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RESEARCH ARTICLE

Nutrient enhancement of chickpea grown with plant growth promoting bacteria in local soil of Bathinda, Northwestern India

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Abstract Plant growth promoting bacteria (PGPB) enhance crop productivity as part of green technology to reduce the use of chemical fertilizers. They also have the capability to enhance macro- and micronutrient content of plants. In the present study, PGPB isolates belonging to *Pseudomonas citronellis* (PC), *Pseudomonas* sp. RA6, *Serratia* sp. S2, *Serratia marcescens* CDP13, and *Frateuria aurantia* (Symbion-K) were tested on two chickpea varieties, PBG1 and PBG5 grown for 30 days in local soil from Bathinda region in Northwestern India. PC and CDP13 were found to be better chickpea growth stimulators compared to the commercial Symbion-K based on shoot length and biomass. Most PGPB enhanced macro- and micronutrients in shoots to varying degrees compared to the control. PBG5 gave better response compared to PBG1 with reference to plant growth attributes and enhancement of the macronutrients, calcium, nitrogen and phosphorus and micronutrients, boron, copper, iron, and zinc. PBG5 is a high yielding variety with better resistance compared to PBG1. Overall, PGPB isolated from the local soil and PGPB from other parts of India were shown to be useful for enhancement of nutrient content and plant growth.

Keywords Bomass · Chickpea · Inductively Coupled Plasma Mass Spectrometry · Nutrient Content · Plant Growth Promoting Bacteria · Biofertilizer · Macronutrient · Micronutrient · *Pseudomonas*

Introduction

The use of pesticides, herbicides, and other chemicals to increase the productivity of crops have greatly increased the quantity of food to feed the growing population of the world. However, the use of these chemicals reduced the quality of soil in terms of heavy metal deposition, fertility, water absorption, etc. It has affected the health of not only humans but also other animals including birds and fishes by biomagnification (Marrugo-Negrete et al. 2017). The soil is extensively contaminated with heavy metal deposition (Cd, Cu, Pb, Cr, Hg, U). It is important to get rid of these heavy metals as they are non-biodegradable and harmful to humans and the microbial population in the soil. To deal with this problem, phytoremediation technique is employed in which the metal resistant plants precipitate metal ions around the roots. This adaptation of plants in the contaminated area is facilitated by microorganisms colonizing both the rhizosphere and phyllosphere, so that they can provide nutrients to plants and can reduce the toxic effects of contaminants. Such bacteria associated with plant growth are referred as plant growth promoting bacteria (PGPB) and most of them also harbor the property of metal tolerance (Navarro-Torre et al. 2016). Previous studies have shown growth promoting and nutrient enhancement in

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maize, sorghum and millets (Li and Ramakrishna 2011; Li et al. 2014; Dhawi et al. 2015, 2016, 2017, 2018).

PGPB can stimulate plant growth by either direct or indirect way (Glick 1995; Premachandra et al. 2016). In direct way, these bacteria produce plant growth regulating hormones and maintain nutritional balance. On the other hand, they induce resistance against plant pathogens and solubilize nutrients for easy uptake by plants to boost their growth. PGPB produce or alter the concentration of growth regulators such as indole acetic acid, gibberellin, cytokinin, and ethylene (Vejan et al. 2016). These growth regulators, also known as phytohormones are found in extremely low amounts and they exert influence on the biochemical, physiological, and morphological processes in plants. These include primary root elongation (auxin), seed germination, floral induction, floral and fruit development, stem and leaf growth, cell division, vascular cambium sensitivity, root hair proliferation (cytokinin), ripening of fruits and the abscission of leaves (ethylene), and increase in stem length and delay in senescence (gibberellin) (Vejan et al. 2016). PGPB can fix the nutrients present in the soil thereby preventing leaching out of nutrients (Mantelin and Touraine 2004). Nitrogen is required for the synthesis of proteins, amino acids, and nucleic acids. Plants take the required nitrate and ammonia produced by nitrifying bacteria from atmospheric nitrogen and the rest is converted back to nitrogen dioxide by denitrifying bacteria and liberated back into the atmosphere (Butterbach-Bahl et al. 2013). Most PGPB present in the soil are known for their ability to solubilize phosphate, which is readily taken up by plants (Yadav et al. 2014). Some PGPB produce enzymes which have antibiosis and anti-fungal properties used for defense purpose against harmful pathogenic microbes present in the soil. These enzymes are hydrolytic in nature, e.g. chitinase and glucanase produced by *Sinorhizobium fredii* KCC5 and *Pseudomonas fluorescens* LPK2, respectively, which act by degrading the cell wall of the pathogens (Arora et al. 2008). Other ways by which PGBP promote plant growth are production of siderophores and volatile organic compounds (Flores-Félix et al. 2015).

Majority of farmers in several states of India including Punjab state use chemical NPK fertilizers rich in nitrogen, phosphorus, and potassium. Due to the changing soil health of these regions, ways to restore soil health along with the production of crops with reduced heavy metal contamination are required. This can be achieved by employing local PGPB as biofertilizers for chickpea. Chickpea (*Cicer arietinum* L.) is an important food legume with high protein content ($\sim$ 20%) and enriched with calcium, iron, magnesium, phosphorus, and zinc (Gaur et al. 2010). Chickpea improves soil fertility by symbiotic nitrogen fixation. India accounts for the majority of chickpea production in the world. In this study, the effect of several isolates of PGPB on the growth of chickpea cultivars was evaluated. Furthermore, the nutrient content of chickpea plants inoculated with PGPB and grown in local soil was estimated employing inductively coupled plasma–mass spectrometry (ICP-MS).

Materials and methods

Plant growth promoting bacteria and their characterization

Five bacterial strains were tested for their ability to promote growth in two chickpea varieties. Two of these bacteria were previously collected from three areas of Bathinda region and characterized as *Pseudomonas sp. RA6* and *Pseudomonas citronellis* (PC), based on biochemical and DNA sequence analysis (Adhikary et al. 2019; Accession numbers KM594398 and KM594397). The third bacterium, *Serratia marcescens* CDP-13 is a PGPR associated with *Capparis decidua* plant (Singh and Jha 2016; Accession number KJ950714). The fourth one, Symbion-K is a commercial bio-fertilizer based on a selective strain of potash solubilizing, beneficial bacteria, *Frateuria aurantia* (http://www.tstanes.com/products-symbion-k.html). These bacterial strains show plant growth promoting traits such as the production of the plant hormone, indole acetic acid (IAA) and solubilization of phosphate.

The fifth bacterial strain designated as S2 was isolated from the soil sample collected from village Ramsara (latitude—29.2848938°N and longitude—75.298943°E) located in the vicinity of Guru Gobind Singh oil refinery in Bathinda district of Punjab state, India. Three samples were collected in August 2016 up to 10 cm of depth according to three point sampling method. S2 was subjected to various assays which included production of IAA, phosphate solubilization and tolerance to heavy metals. Production of IAA was estimated with the colorimetric technique proposed by Gordon and Weber (1951). In this method, the test isolate was grown in liquid medium with or without tryptophan (100 μg/mL) at 37 °C with continuous shaking at 80 rpm for 72 h. The supernatant of 1.5 mL culture was recovered after centrifugation at 8000 rpm for 5 min. 1 mL supernatant was taken in a test tube, and two mL IAA reagent (1 mL of 0.5 M $FeCl_3$ mixed in 50 mL of 35% $HClO_4$) was added. Incubation for 25 min at room temperature was then followed by recording the absorbance of the sample at 530 nm with the blank medium as negative control. The amount of IAA was calculated using standard of pure IAA that was prepared separately (Li and Ramakrishna 2011). Phosphate solubilization was assayed on Pikovskaya's medium agar plate. After inoculation and

subsequent incubation at 37 °C, the halo and colony diameter were observed. Clear zone development around the spot after incubation was considered as an index of phosphate solubilization (Singh et al. 2015).

Genomic DNA from S2 was isolated using Wizard genomic DNA isolation kit (Promega). PCR was performed using primers (5′-AGAGTTTGATCATGG CTCAG-3′ and 5′-TACGGCTACCTTGTTACGAC-3′) for partial 16S ribosomal DNA amplification (Singh and Jha 2016). The PCR products were purified with HiPurA PCR product purification kit (Himedia). DNA sequencing reactions were performed with BigDye™ Terminator v3.1 cycle sequencing kit (Applied Biosystems). The sequencing reactions were resuspended in 10-µL Hi-Di™ formamide and run on 3730xl DNA analyzer (Applied Biosystems). The high quality sequences were used for BLAST searches (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify similarity to sequences in Genbank.

Preparation of bacterial inoculum

LB media (1 ml) was inoculated with a single colony of the bacterial strains and incubated for 24 h at 37 °C. This culture was inoculated in 250 ml LB and incubated for 24 h at 37 °C. The cell density was adjusted to 10^8 cells/ml based on absorbance at 550 nm. The cells were centrifuged for 10 min at 4000 rpm at a temperature of 4 °C (Bashan et al. 2002). The pellet was resuspended in saline buffer (0.85% NaCl) which was used for coating of the seeds.

Plant material, inoculation and growth conditions

Two chickpea varieties (PBG1 and PBG5) procured from Punjab Agricultural University, Ludhiana were used in this study. Plastic pots (diameter 4.5'' and height 4'') were used to grow plants in the soil collected from the Central University of Punjab, Bathinda city campus. Seeds were kept in Pertri plates with cotton and rinsed with distilled water for germination. After sprouting, seeds were immersed in PGPB suspended in saline buffer for 45 min. Five seeds from each treatment and control were sown in pots. The pots were kept in the greenhouse and allowed to grow for 30 days.

Plant growth measurements

Shoot lengths of plants were measured after the 15th day and 30th day after germination. At the end of each experiment, root length of each plant was measured and compared with control plants used for each strain. The biomass of plants before drying (fresh weight) and after drying (dry weight) was recorded on the 30th day.

Nutrient quantification using ICP-MS

Macro and micronutrient levels except N and P were evaluated using ICP-MS. Samples were dried and exactly 0.5 gm was taken and mixed with 10 ml digesting solution (8 ml 70% HNO_3 and 2 ml H_2O_2) in tubes designed for microwave digestion (Krachler et al. 2002). The tubes were kept in Microwave Digesting System for 4–5 h for digestion. After digestion, the samples were filtered using Whatman filter paper followed by filtration using a syringe filter. The filtered solution was diluted 10,000 times using sterile water (Becker et al. 2008). These samples were given to the CIL (Central Instrumentation Laboratory) for ICP-MS analysis. To determine the nutrient content of the soil used in the experiment, three soil samples were mixed and three replicates of 0.5 g were analyzed by ICP-MS. The soil ICP-MS data was compared with three other soil samples collected from agricultural fields in Bathinda. The same procedure mentioned above was used for ICP-MS analysis of soil used for growing the plants. The level of potassium was 30–40% higher in field soil samples compared to the soil used in this study, which may be due to the use of fertilizers. The concentration of magnesium was 40% higher and calcium was > fourfold (except for Ramsara soil) higher compared to the soil from agricultural fields in Bathinda (Supplementary Table 1).

Nitrogen and phosphorus quantification

Nitrogen estimation percent by weight in the leaf sample was determined using Kjeldahl method (IS 5194 1969). Briefly, 1 g of leaf sample was digested for 3 h in Kjeldahl flask with 10 ml concentrated sulfuric acid in the presence of 3.5 grams of cupric sulfate and potassium sulfate mixed in 1:10 ratio. Boric acid was used to absorb ammonia evolved during distillation. Methylene blue and methylene red were used as indicators. Excess of sulfuric acid was titrated with standard sodium hydroxide solution till the green color changes to steel gray. Percent by weight of nitrogen was estimated using the formula given by the Bureau of Indian Standards (IS 5194 1969).

Total phosphorus of chickpea leaf samples was determined using Allen's method (Jackson 1973). Calibration curve for phosphorus was made by dissolving 0.10975 g of KH_2PO_4 in 500 ml distilled water. This solution contains 25 µg phosphorus/ml. 0, 1, 2, 4, 6 and 10 ml of this solution was taken in separate 25-ml flasks. To each flask, 2.5 ml of extractant solution of $NaHCO_3$, 2.5 ml of molybdate reagent were added and diluted with water to 20 ml. Few drops stannous chloride in glycerol were added and diluted to 25-ml mark. After 10 min at room temperature, blue color appeared which was read using a spectrophotometer at a wavelength of 660 nm.

Leaf tissue from each sample (1 g) was added to 10 ml of 1:2 (v/v) mixtures of nitric acid and perchloric acid and digested till white fumes appeared. 25 ml double-distilled water was added followed by filtration using Whatman filter paper no. 1. The residue was washed multiple times with distilled water. The filtrate was diluted to 100 ml with water and 2.5 ml was transferred to a 50 ml volumetric flask. 2.5 ml ammonium molybdate and 4–5 drops stannous chloride in glycerol were added in each flask with the final volume of 50 ml. The absorbance was recorded at 660 nm after 10-min incubation at room temperature for development of blue color. Total P content was calculated as per Jackson (1973).

Statistical analysis

A pair-wise *t* test was performed for statistical significance ($p < 0.05$) of each treatment with the control. The parameters taken for this analysis were shoot length, root length and nutrient concentration of each element quantified in the plant samples. Similar analysis was performed using ANOVA.

Results

Identification and plant growth promoting attributes of a bacterial strain from the local soil

The PCR product corresponding to partial sequence of 16S ribosomal DNA of S2 was sequenced and 598 bases was submitted to Genbank (Accession number MK282176). This sequence showed > 99% identity with *Serratia marcescens* strain TS1 16S ribosomal RNA gene (accession number GU046543). Evaluation of plant growth promoting traits showed that IAA produced by the isolate S2 was 74.4 ± 8.7 µg/ml based on the standard curve of pure IAA. The strain S2 showed clear zone around the colonies grown on Pikovskaya's medium indicating its ability to solubilize phosphate.

Chickpea varieties showed best growth response to PGPB (PC) isolated from the local soil

PBG1 variety plants treated with the strains PC and RA6 showed increased growth but no growth promoting activity was seen with S2, Symbion-K and CDP-13, in the first 15 days (Fig. 1a). On the 30th day, the height of the plants treated with PC, Symbion-K and CDP-13 increased compared to the control plants where CDP-13 and PC treatment showed significant growth in the plants. In case of PBG5 variety, the shoot length measured on the 15th day showed that out of all the bacterial strains tested, S2, PC, RA6 and CDP-13 promoted the plant growth by increasing the shoot length but a significant growth was seen only in S2 and CDP-13 treated plants (Fig. 1b). On the 30th day, all the treated plants showed higher shoot length compared to the control and this increase was significant in case of S2, PC, RA6 and CDP-13. PC treated PBG1 and PBG5 plants showed the highest shoot lengths with 17% and 15% increase, respectively, compared to control plants. The root length of treated plants did not show significant increase compared to control plants of both PBG1 and PBG5 varieties.

The next step was to determine the biomass of the plants harvested. PBG1 variety plants showed higher fresh weight in the CDP-13 and PC treated plants whereas the dry weight measurements showed increased weight only in CDP-13 treated plants (Fig. 2a). In case of PBG5 variety, the fresh and dry weight of all the treated plants increased when compared to the control plants (Fig. 2b). However, fresh weight increase was statistically significant ($p < 0.05$) in case of S2, PC and CDP13 treatments and dry weight increase was significant in case of the treatment with PC.

PGPB enhance macro and micronutrients of PBG1 and PBG5

Macronutrients (N, K, Ca, Mg, P, and S) are needed in higher quantities and micronutrients (Cl, Fe, B, Mn, Zn, Cu, Mo, and Ni) are needed in lower quantities for optimal plant growth. Levels of the macronutrient, nitrogen in PBG1 and PBG5 were twofold or higher compared to control in PC and Symbion-K inoculated plants (Fig. 3). Similarly, phosphorus levels in PBG5 inoculated with PC and RA6 were about twofold compared to control (Fig. 4). The increase in phosphorus levels was less pronounced in case of PBG1 with a maximal increase of about 50% in RA6 inoculated plants. Increase in magnesium levels in PBG5 plants treated with CDP-13 and Symbion-K was observed but they were not significant (data not shown). Calcium levels in PBG1 variety treated with CDP-13 and PBG5 variety treated with Symbion-K and CDP-13 were twofold compared to control plants (Supplementary Fig. 1). PC-treated plants of PBG5 variety also showed significant increase in calcium levels.

Micronutrient analysis showed significant increase in boron in all treated plants compared to control in both PBG1 and PBG5 (Supplementary Fig. 2). Increase in iron accumulation was observed in PC, RA6, Symbion-K and CDP-13 treated PGB5 and CDP-13 treated PBG1 plants (Fig. 5). Iron levels reached about twofold in PC, RA6 and CDP-13 treated PBG5 compared to control plants. Significant accumulation of zinc was seen only in shoots of PBG5 variety plants treated with RA6, Symbion-K and

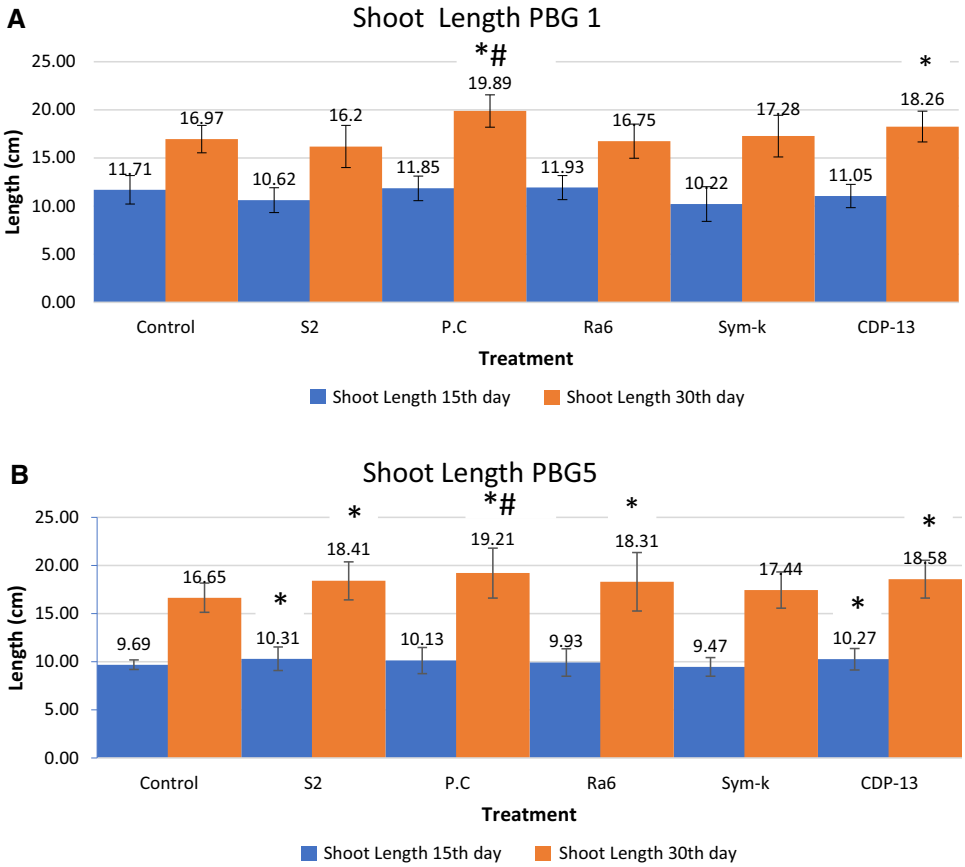


Fig. 1 Shoot length of chickpea varieties inoculated with PGPB. **a** PBG1 and **b** PBG5. Readings were taken after 15 and 30 days of germination. * and # represent p value < 0.05 using t-test and ANOVA, respectively for comparisons of treatments with the control. Error bars show standard deviation

CDP-13 (Supplementary Fig. 3). Zinc levels in RA6 and CDP-13 treated plants increased approximately 50% compared to control plants. In case of manganese, significant increase was seen only in PBG5 treated with Symbion-K (data not shown). Nickel levels were significantly higher in Symbion-K and CDP-13 treated PBG1 plants and RA6, Symbion-K and CDP-13 treated PBG5 plants (Supplementary Fig. 4). Significant increase in copper levels were seen in PBG5 variety shoots in RA6 and CDP-13 treated plants compared to control plants (Supplementary Fig. 5).

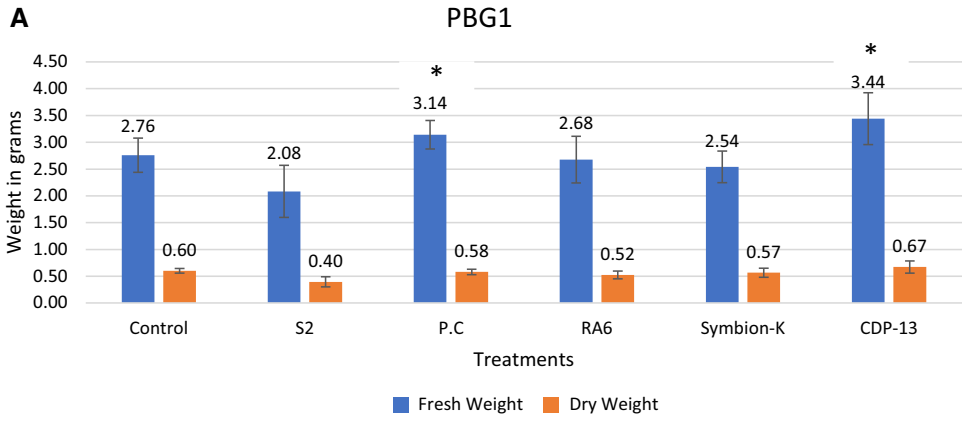
Cobalt and sodium are neither macronutrient nor micronutrient. However, they have functional significance in some plants (cobalt) and under some conditions (sodium). Levels of cobalt increased significantly in RA6, Symbion-K and CDP-13 treated PBG5 plants (Supplementary Fig. 6). In case of sodium, significant increase was observed in S2, PC and Symbion-K treated PBG1 plants (Supplementary Fig. 7). In comparison, significant accumulation was seen in all the treated plants compared to control plants of PBG5 variety.

Our study showed that the levels of all the elements reported except nitrogen, copper and sodium were higher in control plants of PBG5 variety compared to PBG1 variety. PGPB enhanced the macronutrients, nitrogen, phosphorus, and calcium in both PBG1 and PBG5, and magnesium only in PBG5. The levels of micronutrients, iron, boron, manganese, zinc, copper and nickel were higher in one or more PGPB treated plants in both PBG1 and PBG5. Symbion-K showed the best effect in PBG5 with maximum increase in the level of eight elements. CDP-13 showed the best effect in PBG1 with the highest levels of four elements compared to control.

Discussion

There is a need to isolate and characterize bacterial strains which are suited for enhancing productivity of plants grown in specific land regions. In our study, a local isolate, PC gave the best results with reference to chickpea growth. Plant growth enhancement based on shoot length by PC was about 15% higher compared to the commercial Symbion-K. Biomass of PC and CDP13 inoculated chickpea varieties was about 20% higher than Symbion-K.

The use of naturally occurring plant growth promoting bacteria as part of biofertilizers to improve the nutritional quality of crops is an approach with multiple benefits. It can meet the growing demand for food in addition to removing micronutrient malnutrition and preventing soil



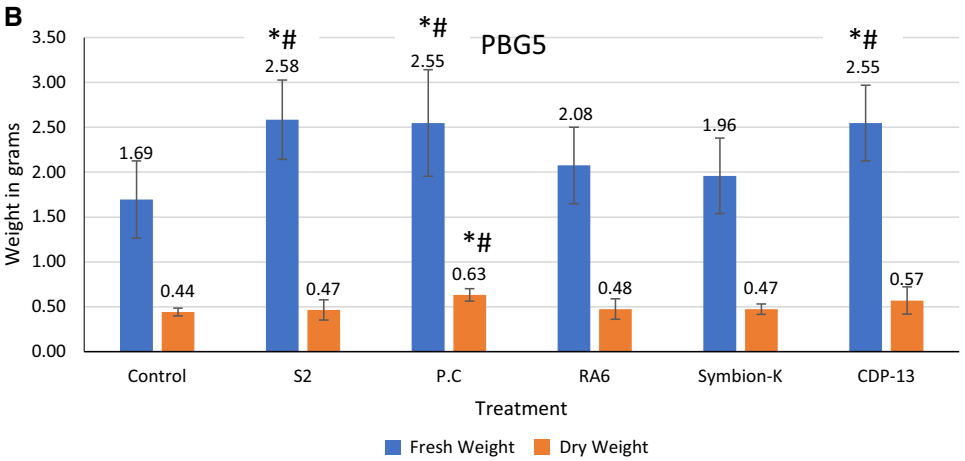


Fig. 2 Comparison of fresh and dry weight of **a** PBG1 and **b** PBG5 shoots

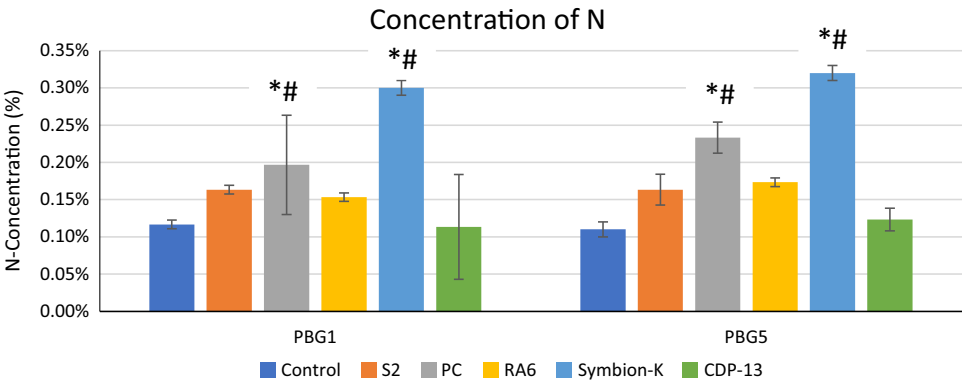


Fig. 3 Comparison of nitrogen levels in PBG1 and PBG5 shoots

health degradation by the excessive use of fertilizers (Garcia-Casal et al. 2016; Tarafdar et al. 2013). Biofortification of crop plants using PGPB is an environmental-friendly and economical approach which is yet to be exploited on a commercial scale (Prasanna et al. 2016; Singh et al. 2018). Development of local PGPB suitable for a particular region is needed as they tend to perform better and blend well with local soil microbial communities (Laditi et al. 2012).

All macronutrients have important cellular, molecular or physiological functions in plants (Maathuis 2009). Three of the four macronutrients tested in our study showed a significant increase with multiple PGPB. Of these, nitrogen and phosphorus are part of chemical fertilizers. Nitrogen has an important role in photosynthesis and nucleic acid synthesis as it is part of chlorophyll and ATP as well as all nucleic acid bases (Leghari et al. 2016). Phosphorus is required for plant growth and developments as it is an essential element of DNA, RNA, ATP and NADPH and is

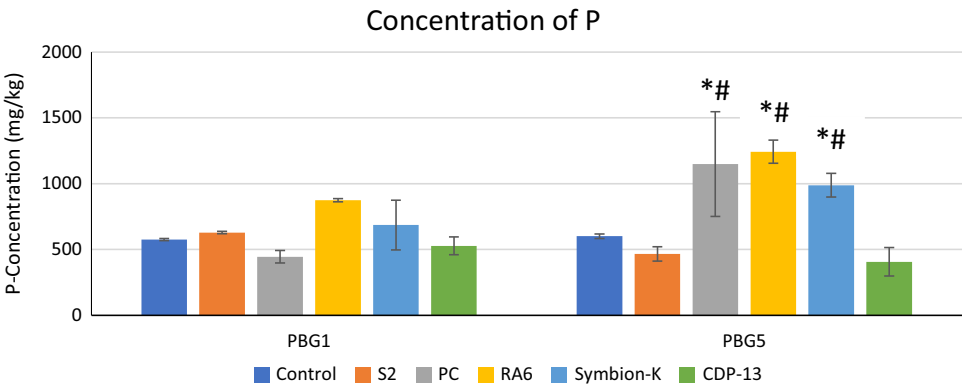


Fig. 4 Comparison of phosphorus levels in PBG1 and PBG5 shoots

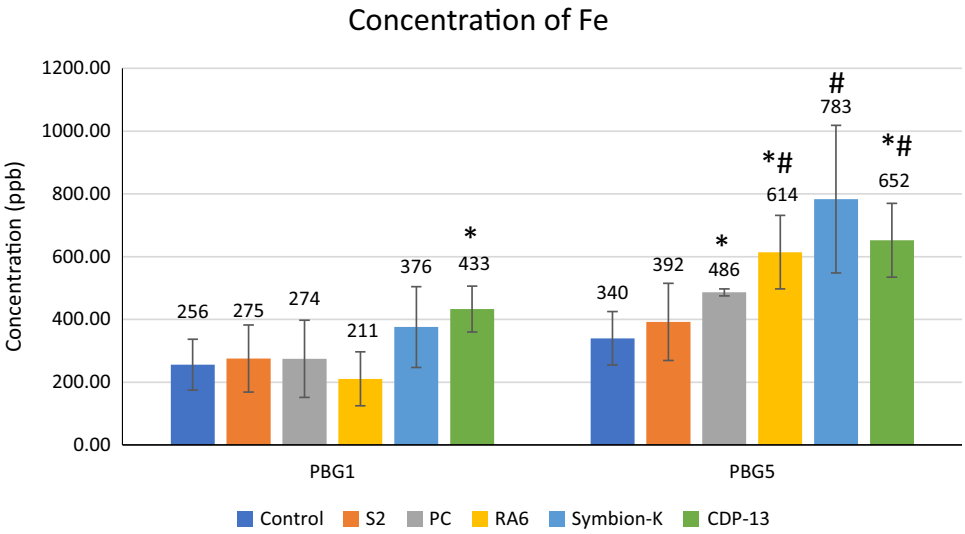


Fig. 5 Comparison of iron concentration in shoots of PBG1 and PBG5

involved in the activation of enzymes by phosphorylation (Młodzińska and Zboińska 2016). Calcium is not only an important structural component of cells walls crosslinking pectin residues but also regulates root growth and membrane permeability (Hepler 2005).

Micronutrients are needed by plants in minute quantities and they play important roles in various physiological and biochemical functions. Iron is a key component in electron exchange and is part of biomacromolecules involved in photosynthesis, DNA synthesis and respiration (Connorton et al. 2017). Biofortification of chickpea with iron using PGPB would result in ameliorating iron deficiency prevalent in both developing and developed countries (Tan et al. 2017). It will avoid the use of GM food crops which are not allowed in many developing countries. Boron along with calcium contributes to cell wall strength and is essential for cell division in meristematic tissues, water uptake and transport of sugars (Wimmer and Eichert 2013). The major copper-containing proteins in plants are plastocyanin which is part of photosynthetic electron transport chain and Cu/Zn superoxide dismutase which is an antioxidant enzyme reducing reactive oxygen species formed under stress conditions (Yruela 2005). Zinc is part of several enzymes and proteins involved in plant metabolism (Hafeez et al. 2013). Zinc is essential for tryptophan synthesis which is required for the biosynthesis of the plant hormone, indole acetic acid (IAA). It is also part of zinc finger transcription factors with a role in reproductive development and zinc is required for the maintenance of the integrity of cell membrane. Nickel is part of urease which converts urea into ammonia which serves as a source of nitrogen (Fabiano et al. 2015). Plant glyoxalase I involved in detoxification of methylglyoxal requires nickel for its activity which points to a role for nickel in combating abiotic stress (Mustafiz et al. 2014). It is likely that the higher levels of manganese, nickel and copper observed in plants incoculated with some PGPB enhance the biological functions of proteins associated with these elements.

Cobalt and sodium are not essential nutrients for all plants. Cobalt is a cofactor of cobalamin, which is a coenzyme required for symbiotic nitrogen fixation in leguminous plants (Minz et al. 2018). It also improves nodule formation and regulates overall plant growth which

is related to nitrogen production. PGPB may enhance the key function of nitrogen fixation in chickpea. Sodium is considered as a non-essential or functional nutrient as it can take over part of potassium function when there is a deficiency of this nutrient (Maathuis 2014). It is important to understand the mechanism of sodium sensing, uptake and signaling which will enable us to develop a plant-microbial combination to deal with salinity stress relevant to chickpea.

Overall, the ICP-MS data showed an increase in many essential macro and micronutrients in both PBG1 and PBG5 but the effect of PGPB on nutrient enhancement was more pronounced in PBG5 variety. PBG5 is a high yielding variety compared to PBG1 and PBG5 confers better resistance to *Aschochyta* blight and *Fusarium* wilt compared to PBG1 (Singh et al. 2004). PGPB can be considered as one of the modern-day strategies for biofortification of food crops including chickpea to deal with the malnutrition spread in many areas of the world.

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