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ORIGINAL ARTICLE

Endophytic occupation of legume root nodules by *nif*H-positive non-rhizobial bacteria, and their efficacy in the groundnut (*Arachis hypogaea*)

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Abstract Despite the high specificity between *Rhizobium* and legumes, the presence of non-rhizobia in root nodules has been reported. The present investigation was carried out to isolate plant growth-promoting endophytic non-rhizobial bacteria from leguminous crops viz. *Glycine max*, *Vigna radiata*, *Vigna unguiculata*, *Arachis hypogaea* and *Vigna mungo*, and their influence on groundnut growth. Seven endophytic non-rhizobial bacteria were isolated possessing the *nif*H gene showing amplification of a ~380-bp fragment. Analysis by 16S rDNA gene sequence similarity characterized them as *Enterobacter cloacae* AS1 (accession no. KF758544), *Chryseobacterium indologenes* AM2 (KF758545), *Enterobacter cloacae* ACP3 (KF758546), *Klebsiella pneumoniae* AG4 (KF758547), *Pseudomonas aeruginosa* ABG5 (KF758548), *Enterobacter ludwigii* ABG6 (KF758549) and *Klebsiella variicola* ABG7 (KF758550). Qualitative estimation of nitrogen fixation confirmed the efficiency of isolates to fix atmospheric nitrogen in a range of 11.55 to 62.10 mg N g^{-1} of glucose consumed. All strains were found to produce indoleacetic acid ranging from 34.86 to 119.44 $\mu g\ ml^{-1}$. The highest phosphate solubilization index was shown by *K. pneumoniae* AG4 (14.67). Siderophore production was observed only in *P. aeruginosa* ABG5, and the highest 1-aminocyclopropane-1-carboxylate deaminase activity was shown by *C. indologenes* AM2, *E. cloacae* ACP3 and *P. aeruginosa* ABG5. Plant growth stimulation was confirmed by seed application in groundnuts

in a gnotobiotic system and in pots. The results showed that isolates had a positive effect on groundnut growth, with no negative influence. In addition, treatment with 50 % recommended dose of chemical fertilizer demonstrated a positive influence on groundnut growth, but an increase in concentration from 50 to 100 % resulted in adverse effects on populations of non-rhizobial bacteria and groundnut growth. Thus the results of this study clearly suggest that root nodules accommodate endophytic non-rhizobial bacteria, which stimulates plant growth, and that chemical fertilizers greatly affect their number and activity.

Keywords Endophytic non-rhizobial bacteria · Endophyte · Legume · Nitrogen fixation · *nif*H gene

Introduction

Plant growth-promoting bacteria (PGPB) are an important group of bacteria that enhance plant growth and yield through a variety of mechanisms including the production of plant growth-stimulating hormones, the suppression of pathogens, nitrogen fixation, increased availability of nutrients (macro and micro), and improvement in soil structure, thereby enhancing root growth and promoting other beneficial plant–microbe symbiosis (Vessey 2003). These PGPB are associated with plants through colonization of the rhizosphere (rhizobacteria), phyllosphere (epiphytes) or living plant tissues (endophytes).

In recent decades, endophytic bacteria from root nodules have attracted increasing attention as novel resources for the promotion of plant growth and biological control of plant diseases. In the early days, it was believed that the root nodules of leguminous plants accommodated only endophytic bacteria of the genera *Rhizobium*, *Mesorhizobium*,

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Bradyrhizobium, *Ensifer* and *Azorhizobium*—collectively known as rhizobial bacteria—with fixation of atmospheric nitrogen occurring symbiotically. However, root nodules also accommodate various non-rhizobial bacteria, which have a definite influence on the survival, nodulation and grain yield of the crop (Lin et al. 2008; Pandya et al. 2013; Zhang et al. 1996; Ibanez et al. 2009; Tariq et al. 2012; Rajendran et al. 2011 and Egamberdieva et al. 2010).

Over the past decade, several symbionts capable of forming nodules and fixing nitrogen in legume roots have been documented and grouped under α, β and γ Proteobacteria, including *Methylobacterium nodulans*, *Blastobacter denitrificans*, *Devosia* sp., *Ochrobactrum lupini*, *Agrobacterium*, *Phyllobacterium trifolii*, *Herbaspirillum lusitanum*, *Ralstonia taiwanensis*, *Burkholderia tuberum*, *Burkholderia phymatum* and *Burkholderia cepacia* (Balachandar et al. 2007). The occurrence of *Bacillus* species as root nodule endophytes has also been reported in various plants such as pigeon pea, wheat, kudzu and soybean (Zhao et al. 2011). Tariq et al. (2012) reported that endophytic non-rhizobial bacteria acted synergistically with rhizobia to enhance nodulation and growth, possibly through IAA production, phosphate solubilization, nitrogen fixation and siderophore production. Stajkovic et al. (2009) reported 14 bacterial genera associated with wild legume nodules in Tunisia and over 24 non-rhizobial taxa isolated from nodules of different wild legumes.

The groundnut (*Arachis hypogaea*) is an important annual herbaceous, self-pollinated allotetraploid ($2n = 4x = 40$) leguminous plant, having a genome size of 2891 Mb. It is grown on nearly 23.95 million ha worldwide, with total production of 40.12 million metric tons and an average yield of 1068 metric tons per hectare (USDA 2013, available at https://www.nass.usda.gov/Publications/Ag_Statistics/2013/Agricultural_Statistics_2013.pdf). China, India, Nigeria, the USA and Myanmar are the major groundnut-producing countries globally. Groundnut seeds are a rich source of nutrients, providing over 30 essential phytonutrients including niacin, folate, fiber, vitamin E, magnesium (52 %) and phosphorus (48 %), are naturally free of trans fats and sodium, and contain about 25 % protein. The residual oilcake contains 7 to 8 % of N, 1.5 % of P_2O_5, and 1.2 % of K_2O, and is used as a fertilizer. In addition to its nutritional and agricultural importance, the groundnut (as a legume) plays an important role in improving soil fertility through the process of biological nitrogen fixation (BNF).

In BNF, atmospheric nitrogen is converted to ammonia by the nitrogenase enzyme complex containing Fe and Mo-Fe proteins encoded by *nif* genes, which helps maintain and enhance soil fertility. Multiple subunits of nitrogenase enzyme are encoded by the three genes *nif*H, *nif*D and *nif*K (Rubio and Ludden 2002). Of these, the *nif*H gene encoding the dinitrogenase reductase subunit is the most well studied and best sequenced. Therefore, the identification and study of the microflora associated with root nodules possessing the *nif*H gene is essential for understanding their positive or negative impact on leguminous crop growth. To this end, the present study aimed to isolate, characterize and identify endophytic non-rhizobial microflora from various root nodules that are capable of atmospheric nitrogen fixation and that possess the *nif*H gene, in order to determine their effect as PGPB on the growth of the groundnut GG-31, and thus their potential as a sustainable and inexpensive alternative to the use of agrochemicals.

Materials and methods

Isolation and screening of endophytic non-rhizobial bacteria

Root nodule samples of various leguminous crops viz. *Arachis hypogaea* (groundnut), *Vigna unguiculata* (cowpea), *Vigna mungo* (black gram), *Glycine max* (soybean) and *Vigna radiata* (mung bean) were collected for isolation. The samples were grown on the Anand Agricultural University farm on sandy loam soil, with average humidity of 68–75 %, rainfall of 800–1000 mm (Priyan 2015) and temperature of 26–28 °C in the kharif season.

Isolation of endophytic bacteria was carried out according to the method described by Selvakumar et al. (2008), with the following modifications: yeast extract-mannitol agar was replaced with nitrogen-free bromothymol blue, nutrient agar, Jensen's agar, Pikovskaya's medium (PKVK), King's B medium and PCAT selective medium for *Burkholderia* (Di Cello et al. 1997). Nodules excised from the roots were surface-sterilized with 70 % ethanol for 30 s followed by 0.1 % $HgCl_2$ for 2 min, and were washed three times with sterile distilled water for 1 min each under aseptic conditions. The nodules were then crushed using sterile forceps, and 100-μl aliquots of the contents were inoculated onto the different media as described above.

For confirmation of surface sterilization, water from the final rinse was spread onto R2A medium (0.5 g peptone, 0.5 g starch, 0.5 g glucose, 0.5 g yeast extract, 0.5 g casein hydrolysate, 0.3 g dipotassium phosphate, 0.3 g sodium pyruvate, 0.024 g magnesium sulphate anhydrous, 15 g agar, 1000 ml distilled water, pH 7.2) and incubated at 28 ± 2 °C for 36 h. Isolated colonies were further screened for nitrogen fixing capability on Dworkin–Foster (DF) minimal salt nitrogen-free medium and incubated at 28 ± 2 °C for 48 h.

Detection of the *nif*H gene

Fourteen pure isolates were selected after screening on nitrogen-free medium, subjected to PCR amplification of the region coding for Fe protein (component II) of the nitrogenase enzyme complex using degenerate primers described by Poly et al. (2001; Pol F- 5′ TGCGAYCCSAARGCBGACTC 3′

and Pol R-5′ ATSGCCATCATYTCRCCGGA 3′) and using colony PCR (Mirhendi et al. 2007), with PCR amplification conditions modified as follows: initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s for 25 cycles, and final extension at 72 °C for 10 min.

Characterization and identification of endophytic non-rhizobial bacteria

Morphological, cultural and biochemical characteristics of the purified isolates were studied using standard procedures described by Holt et al. (1994). In addition, seven isolates were grown in Luria broth for 24 h, and genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Sambrook et al. 1989; Jhala et al. 2014). The integrity and concentration of the purified DNA was determined by agarose gel electrophoresis. The total genomic DNA extracted was dissolved in Tris-EDTA buffer and stored at 4 °C.

To further confirm the results of morphological and biochemical studies, sequencing of the 16S ribosomal RNA (rRNA) gene was performed. This sequencing has emerged as a powerful technique for phylogenetic placement, identification and diversity analysis of bacteria, and was carried out using the set of primers suggested by Di Cello et al. (1997) (27 F- 5′AGAGTTTGATCCTGGCTCAG 3′ and 1492 R- 5′ GGTTACCTTGTTACGACTT 3′).

16S rDNA amplification was performed in a thermal cycler (Eppendorf Mastercycler; Eppendorf AG, Hamburg, Germany) with a 25-μl reaction mixture containing 50 ng of genomic DNA, 0.2 mM of each dNTPs, 1 μM of each primer (Lee et al. 2005), 2.5 mM of $MgCl_2$, and 1 U of Taq DNA polymerase (Bangalore Genei, India) and the buffer supplied with the enzyme. The output sequences were subjected to BLAST [Basic Local Alignment Search Tool] analysis to identify the cultures and to determine the nearest match (http://www.ncbi.nlm.nih.gov). A neighbor-joining phylogenetic tree was constructed using Phylogeny.fr software (http://www.phylogeny.fr/).

Quantitative estimation of nitrogen fixation

Quantitative estimation of nitrogen was carried out using the Micro-Kjeldahl method (AOAC 1965), and sugar utilization was estimated using Fehling's method (Stiles et al. 1926).

IAA production

IAA production assay was performed as described by Glickmann and Dessaux (1995). Bacterial isolates were grown in glucose phosphate broth containing L-tryptophan (0.005 M) for 3 days on a shaker at 100 rpm, and then centrifuged at 3000 rpm for 20 min. One milliliter of supernatant

was mixed with 2 ml of Salkowski's reagent (2.03 g of 0.5 M $FeCl_3$+ 300 ml Conc. H_2SO_4). An uninoculated control was kept for comparison. The intensity of the pink color that developed within 30 min was measured at 535 nm on a UV/Vis spectrophotometer. The quantity of IAA was determined by comparison with an IAA standard curve and expressed in μg ml^{-1}.

Phosphate solubilization capacity

Phosphate solubilization activity of the selected isolates was determined on Sperber's agar using the method described by Taurian et al. (2010). Bacterial isolates were inoculated using a toothpick on agar plates under aseptic conditions and were incubated at 30 ± 2 °C for 5 days. Observations of colony diameter and solubilization of tricalcium phosphate were recorded every 24 h. The formation of a clear zone around the growing colony indicated phosphate solubilization activity.

Efficiency of siderophore production

The production of siderophores was determined using the chrome azurol S (CAS) agar method (Schwyn and Neilands 1987). Bacterial isolates were inoculated at the center of a CAS agar plate. Siderophore production was assessed by a change in the color of the medium surrounding the colony from blue to orange after incubation at 28 °C for 5 days.

ACC (1-aminocyclopropane-1-carboxylate) deaminase production

ACC deaminase enzyme production capability was assessed based on the ability to use ACC as a sole nitrogen source in a minimal medium (Duan et al. 2009). Cultures were spot-inoculated on plates containing minimal medium supplemented with 3 mM ACC substrate. Plates containing minimal medium without ACC served as negative control, and those with $(NH_4)_2SO_4$ (2.0 gm l^{-1}) as a nitrogen source served as positive control. The plates were incubated for 4 days at 28 ± 2 °C. The growth of isolates on ACC-supplemented plates was compared with that of positive and negative control plates.

Plant growth stimulation in a gnotobiotic system

To evaluate the effect of endophytic non-rhizobial strains, seeds of groundnut cv. GG-31 were surface-sterilized by washing under running tap water and then with 70 % ethanol solution for 30 s, followed by treatment with 0.1 % $HgCl_2$ for 2 min, and were then washed three times with sterile distilled water for 1 min each under aseptic conditions. The surface-sterilized seeds were then treated with individual 24-h-old isolates grown in nutrient broth at 28 ± 2 °C containing approximately 10^8 cells ml^{-1}, keeping untreated seeds as

control. Each treatment was carried out three times and seeds were allowed to grow in 100-ml tubes containing sterilized 40-ml plant nutrient semisolid medium under dark conditions at 28 ± 2 °C. The percentage of germination was recorded after 72 h (Stajkovic et al. 2009). After germination, the plants were exposed to artificial light in a 12-h light/dark cycle in a plant growth chamber. Root and shoot length, fresh weight and dry weight observations were recorded after 30 days.

Plant growth stimulation in pots

An inoculation study was carried out in pots under controlled conditions similar to that in tubes, using deep black loamy sand (Solanki 2012) from Middle Gujarat, India, known locally as "Goradu". The soil had pH 7.0 and electrical conductivity of 0.17 dSm^{-1} at 25°. Seeds of the groundnut cv. GG-31 were surface-sterilized and inoculated with bacteria as described above. The pots used for the experiment were 25 cm × 28 cm, with a capacity of 10 kg soil. The treatments were as follows:

> T1- Control
> T2- 100 % recommended dose of fertilizer (RDF) (6.25:12.5:: N:P mg kg^{-1})
> T3- 75 % RDF (4.69:9.38:: N:P mg kg^{-1})
> T4- 50 % RDF (3.12:6.25:: N:P mg kg^{-1})
> T5- *E. cloacae* AS1
> T6- *C. indologenes* AM2
> T7- *E. cloacae* ACP3
> T8- *K. pneumoniae* AG4
> T9- *P. aeruginosa* ABG5
> T10- *E. ludwigii* ABG6
> T11- *K. variicola* ABG7

The percentage of germination was recorded at day 10 after sowing (DAS). After harvest, quantification was carried out for root and shoot length, fresh weight, dry weight, chlorophyll content, nodules per plant, organic carbon by wet digestion, total nitrogen, phosphorus by Olsen's (NaHCO$_3$) method described by Tandon (1995), and total microbial population in the soil.

Statistical analyses

The data obtained from the above experiments were statistically analyzed using a completely randomized design (CRD), with significance set at a probability level of 0.05. Data were subjected to analysis of variance and means and were compared by Duncan's new multiple range test (DNMRT; Duncan 1955). WASP [Web Agri Stat Package 2.0] software developed by ICAR-Central Coastal Agricultural Research Institute, Goa, India, was used for analysis, with a 5 % level of significance.

Results

Isolation and screening of endophytic non-rhizobial bacteria

A total of 34 isolates were obtained from root nodules of five plants; after screening for nitrogen fixation, 14 were able to grow and flourish on nitrogen-free medium. No bacterial growth was observed on R2A medium plates on which the water from the final rinse after surface sterilization was spread.

Detection of the *nif*H gene

Among the 14 isolates that grew and flourished on nitrogen-free medium, seven produced a single band of ~380 bp (Fig. 1), indicating the capacity for atmospheric nitrogen fixation.

Characterization and identification of endophytic non-rhizobial bacteria

The seven isolates demonstrated variable growth patterns, as shown in Table 1. Isolate AM2 showed yellow pigment and ABG5 showed fluorescent blue pigment, whereas isolates

Fig. 1 *Nif*H gene amplification from promising non-rhizobia. (M) 100-bp DNA ladder; (1) Std. *Rhizobium* sp. (accession no. KC787583); (2) *E. cloacae* AS1; (3) Isolate S5; (4) Isolate M3; (5) *C. indologenes* AM2; (6) Isolate M5; (7) Isolate CP1; (8) Isolate CP5; (9) *E. cloacae* ACP3; (10) Isolate G2; (11) *K. pneumoniae* AG4; (12) *P. aeruginosa* ABG5; (13) *E. Ludwigii* ABG6; (14) *K. variicola* ABG7
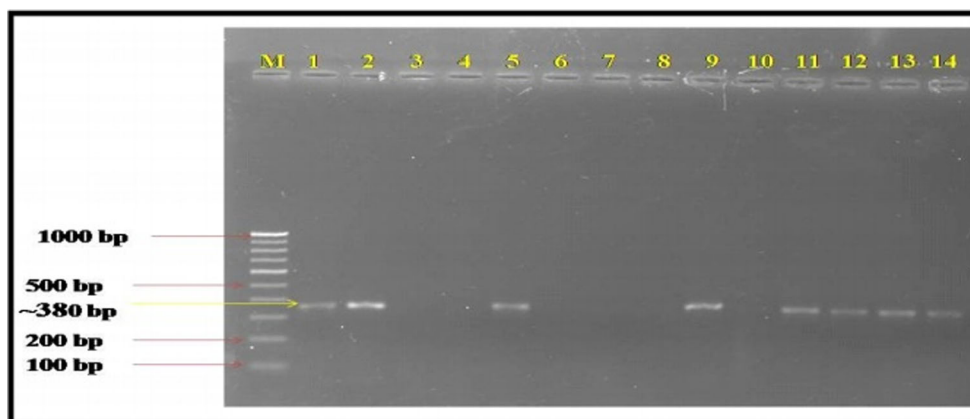
Table 1 Characterization of isolates

Test	AS1	AM2	ACP3	AG4	ABG5	ABG6	ABG7
ortho-Nitrophenyl-β-galactoside	+	+	+	+	+	+	+
Lysine utilization	−	−	−	+	−	−	−
Ornithine utilization	+	+	+	−	−	+	+
Urease	−	−	−	−	−	−	−
Phenylalanine deamination	+	+	+	+	+	+	+
Nitrate reductase	+	+	+	+	+	+	+
H_2S production	+	−	+	+	+	+	+
Citrate utilization	+	−	+	+	+	+	+
Voges–Proskauer	+	−	+	+	−	+	
Methyl red	−	−	−	−	−	−	−
Indole	−	−	−	−	−	−	−
Malonate utilization	+	−	+	+	+	+	+
Esculin hydrolysis	+	+	+	+	−	+	+
Arabinose	+	−	+	+	+	+	−
Xylose	+	−	+	+	+	+	+
Adonitol	−	−	−	+	−	−	−
Rhamnose	+	−	+	+	−	+	−
Cellobiose	+	−	+	+	+	+	−
Melibiose	+	−	+	+	−	+	−
Saccharose	+	−	+	+	−	+	−
Raffinose	+	−	+	+	−	+	−
Trehalose	+	−	+	+	−	+	−
Glucose	+	+	+	+	+	+	+
Lactose	−	−	−	+	−	−	−
Oxidase	+	+	+	+	+	+	+
Growth on Hofer's alkaline medium	+	−	+	+	+	+	+

+ Positive test, − negative test

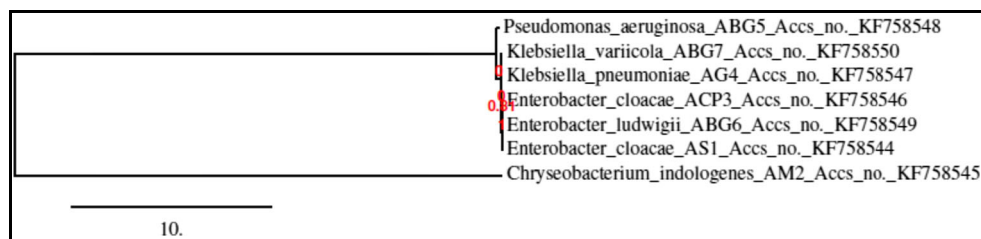AS1, AG4, ACP3, ABG6 and ABG7 showed no pigmentation on DF media.

PCR amplification of the 16S rRNA gene from seven isolates was carried out using universal primers (27 F and 1492 R). All isolates produced a single band of ~1500 bp on 2 % agarose gel. From the 16S rRNA gene sequence, isolate AS1 was identified as *Enterobacter cloacae*, with 100 % similarity and 99 % query coverage to *E. cloacae* strain SDM. Isolate AM2 was identified as *Chryseobacterium indologenes*, showing 97 % identity with *C. indologenes* strain LMG8337, with 95 % query coverage, which confirmed that isolate AM2 belonged to the *Chryseobacterium* genus. Isolate ACP3 was identified as *Enterobacter cloacae*, showing 99 % identity with *E. cloacae* strain 279–56, with 100 % query coverage. Isolate AG4 was identified as *Klebsiella pneumoniae*, showing 99 % identity with *K. pneumoniae* strain MGH 78578, with 100 % query coverage. Isolate ABG5 was identified as *Pseudomonas aeruginosa*, showing 99 % identity with *P. aeruginosa* strain PAO1, with 100 % query coverage. Isolate ABG6 was identified as *Enterobacter ludwigii*, showing 99 % identity with *E. ludwigii* strain EN-119 = DSMZ, with 100 % query coverage. Isolate ABG7 was identified as

Klebsiella variicola, showing 99 % identity with *K. variicola* strain At-22, with 100 % query coverage. The sequence analysis of the partial 16S rRNA gene of isolates AS1, AM2, ACP3, AG4, ABG5, ABG6 and ABG7 were deposited in the NCBI GenBank under accession numbers KF758544, KF758545, KF758546, KF758547, KF758548, KF758549 and KF758550, respectively. The analysis named isolate AS1 as *E. cloacae* AS1, isolate AM2 as *C. indologenes* AM2, isolate ACP3 as *E. cloacae* ACP3, isolate AG4 as *K. pneumoniae* AG4, isolate ABG5 as *P. aeruginosa* ABG5, isolate ABG6 as *E. ludwigii* ABG6 and isolate ABG7 as *K. variicola* ABG7. The neighbor-joining phylogenetic tree of all isolates and related taxa are grouped together in Fig. 2.

Quantitative estimation of nitrogen fixation

The results of quantitative estimation of nitrogen fixing by isolates are listed in Table 2. All isolates demonstrated atmospheric nitrogen fixation in a range of 11.55 to 62.10 mg N g^{-1} of glucose consumed, with isolate *E. ludwigii* ABG6 showing the highest nitrogen fixation capacity (62.10 mg N g^{-1} of glucose).

Pseudomonas_aeruginosa_ABG5_Accs_no._KF758548
Klebsiella_variicola_ABG7_Accs_no._KF758550
Klebsiella_pneumoniae_AG4_Accs_no._KF758547
Enterobacter_cloacae_ACP3_Accs_no._KF758546
Enterobacter_ludwigii_ABG6_Accs_no._KF758549
Enterobacter_cloacae_AS1_Accs_no._KF758544
Chryseobacterium_indologenes_AM2_Accs_no._KF758545

10.

IAA production

All isolates were capable of producing IAA, which ranged from 34.86 to 119.44 µg ml^{-1} (Table 2) at 3 days after incubation. The highest concentration was recorded in isolate *E. ludwigii* ABG6 (119.44 µg ml^{-1}), followed by isolate *K. pneumonia* AG4 (117.96 µg ml^{-1}), while isolates *P. aeruginosa* ABG5 and *E. cloacae* AS1 produced the lowest IAA, at 34.86 and 39.86 µg ml^{-1}, respectively.

Phosphate solubilization capacity

Isolate *K. pneumoniae* AG4 showed the highest solubilization index (SI), at 14.67 mm (Table 2), followed by isolate *E. cloacae* ACP3 (14.00 mm).

Efficiency of siderophore production

Isolate *P. aeruginosa* BG8 produced a yellow-orange color zone on CAS agar plates, and hence was confirmed as a siderophore producer.

ACC deaminase production

All isolates were found to grow luxuriously on positive control plates containing $(NH_4)_2SO_4$ as a nitrogen source, whereas they grew poorly on negative control plates containing nitrogen-free MS media. Isolates *C. indologenes* AM2,

E. cloacae ACP3 and *P. aeruginosa* ABG5 showed luxurious growth on plates with ACC as the sole source of nitrogen, indicating their ability to produce enzyme ACC deaminase (Table 2).

Stimulation of plant growth in a gnotobiotic system

The seed application of all isolates had a notable effect on the growth parameters of groundnut cv. GG-31 compared to the uninoculated control (Table 3). Application with isolates *E. cloacae* AS1, *C. indologenes* AM2, *K. pneumoniae* AG4 and *P. aeruginosa* ABG5 showed 100 % germination.

The greatest increase in shoot length compared to uninoculated control was recorded with *C. indologenes* AM2 (4.85 cm), followed by *K. pneumoniae* AG4 (4.77 cm), *P. aeruginosa* ABG5 (4.23 cm) and *K. variicola* ABG7 (4.20 cm). The greatest root length was demonstrated by *P. aeruginosa* ABG5 (1.46 cm), which was on par with treatment with *E. cloacae* AS1 (1.42 cm), *C. indologenes* AM2 (1.32 cm), *E. cloacae* ACP3 (1.45 cm) and *K. variicola* ABG7 (1.11 cm). The increase in fresh biomass was highest for treatment with *E. Ludwigii* ABG6 (51.40 %). The maximum shoot fresh (1.81 g plant^{-1}) and dry (0.46 g plant^{-1}) weight was observed with *K. pneumoniae* AG4. Significantly higher root fresh (0.69 g plant^{-1}) and dry (0.44 g plant^{-1}) weight was observed with *C. indologenes* AM2. A greater increase in dry biomass was found for treatment with *K. pneumoniae* AG4 (44.63 %) and *E. Ludwigii* ABG6 (44.63 %). The data as a whole show that all the endophytic non-rhizobial bacteria had a positive

Table 2 Quantitative estimation of growth-promoting traits

Isolate	Nitrogen fixation capacity (mg N/g glucose consumed)	IAA concentration (µg ml^{-1})	Phosphate solubilization index (mm)	ACC deaminase
E. cloacae AS1	48.99	39.86[c]	5.33[c]	−
C. indologenes AM2	19.55	87.04[b]	1.33[d]	++
E. cloacae ACP3	15.77	75.21[b]	14.00[a]	++
K. pneumoniae AG4	20.97	117.96[a]	14.67[a]	+
P. aeruginosa ABG5	11.55	34.86[c]	5.50[c]	++
E. ludwigiiABG6	62.10	119.44[a]	12.00[b]	+
K. variicola ABG7	45.62	76.06[b]	1.33[d]	−
S.Em.±	NS	6.86	0.79	−
CV %	11.47	10.67	12.51	−

++ Higher growth, + Moderate growth, − No growth

Different letters in rows indicate significant difference at $P \leq 0.05$

Table 3 Effect of non-rhizobial bacteria on the germination and growth of groundnuts under gnotobiotic conditions

Treatment	Germination (%)	Shoot length (cm)	Root length (cm)	Shoot fresh wt. (g plant^{-1})	Increase in fresh biomass (%)	Root fresh wt. (g plant^{-1})	Shoot dry wt. (g plant^{-1})	Root dry wt.	Increase in dry biomass (%)
Uninoculated control	66.67[b]	3.17[c]	0.51[d]	0.89[f]	–	0.15[e]	0.28[c]	0.024[c]	–
E. cloacae AS1	100.0[a]	3.83[bc]	1.42[a]	1.02[ef]	38.10	0.66[ab]	0.38[b]	0.042[a]	27.96
C. indologenes AM2	100.0[a]	4.85[a]	1.32[ab]	1.62[ab]	54.98	0.69[a]	0.46[ab]	0.044[a]	39.68
E. cloacae ACP3	66.67[b]	3.70[bc]	1.45[a]	1.18[de]	40.23	0.56[c]	0.46[ab]	0.044[a]	39.68
K. pneumoniae AG4	100.0[a]	4.77[a]	0.98[c]	1.81[a]	50.24	0.28[d]	0.46[ab]	0.044[a]	39.68
P. aeruginosa ABG5	100.0[a]	4.23[ab]	1.46[a]	1.33[cd]	44.97	0.56[c]	0.51[ab]	0.039[ab]	44.63
E. ludwigii ABG6	66.67[b]	3.63[bc]	0.96[c]	1.65[ab]	51.40	0.49[c]	0.51[a]	0.039[ab]	44.63
K. variicola ABG7	66.67[b]	4.20[ab]	1.11[ab]	1.53[bc]	50.71	0.58[bc]	0.49[bc]	0.029[bc]	41.43
S.Em.±	12.56	0.38	0.11	0.13		0.048	0.043	0.004	
CV %	18.29	10.71	10.85	10.82		11.03	11.24	12.59	

Different letters in rows indicate significant difference at $P \leq 0.05$

effect on groundnut GG-31 growth and development in the laboratory, and no treatment had a negative impact.

Plant growth stimulation in pots

The results of plant growth stimulation by endophytic nonrhizobial bacteria on groundnut cv. GG-31 in pots are presented in Table 4. Complete germination was observed in *C. indologenes* AM2 and *E. cloacae* ACP3, followed by *K. pneumoniae* AG4 and *K. variicola* ABG7 (94.44 %). Shoot length was greatest in *K. pneumoniae* AG4 (62.67 cm), followed by *C. indologenes* AM2 (61 cm), while the shortest shoots were found in the uninoculated control (42.33 cm) and 100 % RDF (43 cm). The greatest root length was observed in *E. cloacae* ACP3 (17.33 cm), followed by *C. indologenes* AM2 (17 cm).

Significantly higher chlorophyll content was observed with all bacterial treatments and with 50 % RDF, ranging from 0.060 to 0.065 mg cm^{-2}, while the lowest was observed in the uninoculated control (0.056 mg cm^{-2}). The highest number of nodules per plant were noted in *E. cloacae* ACP3 (94.11), followed by *E. cloacae* AS1 (93.78), *C. indologenes* AM2 (89.89) and *K. pneumoniae* AG4 (89.11). However, the lowest number of nodules per plant was observed in 100 % RDF, indicating that chemical fertilizers (CF) exerted a strong adverse effect on nodulation. Significant shoot weight was found in treatments with *C. indologenes* AM2 (22.33 g plant^{-1}), *E. cloacae* ACP3 (22.14 g plant^{-1}), *E. Ludwigii* ABG6 (22.22 g plant^{-1}), *K. variicola* ABG7 (21.84 g plant^{-1}) and *K. pneumoniae* AG4 (21.43 g plant^{-1}).

Shoot dry weight was significantly greater in treatments with *C. indologenes* AM2 (19.1 g plant^{-1}), followed by *E. cloacae* ACP3 (17.84 g plant^{-1}) and *K. variicola* ABG7 (17.35 g plant^{-1}), than in the uninoculated control and the 100 % RDF. Significantly higher root fresh (7.9 g plant^{-1}) and dry weight (4.31 g plant^{-1}) was observed in treatments with *C. indologenes* AM2 compared to uninoculated control. The highest fresh (52.75 %) and dry (68.54 %) biomass was found in treatments with *C. indologenes* AM2.

Overall, significantly higher growth parameters were observed with *C. indologenes* AM2, including seed germination (100 %), shoot length (61 cm), root length (17 cm), chlorophyll content (0.064 mg cm^{-2}), nodules per plant (89.89), fresh (22.33 g plant^{-1}) and dry (19.10 g plant^{-1}) weight of shoots, and fresh (7.9 g plant^{-1}) and dry (4.31 g plant^{-1}) weight of roots of the groundnut cv. GG-31 compared to uninoculated control and 100 % RDF.

There was no significant difference in soil organic carbon content compared to the control, but *K. variicola* ABG7 showed the highest percentage of nitrogen content, at 0.050 %, followed by 50 % RDF and *P. aeruginosa* ABG5, with 0.045 and 0.044 % available nitrogen, respectively. Treatment with *K. pneumonia* AG4 showed the highest

Table 4 Effect of non-rhizobial bacteria on germination and growth parameters of groundnut cv. GG-31 in pots

Treatment	Germination (%)	Shoot length (cm)	Root length (cm)	Chlorophyll content (mg cm^{-2})	Nodules per plant^{-1}	Shoot fresh wt. (g plant^{-1})	Root fresh wt. (g plant^{-1})	Increase in fresh biomass (%)	Shoot dry wt. (g plant^{-1})	Root dry wt. (g plant^{-1})	Increase in dry biomass (%)
Uninoculated control	72.22^{d}	42.33^{e}	8.00^{g}	0.056^{c}	50.22^{c}	15.32^{c}	4.47^{f}	–	12.32^{d}	1.57^{e}	–
100 % RDF (12.5:25::N:P kg ha)	77.77^{cd}	43.00^{e}	9.33^{fg}	0.057^{bc}	45.67^{c}	16.22^{c}	4.50^{f}	4.70	13.46^{cd}	2.13^{d}	12.24
75 % RDF (9.38:18.75::N:P kg ha^{-1})	83.33^{bcd}	44.67^{de}	10.00^{ef}	0.058^{bc}	52.89^{c}	16.33^{c}	5.23^{e}	8.94	13.91^{cd}	2.31^{d}	16.77
50 % RDF (6.25:12.5::N:P kg ha^{-1})	88.89^{abc}	47.33^{de}	10.67^{ef}	0.061^{ab}	53.89^{c}	16.36^{c}	5.48^{e}	10.36	13.91^{cd}	2.42^{d}	17.57
E. cloacae AS1	88.89^{abc}	49.67^{cde}	12.67^{d}	0.062^{ab}	93.78^{a}	16.37^{c}	7.57^{ab}	20.97	14.88^{bc}	3.66^{bc}	33.48
C. indologenes AM2	100.00^{a}	61.00^{a}	17.00^{ab}	0.064^{a}	89.89^{a}	22.33^{a}	7.90^{a}	52.75	19.10^{a}	4.31^{a}	68.54
E. cloacae ACP3	100.00^{a}	58.67^{ab}	17.33^{a}	0.064^{a}	94.11^{a}	22.14^{a}	6.53^{cd}	44.87	17.84^{a}	3.46^{bc}	53.35
K. pneumoniae AG4	94.44^{ab}	62.67^{a}	11.67^{de}	0.060^{abc}	89.11^{a}	21.43^{a}	6.77^{cd}	42.50	15.37^{bc}	3.32^{c}	34.56
P. aeruginosa ABG5	88.89^{abc}	52.00^{bcd}	15.33^{bc}	0.065^{a}	85.22^{a}	18.80^{b}	6.27^{d}	26.68	14.59^{cd}	3.79^{b}	32.33
E. ludwigiiABG6	88.89^{abc}	56.33^{abc}	15.00^{c}	0.063^{a}	65.67^{b}	22.22^{a}	6.66^{cd}	45.93	17.60^{ab}	3.43^{bc}	51.40
K. variicola ABG7	94.44^{ab}	50.67^{cd}	14.67^{c}	0.060^{ab}	66.89^{b}	21.84^{a}	7.19^{bc}	46.69	17.35^{ab}	3.32^{bc}	48.81
S.Em.±	5.55	2.603	0.681	0.0012	3.122	0.60	0.232	–	0.785	0.158	–
CV %	10.83	8.73	9.17	3.54	7.53	5.5	6.46	–	8.68	8.95	–

Different letters in rows indicate significant difference at $P \leq 0.05$

available phosphorus, at 53.16 kg ha^{-1}, followed by treatment with *C. indologenes* AM2, at 51.67 kg ha^{-1} (Table 5). The highest bacterial population was observed in treatment with *C. indologenes* AM2, at 9.5×10^{6}, followed by *K. variicola* ABG7, at 8.9×10^{6}, as shown in Fig. 3.

Discussion

Despite the high specificity between legumes and *Rhizobium*, non-rhizobia such as *K. pneumoniae*, *P. fluorescence*, *Bacillus* sp., *Enterobacter* sp., *Klebsiella* sp. and *Paenibacillus* sp. have been found to be present inside the root nodules (Pandya et al. 2013; Zhang et al. 1996; Ibanez et al. 2009; Tariq et al. 2012; Rajendran et al. 2011; Egamberdieva et al. 2010). *Enterobacter* and *Klebsiella* are members of the family *Enterobacteriaceae*, and many species of these two genera are known to occur endophytically in the root nodules of a number of crops (Pandya et al. 2013; De Meyer et al. 2015; Selvakumar et al. 2008). De Meyer et al. (2015) reported that many of the isolates from root nodules belonged to the genus *Pseudomonas* (15.9 %).

In the present study, seven potential plant growth-promoting non-rhizobial bacteria were isolated from the root nodules of various leguminous plants, and were identified as *Enterobacter cloacae* AS1 (accession no. KF758544), *Chryseobacterium indologenes* AM2 (KF758545), *Enterobacter cloacae* ACP3 (.KF758546), *Klebsiella pneumoniae* AG4 (KF758547), *Pseudomonas aeruginosa* ABG5 (KF758548), *Enterobacter ludwigii* ABG6 (KF758549) and *Klebsiella variicola* ABG7 (KF758550). These bacteria differ in characteristics. For example, *E. cloacae* AS1, *E. cloacae* ACP3, *K. pneumoniae* AG4, *E. ludwigii* ABG6 and *K. variicola* ABG7 are Gram-negative, rod-shaped, oxidase-positive, H$_2$S-producing and citrate-positive bacteria, while *C. indologenes* AM2 is yellow-pigmented, Gram-negative, rod-shaped, chemoorganotrophic (nitrate reductase) and oxidase-positive, and *P. aeruginosa* ABG5 is Gram-negative, rod-shaped, citrate and oxidase-positive.

Bacterial promotion of plant growth is a well-established and complex phenomenon (Selvakumar et al. 2008; Vessey 2003), whereby bacteria may promote growth directly by facilitating resource acquisition or modulating plant hormone levels, or indirectly by reducing the inhibitory effects of various pathogenic agents (Gupta et al. 2014; Figueiredo et al. 2010; Bashan et al. 2008). In the present investigation, all bacteria demonstrated PGP traits of nitrogen fixation and phosphorus solubilization; these two nutrients are major constituents of chlorophyll, proteins, nucleic acid, phospholipids (Vessey 2003; Rodríguez and Fraga 1999) and ACC deaminase production, converting ethylene precursor ACC into 2-oxobutanoate and NH$_3$, and helping to overcome several

Table 5 Effect of non-rhizobial bacteria on soil organic carbon, nitrogen content and available phosphorus

Treatments	Organic carbon (%)	Available nitrogen (%)	Available phosphorus (Kg ha^{-1})
Before sowing	0.37	0.032[c]	29.57[f]
Uninoculated control	0.45	0.039[bc]	33.45[ef]
100 % RDF (12.5:25::N:P kg ha^{-1})	0.49	0.042[ab]	40.02[de]
75 % RDF (9.38:18.75::N:P kg ha^{-1})	0.50	0.043[ab]	44.20[cd]
50 % RDF (6.25:12.5::N:P kg ha)	0.52	0.045[ab]	44.50[bcd]
E. cloacae AS1	0.48	0.041[ab]	48.68[abc]
C. indologenes AM2	0.50	0.043[ab]	51.67[ab]
E. cloacae ACP3	0.46	0.040[bc]	43.01[cd]
K. pneumoniae AG4	0.47	0.040[bc]	53.16[a]
P. aeruginosa ABG5	0.51	0.044[ab]	40.02[de]
E. ludwigiiABG6	0.47	0.040[bc]	48.98[abc]
K. variicola ABG7	0.58	0.050[a]	42.71[cd]
S.Em.±	NS	0.003	3.71
CV %	10.00	10.44	10.15

Different letters in rows indicate a significant difference at $P \leq 0.05$

biotic as well as abiotic stresses. Siderophore-like compounds bind tightly to available iron (Fe^{-3}) in the rhizosphere, thereby preventing the growth of fungal pathogens (Takase et al. 2000; Kloepper et al. 1980). All isolates in the present study were also capable of producing IAA, which plays a crucial role in cell differentiation and regeneration of vascular tissue (Dalal and Kulkarni 2013).

Previous studies have suggested that non-rhizobia have a beneficial effect on the host plant (Vessey 2003; Kuklinsky et al. 2004; Ibanez et al. 2009; Tariq et al. 2012; Pandya et al. 2013; De Meyer et al. 2015; Selvakumar et al. 2008). Many researchers have also reported the beneficial effect of co-inoculating rhizobia with other bacteria (Zhang et al. 1996;

Rajendran et al. 2011; Egamberdieva et al. 2010). In the present study, endophytic non-rhizobial bacteria were found to have a positive effect on the growth attributes of the groundnut. Bhadoria et al. (2011) reported that chemical fertilizers (CF) had a negative influence on bacterial populations. Similarly, the present study demonstrated that an increase in CF level from 0 to 50 % significantly improved bacterial activity and groundnut growth. However, a further increase in CF level to 75 and 100 % resulted in lower bacterial population and growth. These results corroborate the theory that small "starter" doses of CF with bacterial inoculants may stimulate the growth of leguminous crops.
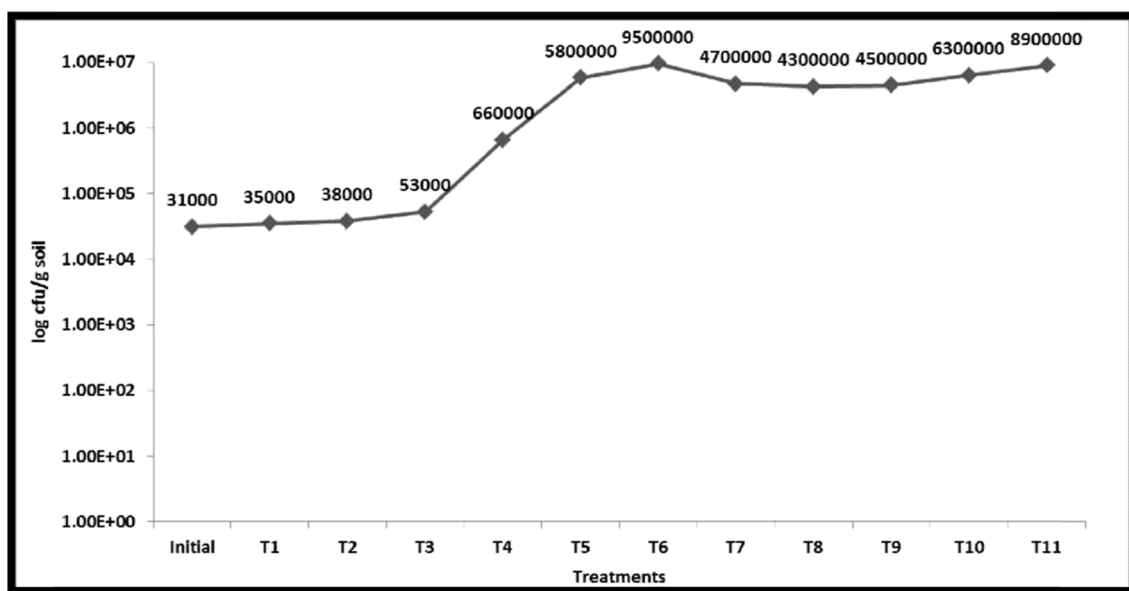

Fig. 3 Population recovery of bacteria from soil after groundnut harvesting

Conclusions

In this study, seven potential plant growth-promoting non-rhizobial bacteria isolated from the root nodules of various leguminous plants were clearly shown to have a positive effect on the growth of groundnut GG-31. Treatment with 50 % RDF of CF also demonstrated a positive influence on groundnut growth, whereas increasing the CF concentration from 50 to 100 % had an adverse effect on both the number of non-rhizobial bacteria and the growth of groundnuts. This suggests that microbial number and activity were significantly affected by chemical fertilizers, and 50 % RDF of CF with microbial inoculants could significantly increase the growth of leguminous plants.

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