



Original article

Isolation, characterization, and effect of phosphate-zinc-solubilizing bacterial strains on chickpea (*Cicer arietinum* L.) growth

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ABSTRACT

Objective: Phosphate (P) and zinc (Zn) are essential plant nutrients required for nodulation, nitrogen-fixation, plant growth and yield. Mostly applied P and Zn nutrients in the soil are converted into unavailable form. A small number of soil microbes have the ability to transform unsolvable forms of P and Zn to an available form. P-Zn-solubilizing rhizobacteria are potential alternates for P and Zn supplement. In the present study, the effect of two P-Zn-solubilizing bacterial strains (*Bacillus* sp. strain AZ17 and *Pseudomonas* sp. strain AZ5) was evaluated on the growth of chickpea plant.

Methodology: Both strains were purified from the rhizospheric soil of chickpea plant grown-up in sandy soil and rain-fed area (Thal desert). *In vitro*, both strains solubilize P and Zn as well both strain produce IAA and organic acids. In the field experiments, conducted in the rain-fed area, the positive influence of inoculation with both bacterial isolates AZ5 and AZ17 on chickpea growth was observed.

Results: The application of inoculum (strains AZ5 and AZ17) resulted in up to 17.47% and 17.34% increase in grain yield of both types of chickpea grown in fertilized and non-fertilized soil, respectively over non-inoculated control. Strain AZ5 was the most effective inoculum, increasing up to 17.47%, 16.04%, 26.32%, 22.53%, 26.12% and 22.59% in grain yield, straw weight, nodules number, dry weight of nodules, Zn uptake and P uptake respectively, over control.

Conclusion: These results indicated that *Pseudomonas* sp. strain AZ5 and *Bacillus* sp. strain AZ17 can serve as effective microbial inocula for chickpea, particularly in the rain-fed area.

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1. Introduction

Worldwide, the majority of soils under chickpea (*Cicer arietinum* L.) cultivation are sandy and deficient in plant nutrients, particularly nitrogen (N), phosphorus/phosphate (P) and zinc (Zn), etc.

Due to deficiency of mineral nutrients, the productivity of chickpea is low. N requirements of chickpea fulfilled by biological N- (atmospheric) fixation. In nodule of chickpea, nodule endophytes (mesorhizobia) fix the atmospheric N by biological N-fixation. Mesorhizobia contributes significantly to N nutrition of chickpea by biological N-fixation. (Laranjo et al., 2014).

P is another important macronutrient due to its critical role in metabolic pathways e.g., nutrient uptake, respiration, biological oxidation, photosynthesis, and cell division for the growth of plants. P is also a structural component of the phospho-proteins, phospho-lipids, coenzymes, nucleic acids, and chromosomes (Gouda et al., 2018).

Another yield and productivity constraint is Zn deficiency as chickpea plant is sensitive to Zn. Approximately 30% of soils of

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49 countries are the deficit in plant-available Zn (Hacisalihoglu and Kochian, 2003). Majority of Pakistani soils are alkaline and calcareous. Alkaline and calcareous soils are likely to Zn-deficiency (Alloway, 2009). The climate of Pakistan (arid and semi-arid) intensifies the Zn-deficiency (Sadeghzadeh, 2013). Zn is an important nutrient for growth and needed in synthesizing of RNA, DNA, auxins, carbohydrates, and nodulation (N-fixation). It plays an important role in the expression of genes, chromatin structure, and metabolism of proteins, photosynthetic carbon, photosynthesis (chlorophyll formation), enzymatic activity, reproductive processes, and production of biomass (Sadeghzadeh, 2013).

Table 1
Soil properties of the experimental field at two depths.

Parameter	Soil depth 0–15 cm	Soil depth 15–30 cm
Rainfall ^a	70 (mm)	70 (mm)
Soil texture	Sandy loam	Sandy loam
Organic matter	0.56 ± 0.05 (%)	0.52 ± 0.02 (%)
pH	8.04 ± 0.1	8.02 ± 0.2
EC	0.72 ± 0.02 (dS m ⁻¹)	0.65 ± 0.03 (dS m ⁻¹)
Total P	570 ± 13 µg g ⁻¹	710 ± 11 µg g ⁻¹
Available P	1.5 ± 0.5 µg g ⁻¹	3.6 ± 0.75 µg g ⁻¹
Available K	164.6 ± 4.76 µg g ⁻¹	168.6 ± 5.33 µg g ⁻¹
Available N	0.007 ± 0.005%	0.009 ± 0.008%
Available Zn	0.5 ± 0.15 µg g ⁻¹	0.6 ± 0.09 µg g ⁻¹

^a Meteorological department (Punjab, Pakistan).

Due to P and Zn deficiency, farmers are bound to apply chemical fertilizer P and Zn for good crop. Frequent use of expensive chemical fertilizer pollutes the environment (Sadeghzadeh, 2013). Therefore, farming communities are shifting from chemical-based agriculture to sustainable organic agriculture. Using PGPR (Plant Growth Promoting Rhizobacteria) as biofertilizer is a big support for environment-friendly crop production (Gouda et al., 2018). PGPR play a key part in P and Zn cycle by converting insoluble form to soluble form. Application of PGPR increases the soil fertility by reduction of chemical use and by producing organic acids. Organic acid production is the key mechanism to transform the unsolvable form of P and Zn into a soluble form. In the rhizosphere, organic acid production acidifies the microbial surrounding. Acetic, citric, formic, gluconic, 2-keto-gluconic, lactic, malic and oxalic acids are the major organic acid produced by PGPR (Ayyaz et al., 2016; Ashraf et al., 2013; Gouda et al., 2018; Rasul et al., 2019; Sadeghzadeh, 2013; Tahir et al., 2013; Zaheer et al., 2016).

Realizing that chickpea is mainly cultivated in nutrient deficient soils in the rain-fed area, the present study was conducted to study the effect of *Pseudomonas* and *Bacillus* strains on nodulation, quality and yield of chickpea in a field experiment. The isolates were characterized by detection of plant growth stimulating traits *i.e.*, P-solubilisation, Zn-solubilisation, IAA production, organic acids productions and used to inoculate chickpea in the field experiment.

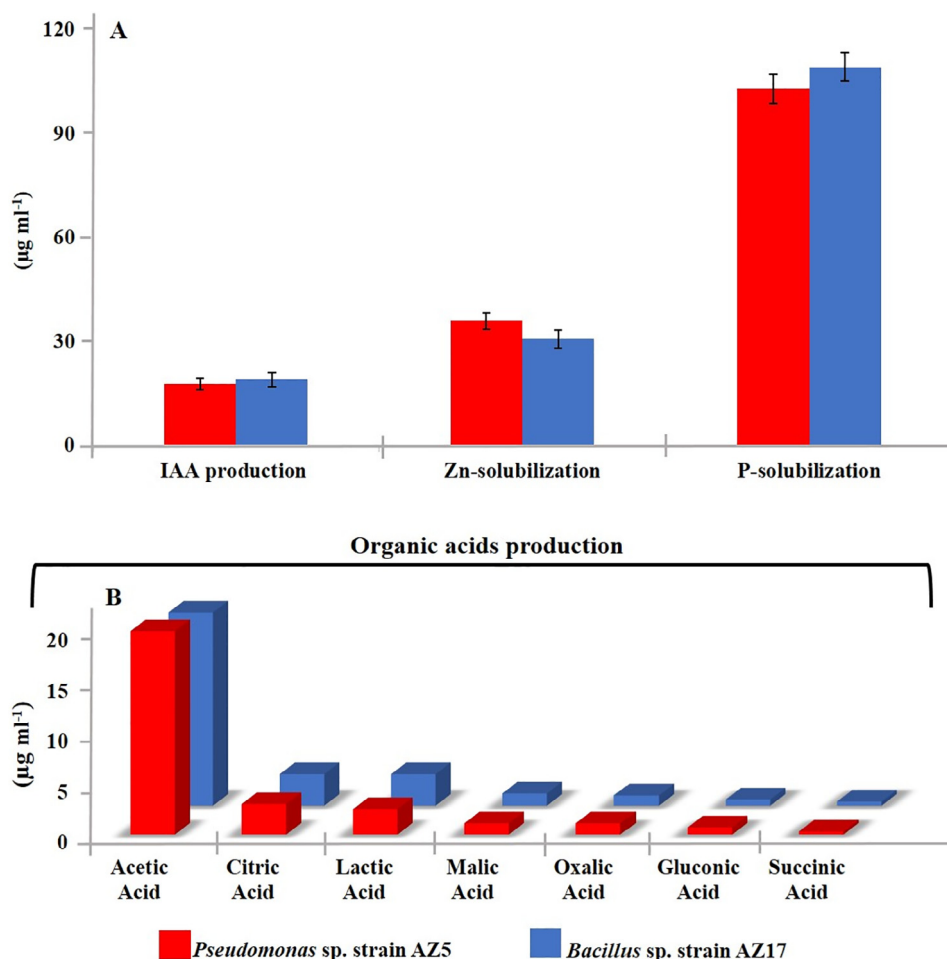


Fig. 1. (A) IAA production, Zn and P-solubilization by *Pseudomonas* sp. strain AZ5 and *Bacillus* sp. strain AZ17. (B) Organic acids productions by *Pseudomonas* sp. strain AZ5 and *Bacillus* sp. strain AZ17.

2. Methodology

2.1. Isolation of bacterial strains

Rhizospheric soil was collected carefully by uprooting the chickpea plant from the farmer's field along with root system at Thal desert. For isolation of P-Zn-solubilization bacteria. Thoroughly mixed and sieved one-gram representative rhizosphere sample was used. A serial dilution (10X) was prepared. 100 μ L of dilution from -4 to -7 were spread on modified Pikovskaya media (Pikovskaya, 1948) plates supplemented with Zn-P-tetrahydrate ($Zn_3(PO_4)_2 \cdot 4H_2O$). Incubation of plates was done at 30 ± 2 °C for two days. The halo zone colonies were selected and were purified by repeating sub-culture of particular single colonies on LB plates.

2.2. Characterization of isolated bacteria

Isolates were cultured in modified Pikovskaya broth medium of 50 mL with Zn-P-tetrahydrate ($Zn_3(PO_4)_2 \cdot 4H_2O$) supplements for seven days at 30 ± 2 °C and constant shaking at 120 rpm. Bacterial culture was centrifuged at 6000 rpm for 10 min, and the cell-free supernatant was used to quantify soluble P and Zn. The spectrophotometric method was employed to quantify the P-solubilization ability of bacterial isolates (Watanabe and Olsen, 1965). Solubilization of Zn by bacterial isolates was measured by Atomic Absorption Spectrophotometry method (Saravanan et al., 2007). The supernatant (10 mL) was concentrated and reduced to 1.5 mL, and filtered by 0.2 μ M filter. This filtered material used

for detection and quantification of organic acids and samples were processed on HPLC as described previously by Tahir et al. (2013).

Bacterial isolates grow in L-tryptophan supplemented LB medium to produce indole-3 acetic acid (IAA) for quantification. Growth conditions and methods for centrifugation was same as described in P-solubilization method. Hydrochloric acid was used to reduce and maintain the pH of cell-free supernatant at 2.8. Ethyl acetate was used to obtain IAA as stated previously by Tien et al. (1979). After the extraction of IAA from acidified supernatant, IAA was re-suspended in methanol (1 mL) and measured using HPLC as described previously (see Qaisrani et al., 2014).

2.3. Identification of bacterial isolates

DNA isolated from the pure culture by modified CTAB method (Wilson, 2001; Zaheer et al., 2016). To identify bacterial isolates, 16S ribosomal RNA gene was amplified using universal PCR primers i.e., PA (5'-AGAGTTTGATCCTGGCTCAG-3') and PH (5'-AAGGAGGTGATCCAGCCGCA-3') (Edwards et al., 1989). Extracted DNA was used for the 25 μ L PCR reaction volume. PCR was done with few changes as described by Zaheer et al. (2016). PCR amplified product was gel purified (Thermo Fisher Scientific, Germany) and sequenced commercially (Macrogen Inc., South Korea). The sequences of bacterial isolates were compared with available type strain sequence in the EzBioCloud database. Obtained and identified sequences (16S rRNA gene) of two isolates i.e., *Pseudomonas* sp. AZ5 and *Bacillus* sp. AZ17 were submitted to GenBank under accession numbers MK125505, MK125506, respectively.

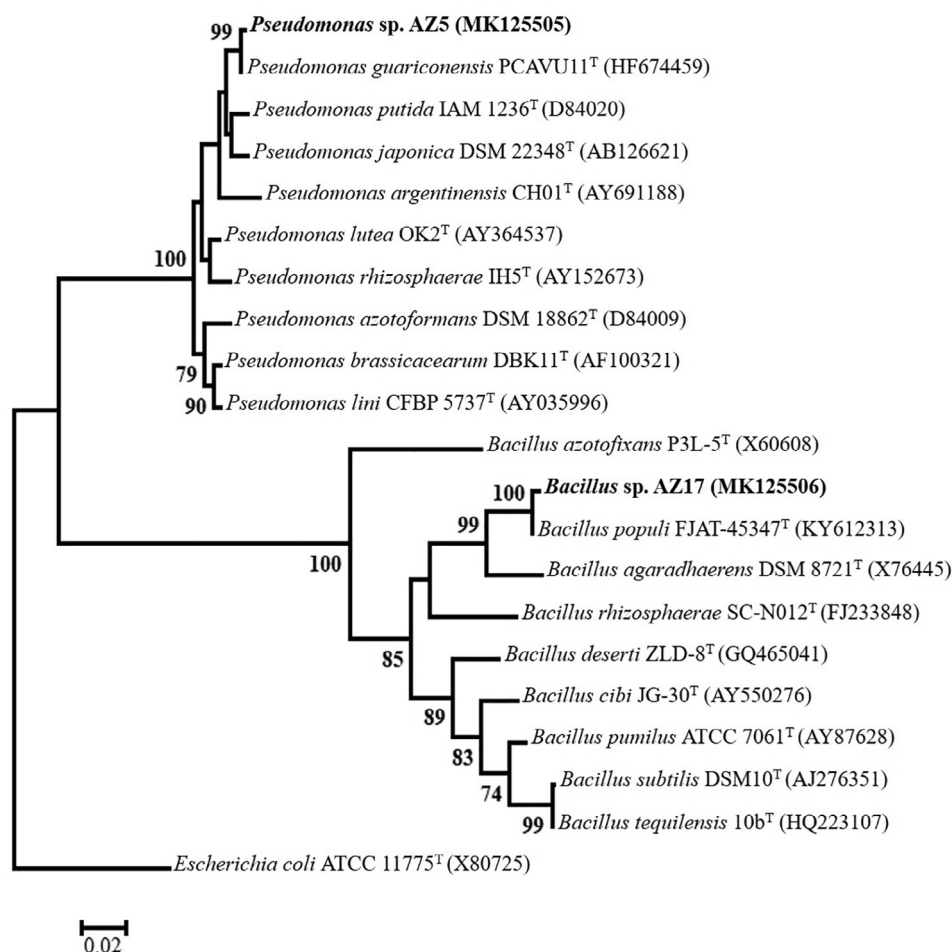


Fig. 2. Molecular phylogenetic analysis of *Pseudomonas* sp. strain AZ5 and *Bacillus* sp. strain AZ17 isolated from the rhizosphere of chickpea. Maximum likelihood method was employed to construct a tree. At the nodes, values ≥ 70 of maximum likelihood bootstrap are shown. The isolates are shown in bold words isolated in the current study.

For phylogenetic analysis of the two bacterial isolates, Sequences of 19 type strain and two bacterial strain isolated in this study were aligned using Clustal X. MEGA (version 7) was used to construct the phylogenetic tree (Kumar et al., 2016). The sequence of *Escherichia coli* ATCC 11775^T (X80725) was used as an outgroup.

2.4. Soil and plant analysis

Before sowing, collected soil samples were air-dried in shadow. Soil samples were ground. Passed the ground soil through a 2 mm sieve. Hydrometer method was employed to determine soil texture. Soil pH was measured from the supernatant of 1:2 soil: water mixture using a combination electrode. The cation exchange capacity, organic matters, total N and P, available P, K and Zn of the soil samples were measured by using standard procedures as described in the literature (Chapman and Pratt, 1962). For estimation of P and Zn in seed, the seed was ground and digested. Zn and P content in plants material was estimated as described previously by Chapman and Pratt (1962).

2.5. Field trial study to check the influence of bacterial inoculation on chickpea plant

Two bacterial strains (AZ5 of *Pseudomonas* sp. and AZ17 of *Bacillus* sp.) were tested as inoculants on two cultivars of chickpea i.e., Desi-type (Bittal-2016) and Kabuli-type (Punjab-Noor-2013) at

experimental field of Bahauddin Zakariya University (BZU), Bahadur sub campus, Thal desert, District Layyah. Soil analysis of the site is given in Table 1. Before sowing, the land was laser leveled and applied the tube-well water to gain optimum moisture contents. Deep plowing was done to prepared soil. Only one time before sowing, irrigation was done. Sowing was done in 5 m × 5 m block according to RCBD at the experimental site. At the time of plowing and sowing, 218 g DAP and 30 g Zn-sulphate monohydrate per block were applied to half blocks (80% of the recommended dose was applied). 25–50–25–5 kg NPKZn ha⁻¹ is recommended dose for chickpea (Aslam et al., 2010; Deolankar and Berad, 1999; Zaheer et al., 2016).

For inoculation, bacterial culture was grown in LB broth and cell pellets were obtained as conditions described earlier. Sterilized water was used to re-suspend the cell pellets. This mixture uses as inoculum; the same amount of sterilized water was used for non-inoculated control. We recorded all data related to the number and dry weight of nodules at the time of flowering. After maturity and harvesting, grain and straw weight, uptake of Zn and P and their contents of grain yield were also recorded.

2.6. Statistical analysis

Effect of bacterial isolates on number and dry weight of nodules, grain, and straw weight, Zn and P content in grain and their uptake by chickpea grain yield was determined. Data obtained

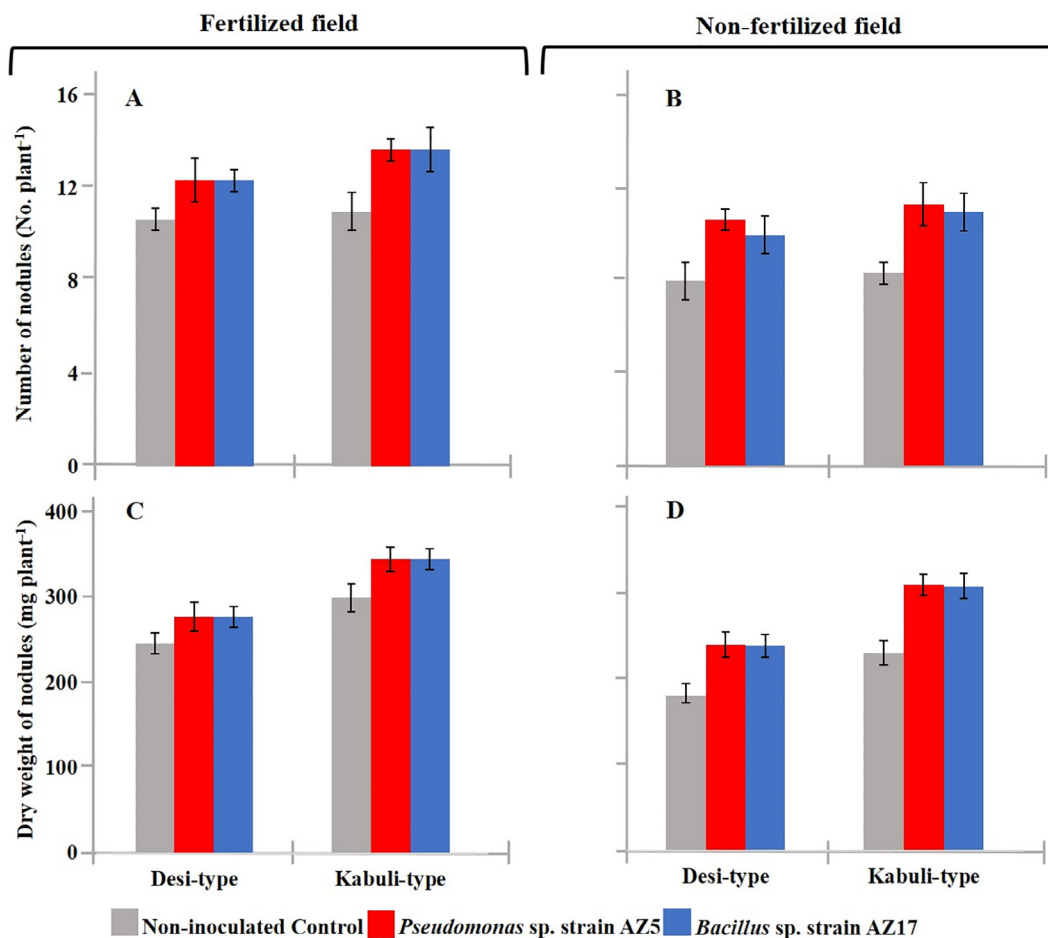


Fig. 3. Bacterial inoculation effect on nodules number (No. plant⁻¹) and dry weight of nodules (mg plant⁻¹) of chickpea cultivar grown in fertilized and non-fertilized soil (Thal desert, District Layyah). A = Number of nodules with fertilizer, B = Number of nodules with no fertilizer, C = Dry weight of nodules with fertilizer and D = Dry weight of nodules with no fertilizer.

was analyzed using *Statistix 8.1* computer software through 4-way ANOVA. LSD test at the level of significance ($P = 0.05$) was applied to compare means on all parameters.

3. Results

Two bacterial isolates *i.e.*, AZ5 and AZ17 were obtained from the rhizosphere of chickpea plant grown-up in farmer field at Thal desert. Both isolated strains *i.e.*, AZ5 and AZ17 showed a comparable concentration of IAA production, P-solubilization, Zn-solubilization, organic acids production in pure culture. The *Bacillus* sp. strain AZ17 produced the maximum amount of IAA ($19 \mu\text{g mL}^{-1}$), followed by *Pseudomonas* sp. strain AZ5 ($17.8 \mu\text{g mL}^{-1}$) in accompanied L-tryptophan LB broth media. The p-solubilization capacity of isolates was measured in Pikovskaya medium. The maximum ($109.4 \mu\text{g mL}^{-1}$) amount of P was solubilized in Pikovskaya medium by *Bacillus* sp. strain AZ17, followed by *Pseudomonas* sp. strain AZ5 ($103 \mu\text{g mL}^{-1}$). In Pikovskaya medium supplemented with Zn-sulphate, the maximum amount of Zn ($36 \mu\text{g mL}^{-1}$) was solubilized by *Pseudomonas* sp. strain AZ5. After seven days of incubation in Pikovskaya medium supplemented with Zn-P-tetrahydrate, many organic acids were noticed in both bacterial cultures. Acetic, oxalic and gluconic acids were produced relatively higher by *Pseudomonas* sp. strain AZ5. Citric, malic, lactic, and succinic acids were produced by *Bacillus* sp. strain AZ17 were slightly higher than *Pseudomonas* sp. strain AZ5. In the cell-free supernatants, media acetic, citric and lactic acids were

detected in higher amounts by both strains as compared to malic, oxalic, gluconic and succinic acids (see Fig. 1).

Sequences analysis of 16S rRNA gene revealed *Pseudomonas* sp. strain AZ5 showed maximum homology (99.73%) with *Pseudomonas guariconensis* PCAVU11^T (HF674459), and *Bacillus* sp. strain AZ17 shared higher sequence homology (99.70%) with *Bacillus populi* FJAT-45347^T (KY612313). Phylogenetic tree analysis, based on 16S ribosomal RNA gene sequences, showed that AZ5 isolate had proximity with *Pseudomonas guariconensis* PCAVU11^T (HF674459) and shared common cluster. The isolate AZ17 make a cluster with *Bacillus populi* FJAT-45347^T (KY612313) (Fig. 2).

To evaluate the role of bacterial isolates in the stimulation of plant growth and yield, a field experiment was conducted at BZU sub-campus Layyah (Thal desert). Soil analysis of experimental filed BZU sub-campus Layyah (Thal desert) indicated that the soil was nutrient-deficient. For inoculation studies, two cultivars of chickpea plant, *i.e.*, Bittal-2016 and Punjab Noor-2013 were grown at the experimental site. The results have shown that *Pseudomonas* sp. strain AZ5 and *Bacillus* sp. strain AZ17 inoculation improved considerably the number and dry weight of nodules, grain, and straw weight, and uptake of P and Zn of the chickpea cultivar compared to non-inoculated control (Fig. 3; Supplementary Tables 1 and 2).

The overall effect of both bacterial isolates *i.e.*, *Pseudomonas* sp. strain AZ5 and *Bacillus* sp. strain AZ17 on both tested varieties of chickpea on fertilized and non-fertilized soil were checked. 1487.1 and 1485.4 kg ha⁻¹ of maximum chickpea grain yields were

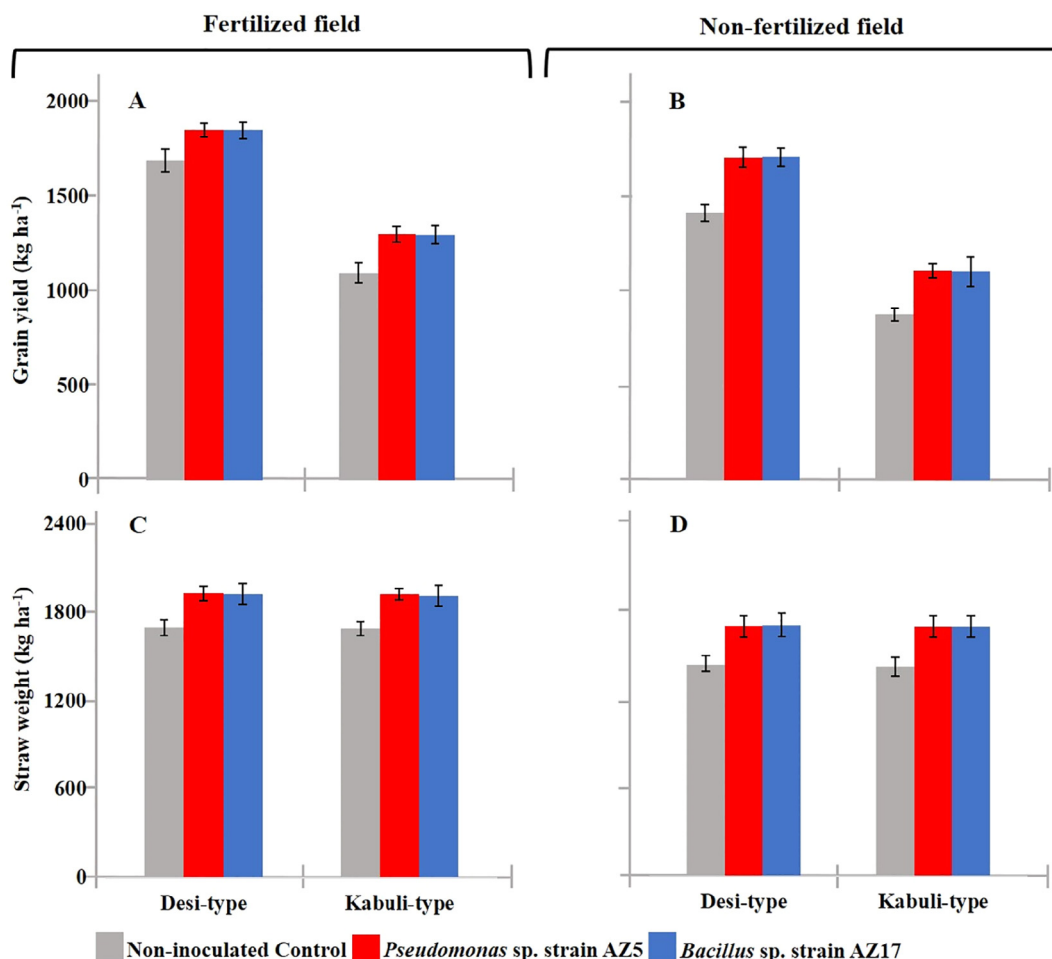


Fig. 4. Effect of bacterial inoculation on grain yield (kg ha⁻¹) and straw weight (kg ha⁻¹) of chickpea grown in fertilized and non-fertilized soil (Thal desert, District Layyah). A = Grain yield with fertilizer, B = Grain yield with no fertilizer, C = Straw weight with fertilizer and D = Straw weight with no fertilizer.

obtained for AZ5 strain *Pseudomonas* sp. and AZ17 strain *Bacillus* sp. respectively, in comparison to 1265.9 kg ha⁻¹ in the non-inoculated control treatment. Desi-type Bittal-2016 yield (1698.6 kg ha⁻¹) was significantly higher than the Punjab Noor-2013 (1127 kg ha⁻¹), irrespective of inoculated or non-inoculated treatments for a tested cultivar of chickpea. Fertilized and non-fertilized soil was used for the sowing of chickpea cultivar, about 15.65% more yield of grain were recorded with fertilized soil over to non-fertilized soil (Fig. 4; Supplementary Tables 3 and 4).

Inoculation of both strains has no significant effect over the type of chickpea i.e., Desi-type and Kabuli-type, fertilized and non-fertilized soil on N and P content in the grains over the control plant (Supplementary Tables 5–7). Among the inoculation effect of AZ5 and AZ17 strain on the uptake of P in grain yield of chickpea. *Bacillus* sp. strain AZ17 have up taken 25.69% more P in grain over the non-inoculated control. Similarly, in the case of Zn uptake, *Pseudomonas* sp. strain AZ5 have up taken 26.12% more Zn over the non-inoculated control (Fig. 5; Supplementary Tables 5–8).

Bacterial strain, *Pseudomonas* sp. strain AZ5 was the most efficient inoculum, increasing up to 17.47%, 16.04%, 26.32%, 22.53%, 26.12% and 22.59% in grain yield, straw weight, number and dry weight of nodules, Zn uptake and P uptake respectively, over control.

4. Discussion

In the current study, isolation of *Bacillus* and *Pseudomonas* strain was done from the rhizosphere of desert-grown chickpea crop,

which is known and well studied for their plant growth characteristics of several agricultural crops growing different regions of the world (Ashraf et al., 2013; Qaisrani et al., 2019; Rasul et al., 2019; Sadeghzadeh, 2013; Tahir et al., 2013). *Bacillus* and *Pseudomonas* were previously obtained and purified from rhizosphere, roots, and nodules of various cereals, legumes, wild and cultivated grasses from the different ecological regions of the world. These genera have positive role for plant stimulation due to P and Zn solubilization and production of IAA, phytase, organic acids, HCN, N-Acyl homoserine lactones and siderophores, which are useful traits for plant growth (Ayyaz et al., 2016; Ashraf et al., 2013; Gouda et al., 2018; Sadeghzadeh, 2013; Shahzad et al., 2017; Tahir et al., 2013; Zaheer et al., 2016).

In vitro, both isolates i.e., *Pseudomonas* sp. strain AZ5 and *Bacillus* sp. strain AZ17 showed comparable indication of plant stimulation by P and Zn-solubilization, production of organic acids and IAA in cell-free supernatant growth media. *Pseudomonas* sp. strain AZ5 was 16.88% more efficient in Zn-solubilization as compared to *Bacillus* sp. strain AZ17. This Zn-solubilization could be due to its capability to produce 16.66% more gluconic acid as over to *Bacillus* sp. strain AZ17, under standard conditions. *Bacillus* sp. strain AZ17 was 6.2% more efficient in P-solubilization as compared to *Pseudomonas* sp. strain AZ5 as AZ17 was capable of yielding 24% higher lactic acid than AZ5, under standard laboratory conditions. The study had shown an association among concentrations of organic acids and P-solubilization. *In vivo*, bacterial isolates with the ability to produce IAA, solubilization of Zn and P can play a major part as plant stimulant when used as bio-inoculum to different crops

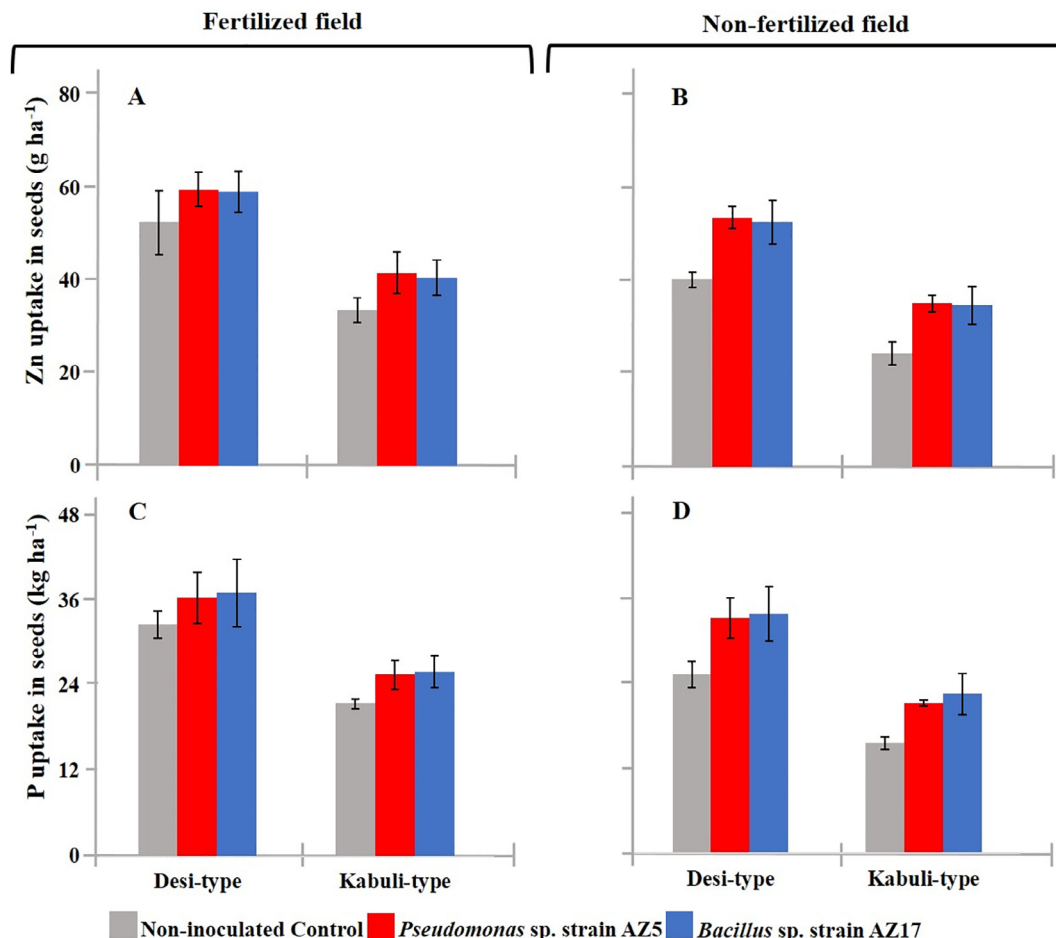


Fig. 5. Effect of bacterial inoculation on Zn uptake in seeds (g ha⁻¹) and P uptake in seeds (kg ha⁻¹) of chickpea grown in fertilized and non-fertilized soil (Thal desert, District Layyah). A = Zn uptake in seeds with fertilizer, B = Zn uptake in seeds with no fertilizer, C = P uptake in seeds with fertilizer and D = P uptake in seeds with no fertilizer.

(Ayyaz et al., 2016; Ashraf et al., 2013; Gouda et al., 2018; Sadeghzadeh, 2013; Tahir et al., 2013; Zaheer et al., 2016).

Entire results of the chickpea inoculation field experiment suggested that inoculation with the *Pseudomonas* sp. strain AZ5 and *Bacillus* sp. strain AZ17 could significantly increase plant growth through their Zn-solubilization, P-solubilization potential, and production of phytohormone. Both bacterial isolates i.e., *Pseudomonas* sp. strain AZ5 and *Bacillus* sp. strain AZ17 on chickpea yield have shown a positive effect over non-inoculated control, however, *Pseudomonas* sp. strain AZ5 inoculated plants exhibited slightly more grain yield and straw weight as matched to *Bacillus* sp. strain AZ17 inoculated plants. This could be due to higher Zn-solubilization activity by *Pseudomonas* sp. strain (Sadeghzadeh, 2013).

Application of fertilizer (80% of the recommended amount of N-P-Zn chemical fertilizer) exhibited a rise in the yield of chickpea, irrespective of both cultivars. This is possible that added fertilizer has delivered a timely enhancement to the chickpea plants over to non-fertilized chickpea plants. Whereas, detected growth stimulant was due to the plant stimulant abilities of *Pseudomonas* sp. strain AZ5 and *Bacillus* sp. strain AZ17 (Ayyaz et al., 2016; Sadeghzadeh, 2013; Zaheer et al., 2016).

Overall results of the field experiments confirmed the positive impact of *Pseudomonas* sp. strain AZ5 and *Bacillus* sp. strain AZ17 on both cultivars of chickpea plant i.e., Bittal-2016 (Desi-type) and Punjab-Noor-2013 (Kabuli-type). Assessment of the total effect of these bacterial inoculate i.e., *Pseudomonas* sp. strain AZ5 and *Bacillus* sp. strain AZ17 improved grain yield of chickpea plant up to 17.47% and weight of straw up to 16.04% of chickpea cultivar over to non-inoculated control chickpea plants. Adesemoye et al. (2008) have described the isolation of *Bacillus* and *Pseudomonas* from the soil of botanical garden and the bacterial strains showed plant stimulant effect on three vegetables i.e., African spinach, okra, and tomato. In the current study, due to the use of fertilizer in desert condition (nutrient-deficient soil) the efficiency of the *Pseudomonas* sp. strain AZ5 and *Bacillus* sp. strain AZ17 was enhanced. Zaheer et al. (2016) confirmed that the combined use of chemical fertilizer and PGPR enhanced the growth stimulant effect of PGPR.

Both bacterial isolates i.e., *Pseudomonas* sp. strain AZ5 and *Bacillus* sp. strain AZ17 have shown beneficial inoculation effect on uptake of Zn and P in chickpea grain yield. Inoculation with *Pseudomonas* sp. strain AZ5 showed 26.12% higher uptake of Zn in grain as compared to *Bacillus* sp. strain AZ17 inoculated plants. *Bacillus* sp. strain AZ17 showed 25.69% higher uptake of P in grain as compared to *Pseudomonas* sp. strain AZ5. This could be due to higher Zn-solubilization activity by *Pseudomonas* sp. strain AZ5 and higher P-solubilization activity by *Bacillus* sp. strain AZ17 (Ayyaz et al., 2016; Gouda et al., 2018; Rasul et al., 2019; Sadeghzadeh, 2013; Zaheer et al., 2016).

5. Conclusion

Both bacterial strains used in the present study i.e., AZ5 and AZ17 exhibited plant growth promoting traits i.e., production of IAA and solubilization of P and Zn in pure culture. In a field experiment, when applied as inoculant for chickpea grown at Thal desert (nutrient-deficient) in the rain-fed area improved plant growth. Therefore, both the isolates qualify as efficient strains to apply as bio-fertilizers at Pakistan particularly in desert conditions.

Conflict of interest

None.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.sjbs.2019.04.004>.

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