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

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ABSTRACT

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

1. Introduction

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

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

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

2. Materials and methods

2.1. Root nodule bacteria isolation and preservation

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

2.2. Symbiotic tests

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

2.2.1. Acetylene reduction activity

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

2.3. Field inoculation trial

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

2.4. PGP traits of the root nodule bacteria

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

2.5. Molecular identification of the root nodule bacteria

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