tobacco (Haas et al., 1991). Rhizobia are widely known to produce siderophores, IAA, β-1,3-glucanase, HCN, ACC deaminase and antibiotics (Holt et al., 1994; Kumar et al., 2011) but not such reports are available for diazotrophic bacteria. It is concluded that the three diazotrophic bacteria possess multiple traits of PGP and biological control of plant pathogens.

Beneficial bacteria also exhibit direct mechanisms of PGP activities often, including ability to fix N_2. In the current study, all the three diazotrophic bacteria were found to have the nitrogen fixing genes and nodulated the chickpea plants under greenhouse conditions. These diazotrophic bacteria not only enhanced nodulation in chickpea, both in terms of nodule number and nodule weight, across all the five cultivars but also enhanced the shoot and root weights and the total chlorophyll content under greenhouse conditions over the un-inoculated control (Table 2). Among the three diazotrophic bacteria, based on nodule weight and nodule number, IC-76A and IC-2002 were found to be better than IC-59 in 4 out of the 5 chickpea cultivars studied (except BG256). Further, the nitrogenase activity of the three diazotrophic bacteria were also demonstrated by ARA which is an indirect method to quantify SNF since it measures the conversion of C_2H_2 to C_2H_4 by the nitrogenase enzymes similar to the reduction of N_2 to ammonia (NH_4) by diazotrophs. Nitrogenase enzymes are the only family of enzymes known to catalyze this reaction, which is a key step in the process of SNF. In the present study, among the five cultivars tested, BG256 and K850 were found to exhibit more nitrogenase activity (4.88 and 5.11 μ mol mg^{-1}, respectively) compared to RSG888 (2.19 μ mol mg^{-1}), Subhra (3.19 μ mol mg^{-1}) and ICCV2 (2.80 μ mol mg^{-1}) (Table 2). Diazotrophic bacteria such as *Gordonia* sp., *Brevundimonas* sp., *Dyadobacter* sp. and *Sphingomonas trueperi* were reported to have N_2 fixing ability (Kayasth et al., 2014; Kumar and Gera, 2014; Kumar et al., 2018; Xu et al., 2018). A rare endophytic diazotrophic *Lysinibacillus sphaericus* isolated from rice stem was reported to have N_2 fixing ability (Shabanamol et al., 2018). The presence of diazotrophic bacteria including *Pantoea dispersa*, *Chryseobacterium indologenes*, *Pseudomonas geniculata* and species of *Stenotrophomonas* were reported in the nodules of chickpea and their ability to nodulate and to fix N_2 by ARA and *nifH* genes (Gopalakrishnan et al., 2017).

In the present study, in greenhouse, all the three diazotrophic bacteria significantly enhanced the agronomic and yield performances of all the five cultivars over the un-inoculated control. At 30 and 45 DAS, all the three diazotrophic bacteria significantly enhanced the nodule number, nodule weight, shoot weight and root weight over the

Table 5
Effect of the three diazotrophic bacteria on plant growth-promoting traits on five cultivars of chickpea under greenhouse conditions, at 45 days after sowing.

	Plant height (cm)					Nodule number (plant^{-1})					Nodule weight (mg plant^{-1})				
Isolate	BG256	RSG888	Subhra	K850	ICCV2	BG256	RSG888	Subhra	K850	ICCV2	BG256	RSG888	Subhra	K850	ICCV2
IC-59	36.8	35.3**	43.2**	30.8**	28.7*	66	85	45	82**	54**	1026	1417**	1429	1575**	1136
IC-76A	40.2*	32.5	44.3**	28.3	26.5	82**	75	43	84**	47	1068*	1582**	1182**	1388	1137
IC-2002	40.2*	34.2	38.7**	28.8	26.7	79**	63**	57*		46	1149**	1425**	1623*	1420*	1105
Control	38	32.7	35.2	28.2	26.7	64	79	47	60	41	991	1174	1498	1289	1057
Mean	38.8	33.7	40.4	29	27.1	73	75	48	73	47	1059	1400	1433	1418	1109
SE ±	0.52	0.6	0.46	0.57	0.38	5.8	4.1	1.7	3.6	0.9	13.7	49.5	60.3	40.7	16.3
LSD (5%)	1.79	2.07	1.61	1.97	1.32	20.2	14	5.9	12.4	3.2	47.5	171.2	208.6	141	56.5
CV%	2	3	2	3	2	14	9	6	9	3	2	6	7	5	3

	Shoot weight (g plant^{-1})					Root weight (g plant^{-1})				
Isolate	BG256	RSG888	Subhra	K850	ICCV2	BG256	RSG888	Subhra	K850	ICCV2
IC-59	1.11	1.12**	1.49*	1.34**	1.09	0.68	0.92**	0.79	1.09	0.64
IC-76A	1.13	1.29**	1.45*	1.24**	1.05	0.63	0.99**	0.9	0.99	0.83*
IC-2002	1.07	1.11*	1.45	1.21*	1.19**	0.74*	0.97**	1.30**	1.23**	0.89**
Control	1.05	0.95	1.33	1.05	1.01	0.56	0.69	0.93	0.97	0.62
Mean	1.09	1.12	1.43	1.21	1.09	0.65	0.89	0.98	1.07	0.75
SE ±	0.039	0.078	0.017	0.04	0.035	0.099	0.089	0.037	0.043	0.034
LSD (5%)	0.135	0.269	0.06	0.139	0.124	0.346	0.307	0.127	0.149	0.117
CV%	6	12	2	6	6	29	17	7	7	8

*= Statistically significant at 0.5; **= statistically significant at 0.01; SE = Standard error; LSD = least significant differences; CV= coefficients of variation.

Table 6
Analysis of Variance of mean sum of squares for the three diazotrophic bacteria on plant growth-promoting traits on five cultivars of chickpea under greenhouse conditions, at 45 days after sowing.

Source	DF	Plant height	Nodule number	Nodule weight	Shoot weight	Root weight
Culture	3	22.35**	698.53**	80,796.56**	0.080**	0.18**
Cultivars	4	404.45**	2499.47**	405,300.71**	0.25**	0.33**
Culture*Cultivars	12	13.12**	113.57**	42,056.15**	0.01*	0.003*
Error	40	1.44	35.63	4708.12	0.005	0.01

un-inoculated control (Tables 3–6). At crop maturity, the diazotrophic bacteria treated pots exhibited enhanced shoot weight, pod number, pod weight, seed number and seed weight over the un-inoculated control pots. Based on the shoot weight, root weight, nodule weight, nodule number, pod number, pod weight, seed number and seed weight, IC-76A and IC-2002 were found to be more promising than IC-59 in all the 5 cultivars studied (Tables 7 and 8). The mechanism by which the three diazotrophic bacteria enhanced the plant growth and grain yield could be collectively through their PGP abilities including cellulase, protease, β-1,3-glucanase, IAA, siderophore, HCN and ACC deaminase (Table 1).

The effect of the PGP bacteria on nodule and root development has been widely reported (Birkhofer et al., 2008; Uphoff et al., 2009; Gopalakrishnan et al., 2014). In the present study, though chickpea roots were not inspected for colonization, an observation at the root morphology (including nodule number, nodule weight and root weight) had strongly suggested that the three diazotrophic bacteria multiplied and colonized the roots of chickpea plants. 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 (Gopalakrishnan et al., 2015, 2016; Sreevidya and Gopalakrishnan, 2017). Diazotrophic bacteria isolated from switch grass and giant reed such as *Sphingomonas trueperi*, *Psychrobacillus psychrodurans* and *Enterobacter oryzae* were reported to have N_2 fixing and PGP traits in wheat and maize (Xu et al., 2018). Shabanamol et al. (2018) reported an endophytic diazotrophic bacteria *Lysinibacillus sphaericus* having PGP and biocontrol potentials in rice. Kumar et al. (2018) reported a psychrotolerant bacterium *Dyadobacter* sp., isolated from Himalaya, was reported to have N_2 fixing and PGP traits in chickpea, black gram (*Vigna mungo*), green gram (*Vigna radiata*), pigeonpea (*Cajanus cajan*) and finger millet (*Eleusine coracana*). Few other diazotrophic bacteria, such as *P. dispersa*, *C. indologenes*, *P. geniculata*, and *Stenotrophomonas* sp., were also reported to enhance plant growth and yield in chickpea (Gopalakrishnan et al., 2017). Bacteria are known to be chemo-attracted and move toward the root exudates, released by the host plants, allowing them to colonize and multiply in the rhizosphere and enhance plant growth and yield (Lugtenberg and Kamilova, 2009). Legumes show improved nodulation and grain yield when co-inoculated with PGP bacteria compared to inoculation with rhizobia alone (Rokhzadi et al., 2008; Yang et al., 2009). Co-inoculation of legumes, chickpea in particular, with rhizobia and PGPR are reported to enhance nodulation and nitrogen fixation (Sindhu and Dadarwal, 2001; Garcia et al., 2004; Valverde et al., 2006; Kaur et al., 2015). Hence, the synergistic benefits of these three diazotrophic bacteria could be exploited for improving grain yield in chickpea through better nodulation and N_2 fixation.

In the present study, all the three diazotrophic root nodule bacteria were identified up to species level by 16 S rDNA analysis. The sequences of 16 S rDNA gene of IC-59, IC-76A and IC-2002 were found to match the maximum with *R. pusense*, *P. kururiensis* and *S. maltophilia*, respectively and these were not identified as plant or animal pathogens. Most nodule-associated bacteria are generally non-pathogenic although some of them such as *Staphylococcus* sp., *Burkholderia* sp. and *Bordetella* sp. were reported to be human and/or animal pathogens (Xu et al., 2014; Martinez-Hidalgo and Hirsch, 2017).

The use of PGP bacteria was recommended by several researchers globally due to their significant contribution not only in plant growth but also yield improvement, that had been demonstrated in grain crops including rice, wheat, bean, pea and chickpea (Figueiredo et al., 2008; Sadeghi et al., 2012; Gopalakrishnan et al., 2014, 2017). Symbiotic

Table 7
Effect of the three diazotrophic bacteria on plant growth-promoting traits on five cultivars of chickpea under greenhouse conditions, at crop maturity.

	Shoot weight (g plant^{-1})					Pod number (plant^{-1})					Pod weight (g plant^{-1})				
Isolate	BG256	RSG888	Subhra	K850	ICCV2	BG256	RSG888	Subhra	K850	ICCV2	BG256	RSG888	Subhra	K850	ICCV2
IC-59	1.47	2.67	3.44	2.67	1.68	10.9	15.6*	14.7	13.7**	11.6	2.82	3.27*	4.53	4.45*	2.81
IC-76A	2.01	3.69**	3.88*	3.20**	2.08	16.7**	18.7	14.7	16.3**	17.9**	4.60**	4.53	5.24**	4.72**	4.71**
IC-2002	1.79	2.56	3.7	2.92*	2.13	14.6**	23.0*	15.3	15.3**	19.3**	4.42**	4.28	5.06**	4.23	4.85**
Control	1.56	2.63	3.39	2.31	1.87	11	19.3	13.7	10	10.6	3.38	3.98	4.24	3.81	3.26
Mean	1.71	2.89	3.6	2.78	1.94	13.3	19.2	14.6	13.8	14.9	3.81	4.02	4.77	4.3	3.91
SE ±	0.211	0.18	0.171	0.138	0.118	0.95	0.66	0.37	0.91	1.32	0.322	0.246	0.152	0.156	0.301
LSD (5%)	0.731	0.63	0.589	0.479	0.408	3.28	2.28	1.29	3.14	4.56	1.114	0.851	0.527	0.542	1.04
CV%	21	11	8	7	11	12	6	4	11	15	15	11	6	6	13

	Seed number (plant^{-1})					Seed weight (g plant^{-1})				
Isolate	BG256	RSG888	Subhra	K850	ICCV2	BG256	RSG888	Subhra	K850	ICCV2
IC-59	10.7	18.3	13.7*	13.3*	9.9	2.13*	2.65	3.57	3.62	1.93
IC-76A	15.3**	20.6	14.0*	13.3*	14	3.77**	3.62	4.35**	3.89*	3.57**
IC-2002	14.3*	26.0**	14.3*	15.3**	17.7*	3.08	3.46	4.14*	3.37	3.78**
Control	11.6	19.3	12	11	13.1	2.75	3.12	3.55	3.17	2.29
Mean	13	21.1	13.5	13.2	13.7	2.93	3.21	3.9	3.51	2.89
SE ±	0.84	1.31	0.43	0.65	1.15	0.254	0.242	0.189	0.221	0.219
LSD (5%)	2.9	2.53	1.49	2.23	3.98	0.881	0.837	0.654	0.766	0.757
CV%	11	11	6	8	15	15	13	8	11	13

* = Statistically significant at 0.5; ** = statistically significant at 0.01; SE = Standard error; LSD = least significant differences; CV = coefficients of variation.

Table 8
Analysis of Variance of mean sum of squares for the three diazotrophic bacteria on plant growth-promoting traits on five cultivars of chickpea under greenhouse conditions, at crop maturity.

Source	DF	Shoot weight	Pod number	Pod weight	Seed number	Seed weight
Culture	3	1.22**	1.43**	5.16**	0.99**	3.57**
Cultivars	4	7.07**	1.01**	1.75**	2.10**	2.14**
Culture*Cultivars	12	0.13*	0.20**	0.51**	0.12*	0.39**
Error	40	0.08	0.04	0.14	0.04	0.13

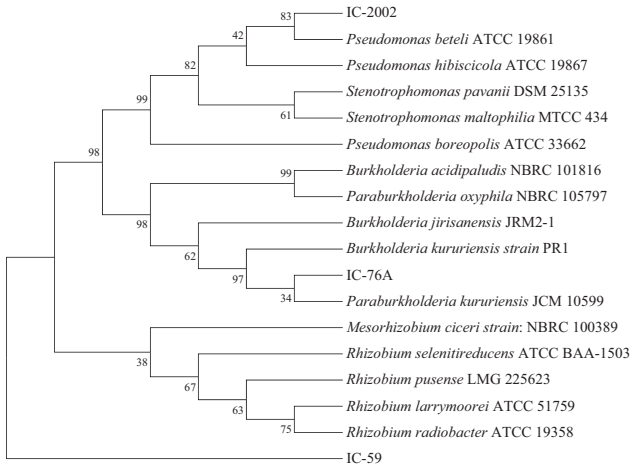

Fig. 1. Phylogenetic relationship between the three diazotrophic bacteria and representative species, by neighbor-joining method.

bacteria including *Bradyrhizobium* sp., *Mesorhizobium* sp. and *Rhizobium* sp. were reported to enhance plant growth and yield in legumes (Pandey and Maheshwari, 2007; Joshi et al., 2008; Kumar et al., 2011). Non-symbiotic bacteria such as *Pseudomonas*, *Bacillus*, *Klebsiella*, *Azotobacter*, *Azospirillum* and *Azomonas* were also reported to enhance the plant growth and grain yield by similar mechanisms followed by symbiotic bacteria (Glick, 1995; Ahemad and Kibret, 2014). This study concludes that nodule-associated diazotrophic bacteria could be a valuable bio-agent for selection of effective PGP strains. Among the five cultivars studied in this investigation, Subhra was found to respond well by most of the PGP traits when treated with the three diazotrophic bacteria, IC-59, IC-76A and IC-2001. However, the other four cultivars were also not far behind in their PGP response. Hence, the three diazotrophic bacteria need to be exploited further for improving nodulation, nitrogen fixation, PGP and yields of chickpea. The selected diazotrophic strains of this study are highly valuable in formulation of new inoculants for commercial production. So for, soil-associated bacteria have been only widely used for PGPR formulations. In fact, mining root nodules for PGP bacteria will make it easier to find more compatible bacterial partners, particularly when one looks for developing a consortia. Further, diversification of PGP bacteria having the ability to fix biological N_2 would have an added advantage in counter balancing the loss of N_2 from soils.

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Conflict of interest

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

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