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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