https://doi.org/10.56093/ijas.v90i4.102207

Indian Journal of Agricultural Sciences **90** (4): 708–12, April 2020/Article

Identification and characterization of promising endophytic bacteria for growth promotion in chickpea (*Cicer arietinum*)

PRIYANKA BATRA*, MONIKA BARKODIA, UMANG AHLAWAT, REKHA SANSANWAL, RAJESH KUMAR VAID and LEELA WATI

CCS Haryana Agricultural University, Hisar, Haryana 125 004, India

Received: 09 July 2018; Accepted: 29 August 2019

ABSTRACT

The present study was carried out for the isolation and characterization of endophytic bacteria from chickpea nodules. A total of 107 endophytes were isolated from five districts of Haryana using three media, viz. YEMA for rhizobia, Pikovskaya and TSA for non-rhizobial isolates. The endophytes were then screened for various growth promoting traits like IAA production, Phosphate solubilization, Siderophore production, ACC utilization, Potassium solubilization and tolerance to NaCl concentrations. Total six endophytes, one rhizobium (HM2) and five non-rhizobial endophytes (RE6, BE13, ME3, HE5, HE7) were selected based on of plant growth promoting traits. Furthermore, the most promising non-rhizobial endophytes RE6 and BE13, compatible with Rhizobial isolate HM2 were *Pseudomonas protegens* and *Bacillus boroniphilus* using 16s rDNA sequencing. Both the isolates are non-pathogenic to humans and thus, are potential plant probiotics that can be used as biofertilizers.

Key words*:* Biofertilizers, Endophytes, Non-rhizobia, Rhizobia

Plants are associated with a variety of microorganisms that can be pathogenic, neutral, or beneficial for the host plant. Bacteria beneficial to plants, exhibiting plant growth promoting traits, improving plant health, or preventing disease, are collectively referred to as bacterial probiotics for plants (Haas and Keel 2003, Flores-Félix *et al.* 2015). These microorganisms promote plant growth either indirectly by helping plants to acquire nutrients, *e.g*. via nitrogen fixation, phosphate solubilization, siderophore production, or directly by producing phytohormones such as auxins or cytokinins or by producing the enzyme ACC deaminase, which lowers plant ethylene levels or by supplying essential vitamins to plants (Wakelin *et al.* 2004). Environmental and public health concerns have laid emphasis on the use of biofertilizers for increasing productivity of various crops.

Chickpea (*Cicer arietinum* L.), one of the most important winter crops, is a restrictive host for nodulation by rhizobia. Over the last few decades, the production of chickpea has declined in India because of various biotic and abiotic stress conditions (Khan *et al.* 1989). Despite chickpea being a major crop in India, endophytic bacteria living in its root nodules are relatively unmapped. However, recent studies suggest that several endophytes can effectively nodulate chickpea (Dudeja *et al.*2009). Thus, there is a possibility of increasing chickpea production by combining high yielding chickpea rhizobial endophytes, with effective rhizobial strains. The productivity of chickpea can be further increased using compatible rhizobial and non-rhizobial endophytes. In the present study, rhizobial and non-rhizobial endophytic bacteria were isolated and characterized by various growth promoting traits. The promising rhizobial endophytes were identified and checked for compatibility with rhizobial isolate. These probiotic endophytic bacteria could be successfully used in field applications for growth promotion in chickpea crops.

*Corresponding author e-mail: priyanka2779@gmail.com

MATERIALS AND METHODS

A total of 25 plants, five each from farmer's field of chickpea from different districts of Haryana, (Bhiwani (28.77N, 78.55E), Hisar (29.14N 75.72E), Jhajjar (28.60N, 78.65E), Mahenderagarh (28.27N, 76.14E), and Rewari (28.19N,78.61E)) were collected. The study was conducted at CCS Haryana Agricultural University, Hisar, Haryana in 2015.

Growth conditions and isolation of endophytic bacteria: Endophytic bacteria were isolated from the root nodules of chickpea according to method described by Vincent (1970). The collected nodules were first surface-sterilized with 70% ethanol, then with 0.1% mercuric chloride and finally washed thoroughly with distilled water. Bacterial isolates were obtained by streaking the crushed root nodules on Yeast extract mannitol agar (YEMA) medium for rhizobial endophytes, Pikovskaya's agar medium and Tryptone soya

agar (TSA) medium for phosphate solubilising and other, non-rhizobial bacteria. A surface sterilized nodule was kept on plates to check the proper sterilization of nodules. The plates were incubated at 28±2°C and after 2-5 days of incubation, bacterial colonies obtained were purified on respective media plates.

Stress tolerance and growth promotion assay

Estimation of indole acetic acid (IAA) production: Bacterial endophytic isolates were tested for their ability to produce IAA. Cultures were inoculated in their respective broth supplemented with 100 µg/mL DL- tryptophan and incubated at 30ºC for 72 h. IAA was determined in the culture supernatant by adding Salkowski reagent (Glickmann and Dessaux 1995).

Phosphate (P) solubilizing activity by plate assay and in liquid medium: All the endophytic isolates were inoculated in their respective broth and incubated at 30ºC in BOD incubator for 3 days. Log phase grown culture (3µl) was spotted on Pikovskaya's plates and incubated at 30ºC for 3-4 days. P-solubilization activity was determined by the formation of solubilization zone and calculation of solubilization index (P-SI) (Edi-Premono *et al*. 1996). Solubilization of tricalcium phosphate (TCP) was examined using Pikovskaya liquid medium under stationary conditions. The P-content in the supernatant was determined by the method as described by Jackson (1973).

Siderophore production by plate assay and in liquid medium: All the endophytic bacterial isolates were screened for the siderophore production activity using universal chemical assay (Schwyn and Neilands 1987) on Chrome azurol S (CAS) agar plates.

For liquid medium, all the endophytes were inoculated in respective iron deficient broth, and incubated at 30ºC on shaker for 5 days. To determine siderophore, 0.5 ml of culture supernatant was mixed with 0.5 ml of CAS assay solution. The tubes were observed for a colour change from blue to orange indicating the presence of siderophore.

Nodulation in chickpea by rhizobial endophytes under sterilized conditions: Seeds of chickpea variety HC-5 were surface sterilized. River sand was thoroughly washed with acid followed by 6-7 washings with water and dried in an oven at 80°C overnight. The sand was added to cups and nitrogen-free nutrient solution was added, and cups were covered with paper held. Cups were then sterilized in an autoclave at 15 lbs for 1 h. Surface sterilized seeds of chickpea were then transferred to sterilized cups containing sand along with 1-2 ml broth of different endophytic mesorhizobial isolates and control was also kept. After 4-5 days seeds germinated in cups, and transferred in pot house and watered with sterile distilled waterwhen required and with sloger's watering solution once in a week. After 75 days plants were recovered and checked for nodulation by mesorhizobial endophytes.

ACC utilization by plate assay: The medium plates were prepared with minimal medium supplemented with 3mM ACC (Penrose D M and Glick B R 2003). Log phase grown culture (5µl) of the endophytic isolates was spotted on the medium plates and growth of bacterial isolates was recorded after 2-5 days of incubation at 30ºC. Bacterial cultures showing good growth on ACC supplemented medium plates and capable of utilizing ACC as nitrogen source were selected. The growth on minimal medium plates supplemented with ammonium sulfate was used as a control.

Salt tolerance activity: Selected endophytes were checked for their ability to grow at different concentrations of NaCl, i.e. 1, 2, 3, 4% (w/v), on respective media containing 20 mM HEPES (Marsudi *et al.* 1999). The plates containing medium were spotted with loopful of bacterial isolates and incubated for 3-4 days at 28± 2ºC in BOD incubator. The susceptibility of a bacterial isolate to salt was recorded as a positive or negative result.

Potassium solubilisation activity on plate: Potassium solubilization by selected endophytes was studied on modified Aleksandrov medium plates by the spot test method. A loopful of 48 h old growth of endophytic isolates was spotted on above-prepared plates and incubated at 28±2ºC for 3 days. Detection of potassium solubilization by endophytic isolates was based upon the ability of solubilization zone formation.

Compatibility test for rhizobial and non-rhizobial endophytes: The rhizobial endophyte was spread on the plate and non-rhizobial endophyte was spotted on it. Inhibition zone formation by endophytes was considered as incompatibility, the endophytes with no inhibition zone were considered compatible.

Partial identification of endophytic bacteria by 16S rDNA sequence analysis: Genomic DNA was extracted from bacteria using the CTAB method. Amplification of 16S rDNA sequence was carried out by polymerase chain reaction using a thermal cycler. The primers 27F and 1378R enable the amplification of 16S rDNA sequences present in endophytic bacterial DNA.The partial sequence of 16S rRNA gene of bacterial isolates was obtained after sequencing from Central Instrumentation Facilities, University of Delhi, South Campus.

RESULTS AND DISCUSSION

Isolation of bacterial nodule endophytes from chickpea: Using biofertilizers in agriculture is important as they may replace the chemical fertilizers and keep the environment safe. But many potentially plant growth promoting bacteria cannot be considered as useful plant probiotics because they are either pathogenic or inhibit the each other's growth when coinoculated (García-Fraile *et al*. 2012). Accordingly, it is necessary to select the plant probiotics among that are not related to human diseases. In our study, a total of 107 endophytic bacterial isolates were obtained from healthy pink nodules of chickpea plant after surface sterilization as the method described in the material and methods section.

Out of 107 endopytes, 24 were mesorhizobia, 29 were phosphate solubilisers and 54 were other endophytes (Table 1).

Screening of endophytic bacteria for plant growth

Table 1 Chickpea nodule endophytes isolated from different districts of Haryana

District	Rhizobial isolates (YEMA)	Phosphate solubilisers (Pikovskaya)	Other endophytes (TSA)	Total
Bhiwani	7 BM1, BM2, BM3, BM4, BM5, BM6, BM7	10 BP1, BP2, BP3, BP4, BP5, BP6, BP7, BP8, BP9, BP10	14 BE1, BE2, BE3, BE4, BE5, BE6, BE7, BE8, BE9, BE10, BE11, BE12, BE13, BE14	31
Hisar	3 HM1, HM2, HM3	3 HP1, HP2, HP3	8 HE1, HE2, HE3, HE4, HE5, HE6, HE7, HE8,	14
Jhajjar	9 JM1, JM2, JM3, JM4, JM5, JM6, JM7, JM8, JM9	2 JP1, JP2	12 JE1, JE2, JE3, JE4, JE5, JE6, JE7, JE8, JE9, JE10, JE11, JE12	23
Mahendargarh	3 MM1, MM2, MM3	10 MP1, MP2 ,MP3, MP4, MP5, MP6, MP7, MP8, MP9, MP10,	9 ME1, ME2, ME3, ME4, ME5, ME6, ME7, ME8, ME9	22
Rewari	2 RM1, RM2	4 RP1, RP2, RP3, RP4	11 RE1, RE2, RE3, RE4, RE5, RE6, RE7, RE8, RE9, RE10, RE11	17
Total	24	29	54	107

promoting traits: All the endophytes from chickpea nodules were screened for the presence of IAA production, P solubilisation and siderophore production.

IAA production by chickpea nodule endophytes: IAA helps by direct mechanism in production of longer roots with increased number of root hairs which are involved in nutrient uptake (Datta and Basu 2000). In the present study, all the chickpea nodule endophytic bacterial isolates were screened for quantitative production of IAA using salkowski reagent. Out of 107 chickpea endophytic isolates tested, about 80 endophytes were IAA producers, however, large variation was observed in the amount of IAA produced. IAA production was in the range of 0.33-19.56 µg/ml among various endophytic isolates. The endophyte RE2 produced maximum IAA (19.56 µg/ml), followed by BE6 and BE13 that produced 19.08 µg/ml and 17.67 µg/ml IAA. Phetcharat and Duangpaeng (2011) showed that out of 71 endophytic bacteria of rice crop, 66 isolates produced IAA and isolate O-1-R-4 (2) produced the highest amount of IAA (14.58 µg/ml).

On categorization of chickpea endophytic isolates with different range of IAA produced, it was observed that 55% of the endophytes were low IAA producers and their IAA production ranged between 0.33-7.0 µg/ml, 14% were moderate producers with a range of 7.01-14 µg/ml IAA and only 6% were high IAA producers, which produced more than 14 µg/ml of IAA. Similarly, Mbai *et al.* (2013) showed that out of 73 isolated bacterial endophytes from Kenyan basmati rice, 10 isolates (14%) were able to produce IAA, had potential to enhance plant growth.

Phosphate solubilization on solid and liquid medium: Phosphorus solubilisation is another important character as phosphate solubilising microorganisms have the capability to solubilise insoluble phosphate thus making it available for plants. In present study, phosphate solubilisation Index (P-SI) of endophytes varied from 1.0 to 3.4. The maximum P-SI was recorded in the endophyte HE6 (3.4) followed by RE6 (3.0) and ME3 (2.9) (Table 2). Categorization of chickpea endophytic isolates on the basis of their P solubilization capacity in liquid medium indicates that high P solubilisation was observed in only 12% isolates, which solubilized more than 40 µg/ml of the insoluble TCP, moderate solubilization (20-40 µg/ml) was observed in 6%, and low solubilization (4.0-25 µg/ml) was observed in 34% of the isolates. Similar study by Kumar *et al.* (2013) reported that, 56% endophytic bacterial isolates from nodules of legumes and non legumes were able to solubilise phosphates, can help in plant growth promotion.

Siderophore production by chickpea nodule endophytes: Ability to produce siderophores by an organism under iron limiting conditions can promote plant growth by directly supplying iron for plant utilization and by removing iron from the environment for the growth of phytopathogens thereby, reducing their competition and enhance the plant growth. In present study, 16 out of 107 endophytic isolates, showed orange color on CAS plates and liquid medium. Both the liquid assay and solid assay for siderophore exhibited similar positive results of siderophore production. Matsuoka *et al.* (2013) found that endophytes belonging to different genera isolated from *Carex kobomugi* roots, exhibited siderophore production activity under Fe limiting conditions.

Nodulation in chickpea by mesorhizobial endophytes under sterilized conditions: Nodulation is one of the most important characters of plant growth promotion and confirms the authentication of the rhizobial strains. Therefore, all the 24 mesorhizobial endophytes were tested for nodulation of chickpea variety HC-5 under sterilized conditions. Out of 24 mesorhizobial isolates, 21 (87.5%) endophytes showed positive nodulation in sterilized cups. Similarily Waswa *et al.* (2014) reported, that out of 100 rhizobial isolates of soybean varieties, 80% formed nodules in soybean in sterile vermiculite and highly effective rhizobial strains in

Table 2 Plant growth promoting traits of chickpea nodule endophytes

Isolate	IAA (µg/ml)	P -S.I.	Siderophore production
BE10	5.48	1.7	+
BE13	17.67	2.6	+
HE1	10.04	1.5	+
HE5	9.07	2.7	+
HE7	6.33	2.2	+
HE8	6.33	1.7	+
ME2	1.35	1.3	+
ME3	11.80	2.9	+
ME9	2.50	2.1	+
RE6	9.74	3.0	+
HM2	7.32	2.4	+

pot house conditions showed more nodulation and plant growth of soybean.

Screening of chickpea endophytic isolates for multi traits: On the basis of different plant growth promoting traits, IAA production, P solubilisation and siderophore production for non-rhizobial endophytes and including nodulation test for mesorhizobial endophytes, out of 107 endophytes, 11 possessed all the three beneficial plant growth promoting traits (Table 2). Among the 11 endophytes, non-rhizobial isolate, BE13 had higher IAA (17.67) production activity and good P-solubilizing ability, RE6 had highest P-solubilising activity and was good IAA producer while other non-rhizobial endophytes ME3, HE5 and HE7 had sufficient P-solubilizing as well as good IAA producing activity. One mesorhizobial endophyte HM2 having all the three activities including positive nodulation in sterilized condition was selected. Among eleven endophytes, five non-rhizobial BE13, HE5, HE7, RE6, ME3 and one mesorhizobial endophyte HM2 were further assessed for more plant growth promoting traits.

Assessment of selected endophytes for other plant growth promoting traits

Utilization of ACC and Potassium solubilisation activity: Ethylene a phytohormone, synthesized from ACC is known to promote ripening of climacteric fruits but acts as inhibitor of nodulation in legumes (Grobbelaar *et al.* 1971). ACC deaminase, a microbial enzyme cleaves ACC to α-ketobutyrate and ammonia, both of which are readily metabolized by endophytic bacteria. In the present study, among six endophytes, all the endophytes showed growth on ACC plates and only RE6 was able to solubilise potassium as indicated by the formation of the clear zone on a plate containing mica as insoluble potassium. Singh RP and Jha P N (2015) reported that *Enterobacter cloaceae* strain isolated from *Aervaj avanica* showed ACC deaminase activity.

Salt tolerance: Salt-stressed soils are known to suppress the growth of plants but some microorganisms have the capability to survive under salt stress conditions and provide growth to plant by various direct and indirect mechanisms. In our study, all the tested endophytes showed salt tolerance up to 4% NaCl concentration.

Compatibility test for rhizobial and non-rhizobial endophytes: The cooperative interaction between rhizobia and other plant root colonizing bacteria is important for improvement of nodulation and N_2 fixation in legume plants (Barea *et al*. 2005). In the present study, results showed that the three non-rhizobial endophytes (BE13, RE6, and ME3) were compatible with the rhizobial endophyte (HM2) as they did not show inhibition zone formation.

Identification of promising endophytes on the basis of partial 16S rDNA gene sequencing: Sequencing of the 16S rRNA gene has served as an important tool for identification of bacteria over a number of years. In the present study, the DNA of three promising endophytes (BE13, RE6 and ME3) was extracted and approximately 1500 bp DNA fragment was amplified from 16S rRNA gene. The sequences were aligned with NCBI database using BLAST program. The endophyte BE-13 showed 98% similarity with *Bacillus boroniphillus*, isolate RE-6 showed 99% similarity with *Pseudomonas protogenes* and ME3 showed 99% similarity with *Enterobacter cloaca*. All the three sequences were submitted to NCBI and accession number was generated by NCBI as KY773244 for *Pseudomonas protegens*, KY773226 for *Bacillus boroniphilus* and KY773231 for *Enterobacter cloacae*. Bensidhoum *et al*. (2016) showed *Pseudomonas protegens* exhibited plant growth promoting potential which is commonly associated with the production of the phytohormone IAA, siderophores and P solubilisation. Although *Enterobacter cloaca* showed plant growth promoting traits but these species were considered to be an important cause of nosocomial infections (Kellar *et al.* 1998).

Thus, *Pseudomonas protegens* and *Bacillus boroniphillus* along with rhizobium, being capable of plant growth promotion in a safe manner also they do not cause disease to human beings, thus environmentally safe could act as plant probiotics. Endophytic probiotics can improve the plant growth without polluting the environment when applied to different crops. These can easily replace the chemical fertilizers and can contribute to pollution free world.

ACKNOWLEDGEMENTS

This research was supported by CCS Haryana Agricultural University, Hisar, Haryana, India.

REFERENCES

Barea J M, Pozo M J, Azcon R and Azcon-Aguilar C. 2005. Microbial co-operation in the rhizosphere. *Journal of Experimental Botany* **56**(417): 1761-78.

Bensidhoum L, Nabti E, Tabli N, Kupferschmied P, Weiss A, Rothballer M and Hartmann A. 2016. Heavy metal tolerant Pseudomonas protegens isolates from agricultural well water in north eastern Algeria with plant growth promoting, insecticidal and antifungal activities. *European Journal of Soil Biology* **75**: 38-46.

Datta C and Basu P S. 2000. Indole acetic acid production by a

Rhizobium species from root nodules of a leguminous shrub, Cajanuscajan. *Microbiological Research* **155**(2): 123-27.

Dudeja S S, Narula N and Anand R C. 2009. Plant Microbe Interactions–A practical manual for laboratory studies. Published by Director HRM, CCS Haryana Agricultural University. Hisar, 125004.

Edi-Premono, Moawad M A and Vleck L G. 1996. Effect of phosphate solubilizing *Pseudomonas putida* on the growth of maize and its survival in the rhizosphere. *Indonesian Journal of Crop Science* **11**:13–23.

Flores-Félix J D, Silva L R, Rivera L P, Marcos-García M, García-Fraile P, Martínez-Molina E and Rivas R. 2015. Plants probiotics as a tool to produce highly functional fruits: the case of Phyllobacterium and vitamin C in strawberries. *PloS One* **10**(4): e0122281.

García-Fraile P, Carro L, Robledo M, Ramírez-Bahena M H, Flores-Félix J D, Fernández M T and Peix Á. 2012. Rhizobium promotes non-legumes growth and quality in several production steps: towards a biofertilization of edible raw vegetables healthy for humans. *PLoS One* **7**(5): e38122.

Glickmann E and Dessaux Y. 1995. A critical examination of the specificity of the salkowski reagent for indolic compounds produced by phytopathogenic bacteria. *Applied and Environmental Microbiology* **61**(2): 793-96.

Grobbelaar N, Clarke B and Hough M. 1971. The nodulation and nitrogen fixation of isolated roots of *Phaseolus vulgaris* L. III. The effect of carbon dioxide and ethylene. *Plant Soil* Special Volume: 215–21.

Haas D and Keel C. 2003. Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. *Annual Review of Phytopathology* **41**(1): 117-53.

Jackson M L. 1973. *Methods of Chemical Analysis*, Prentice Hall of India (Pvt) Ltd, New Delhi.

Keller R, Pedroso M Z, Ritchmann R and Silva RM. 1998. Occurrence of virulence-associated properties in *Enterobacter cloacae*. *Infection and Immunity* **66**(2): 645-49.

Khan A R, Qayyum A and Chaudhary G A. 1989. A country paper on soil, water, and crop management systems for dryland agriculture in Pakistan. Soil, Water, and Crop/Livestock Management Systems for Rainfed Agriculture in the Near East Region: (In) *Proceedings of the Workshop at Amman*, Jordan, Washington DC, USA. January 18-23.

Kumar V, Pathak D V, Dudeja, S S, Saini R, Giri R, Narula S and Anand R C. 2013. Legume nodule endophytes more diverse than endophytes from roots of legumes or non legumes in soils of Haryana, India. *Journal of Microbiology and Biotechnology Research* **3**(3): 83-92.

Marsudi N D, Glenn A R and Dilworth M J. 1999. Identification and characterization of fast-and slow-growing root nodule bacteria from South-Western Australian soils able to nodulate Acacia saligna. *Soil Biology and Biochemistry* **31**(9): 1229-38.

Matsuoka H, Akiyama M, Kobayashi K and Yamaji K. 2013. Fe and P solubilization underlimiting conditions by bacteria isolated from *carexkobomugi* roots at the Hasaki coast. *Current Microbiology* **66(3):** 314–21.

Mbai F N, Magiri E N, Matiru V N, Ng"an"a J and Nyambati V C S. 2013. Isolation and characterisation of bacterial root endophytes with potential to enhance plant growth from Kenyan basmati rice. *American International Journal of Contemporary Research* **3**(4): 25–40.

Penrose D M and Glick B R. 2003. Methods for isolating and characterizing ACC deaminase containing plant growth promoting rhizobacteria. *Physiologia Plantarum* **118**(1): 10-15.

Phetcharat P and Duangpaeng A. 2012. Screening of endophytic bacteria from organic rice tissue for indole acetic acid production. *Procedia Engineering* **32:** 177–83.

Schwyn B and Neilands J B. 1987. Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry* **160**(1): 47-56.

Singh R P and Jha P N. 2015. Plant growth promoting potential of ACC deaminase rhizospheric bacteria isolated from *Aerva javanica*: A plant adapted to saline environments. *International Journal of Current Microbiology and Applied Sciences* **4(7):** 142-52

Vincent J M. 1970. *A manual for the practical study of the root-nodule bacteria,* International Biological Programme, Blackwell Scientific.

Wakelin S A, Warren R A, Harvey P R and Ryder M H. 2004. Phosphate solubilization by *Penicillium spp*. closely associated with wheat roots. *Biology and Fertility of Soils* **40**(1): 36-43.

Waswa M N, Karanja N K, Woomer P L and Mwenda G M. 2014. Identifying elite rhizobia for soybean (*Glycine max*) in Kenya. *African Journal of Crop Science* **2(2):** 60-66.