Isolation and characterization of phosphate solubilizing bacteria in Erbil soil and study their effects on *Cicer arietinum* plant growth and phosphorus uptake

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**1. INTRODUCTION**

Phosphorous (P) is one of the essential macronutrients required for plant growth and development (Gyanshwar *et al.*, 2002). It plays an indispensable biochemical role in photosynthesis, respiration, cell division, energy storage and other processes in living plant (Amit *et al.*, 2012).

A greater part of soil phosphorous present in the form of unavailable phosphates and hence cannot be utilized by the plants (Panhawr *et al.*, 2012). The problem of phosphorous availability is becoming a great matter of concern and major constraint for soil fertility in the majority of agricultural soils due to acidic or alkali nature of these soils. In these regions, phosphate ions are either fixed as insoluble phosphate by iron, aluminum and calcium or adsorbed on to the surface of soil minerals this leads to widespread phosphorous deficiency. Since deficiency of P is the most important chemical factor restricting plant growth. There are some microorganisms which are capable of solubilizing and mineralizing inorganic and
organic phosphorous in soil. Assimilation of phosphate from organic compounds by plant and microorganisms take place through the enzyme phosphatase which is present in wide variety of soil microorganisms. Plants can absorb phosphate only in soluble form. The transformation of insoluble phosphate in to soluble form is carried out by a number of microbes present in the soil (Sharma et al., 2011). A large fraction of soil microbes can dissolve insoluble inorganic phosphates, called phosphate solubilizing microorganisms (PSM), and make them available to plants through production of different organic acids and phosphatase enzyme. In recent decades, increasing evidences indicate that PSM besides to increasing nutrient uptake, they synthesis and export phytohormones, auxins, cytokinins, and gibberellins (Gutierrez-Manero et al., 2001), releasing siderophores (Wani et al., 2007), hydrogen cyanide (Kang et al., 2010), enzymes and/or fungicidal compounds such as chitinase, cellulase, and protease, which ensure antagonism against phytopathogenic microorganisms. Therefore, it is worth to believe that production of plant growth promoting substances by PSMs may effectively contribute to their effect on the enhancement of the plant performance (Nguyen et al., 1992).

Due to phosphorous solubilizing ability from insoluble inorganic pools of total soil phosphorous, PSMs have been widely used as inoculants to increase phosphorous uptake and crop yield (Chen et al., 2008). Plant growth promotion and increased phosphorous availability due to inoculation of PSMs have been assessed in several studies under green house as well as field conditions (Reyes et al., 2002, Zaidi et al., 2003). In the present study, twenty phosphate solubilizing bacterial strains were isolated and out of them, two efficient PSB strains (*Bacillus megaterium* and *Pseudomonas putida*) were employed their effect on the growth and phosphorous uptake of *Cicer arietinum* grown under greenhouse conditions.

2. **MATERIALS AND METHODS**

2.1. **Collection of Soil Samples**

This study was carried out to isolate and identify phosphate solubilizing bacteria (PSB) from soil of different agro climatic zones of Erbil governorate. Thirty soil samples of 300g each were collected randomly from rhizosphere of each agro climatic zone (Ankawa, askikalak, soran, degala, gdarasha, maxmur, Salahaddin, shaqlawa, bnaslawja, kwasinjak, Dargalla, Harir, pirzeen, kawrgosik, dugurtkan, rwandiz, choman, piromar) in Erbil governorate.

2.2. **Isolation of PSB strain**

At the laboratory, field moist soil samples were mixed with sterile 0.85%NaCl solution and shacked for 20min., an aliquot dilutions were inoculated using NBRIP (national botanical research institute phosphorous) medium (containing 10g glucose, 5g Ca3(PO4)2, 5g MgCl2.6H2O, 0.25g MgSO4.7H2O, 0.2g KCl, 0.1g(NH4)2SO4, in 1L distilled water) using pour plate technique and incubated for 5 days at 30°C. The colonies with clear halo zone were considered to be phosphate solubilizing colonies. Predominant colonies were purified by re-streaking on NBRIP agar. Single colonies appearing on NBRIP agar plates were transferred in to liquid broth of NBRIP and on agar slants for further study.

2.3. **Identification of PSB**

Identification of isolated PSB strains was performed according to their microsopical, cultural, physiological and biochemical characteristics (Kandler and Weiss, 1986): gram reaction, production of catalase, oxidase test, gelatin hydrolysis, IMVIC test, motility, nitrate reduction test and carbohydrate fermentation patterns.
2.4. Analysis of phosphate solubilizing activity

Phosphate solubilizing ability of all isolated bacterial strain was assayed using plate screening method and broth culture method.

All the suspected colonies were screened for phosphate solubilization on Pikovskayas (PVK) agar medium (10g glucose, 5g Ca(PO₄)₂, 0.5g(NH₄)₂SO₄, 0.2g NaCl, 0.1g MgSO₄.7H₂O, 0.2g KCl, 0.5g yeast extract, 0.002g MnSO₄.H₂O and 0.002g FeSO₄.7H₂O). Spot inoculation at the center of PVK plate was done and incubated at 30°C. Diameter of clear halo was measured successively after 24hr, up to 7 days. Phosphate solubilization efficiency (PSE) of each isolates was evaluated according to the following equation (Nguyen et al., 1992):

\[
PSE= \frac{\text{solubilization diameter}}{\text{Growth diameter}} \times 100
\]

Broth culture method also was used to determine phosphate solubilizing activities of bacterial isolates. Hundred ml aliquots of PVK broth was transferred in to 250ml conical flask, after sterilization, tricalcium phosphate (0.5g/100ml) was added. Each flask was inoculated with 1ml of active culture suspensions of each PSB isolate. A sterilized non-inoculated medium was prepared for the control treatment. Then all inoculated treatments and non-inoculated control treatment were kept on a rotary shaker (121rpm) for 7 days. At the end of incubation period all the broth culture were centrifuged at 11,000 rpm for 10 min to remove bacterial cell and other insoluble materials. The supernatant was taken to determine available phosphorous using spectrophotometer at 882nm.

2.5. Inoculum preparation for pot experiments

Based on the performance of above, two efficient PSB strains identified as Ps.putida and B. megaterium were selected for the pot trials.

Bacterial inoculum was prepared by transferring of a single colony in to 500ml flasks containing broth medium and grown aerobically on a rotating shaker (121rpm) for 48hr at 30°C. The bacterial suspension was then diluted in sterile distilled water to a final concentration of 106CFU.ml⁻¹ and resulting suspensions were used to inoculate the (Cicer arietinum) seeds.

2.6. Pot experiment

Pot experiment was carried out in a greenhouse located at Ankawa research center/Erbil. The pots (45cm height, 30cm diameters) were filled with sterilized soil (silty clay loam, pH 7.9, EC 0.41ds.m⁻¹, organic matter 2.02%, CaCO₃ 35%, total nitrogen 0.14%, available-P 2.9 μg.gm⁻¹, total-P 5628 mg.kg⁻¹, soluble ions: Ca+2 1meq-1, Mg+2 0.8 meq-1, Na+1 1.02 meq-1, K+1 0.32 meq-1).

Growth promoting effects of isolates were studied on chickpea. Surface sterilized seeds were soaked in prepared inoculum suspensions of PSB isolates for 3hr and were sown in pots. The pots were arranged in a completely randomized block design with three replications per treatments. The experimental plan was based on (soil + Ps.p., soil + Ps.p. + tcp, soil + B.m., soil + B.m.+ TCP, soil + Ps.p + B.m., soil + Ps.p + B.m. + TCP, soil+ TCP, control: soil without PSB and TCP). Chickpea seedlings were watered daily to maintain the water holding capacity of the soil during the study. Growth promoting effects of PSB treatments were assessed by measuring plant height, root length, shoot and root dry weight.
and P-uptake of chickpea plants after seven weeks of planting.

2.7. Soil analysis

The samples of rhizosphere were aseptically separated from plant roots of all treatments to measure soil P- content. Available P extracted by the bicarbonate method (Olsen et al., 1954) was determined following the molybdate blue color method.

2.8. Statistical analysis

The data were subjected to analysis of variance (ANOVA) using SAS version 9.1.3 statistical software with LSD (significance of treatment means at \( P \leq 0.05 \)).

3. RESULTS AND DISCUSSION

3.1. Isolation and identification of phosphate solubilizing bacteria

In this study sixteen bacterial isolates were obtained and screened from rhizosphere soil of different agro climatic zone of Erbil governorate. The isolated strain (PB1, PB2, PB3, PB4, PB5, PB6, PB7, PB8, PB9, PB10, PB11, PB12, PB13, PB14, PB15, PB16, PB17, PB18, PB19and PB20) had marked phosphate solubilizing abilities as visualized by the clear halo zone development around the colonies after 7 days of incubation on NBRIP agar. There was significant difference among soil samples in PSB population. The highest population was found in the shaqlawa soil samples, while the lowest PSB number was observed in the maxmur soil samples. Generally, the results showed that the north area of Erbil city contained higher number of PSB than south area.

All isolates were identified by performing different morphological, physiological and biochemical tests on the isolated cultures and were confirmed by using bergy’s manual of determinative bacteriology. The performed tests showed that 11 isolates (PB1, PB4, PB8, PB9, PB10, PB12, PB14, PB15, PB16, PB19, and PB20) were gram negative, aerobic, had activity of catalase and oxidase, did not hydrolysis gelatin and starch, could utilize glucose but did utilize arabinose, xylose, lactose, mannitol, rafinose, ribose and maltose, their colony on common agar plate appeared as round and creamy, did not spore former, non-motile, could not grow at 4°C and 44°C but could grow very well at 37°C and 40°C. Based on above characteristics, these isolates identified as Ps. putida, while isolates (PB2, PB3, PB5, PB6, PB7, PB11, PB13, PB17, and PB18) were gram positive, motile, spore former, aerobic, with irregular and yellow colonies, positive results for catalase, oxidase and hydrolysis of gelatin with starch, could utilize arabinose, galactose, fructose, mannitol, raffinose, ribose and xylose, but could not utilize mannose and rhamnose. Also they could grow at 4 and 45°C. Depending on mentioned characteristics, these 9 isolates were identified as \textit{B. megaterium}.

3.2 Phosphate solubilizing activities

All isolated PSB strains were tested for P-solubilizing activity in broth and solid PVK media. Production of clear halo zone on agar plates indicating P-solubilizing activity. The results (Table 1) showed that all isolates were able to solubilize P on PVK media plates. The highest P-solubilizing activity (91.2%) was found in PB16 treatment followed by PB15 (87.93%), while isolate PB18 recorded minimum solubilization index (26.91%), which differed significantly from other isolates.

Results of assessing phosphate solubilizing activity (Table 1) in liquid PVK medium at the end of incubation period showed that all isolates were found to release P from tricalcium phosphate ranging from (31.0 to 115.2µg.ml\(^{-1}\)). Similarly to solid plate method, it was observed that the maximum amount of soluble-P was released by PB16, which
referred to Ps. putida, while least amount of soluble-P was released by B. megaterium PB18 isolate.

These results are in agreement with the findings of (Kumar et al., 2010, Parasanna et al., 2011). This solubility of P might be the activity of certain microbes in preferable phosphate sources or due to the activity of phosphatase enzyme (Woo et al., 2010). Mostly, all PSB are able to produce organic acids from different source in solid and broth cultures and it is known that one of the mechanisms for P solubilization is the production of organic acids (Poonguzhali et al., 2008), and those organic acids can chelate the cation with their hydroxyl and carboxyl groups (Fengling et al., 2011), as well as the positive correlation between soluble phosphorous content and titratable acid production, suggested that acidification of the medium could facilitate phosphate solubilization (Park et al., 2016).

Depending on above results the most effective isolate of each of Ps. putida and B. megaterium selected for pot experiment.

Table 1: Qualitative and Quantitative estimation of phosphate solubilization efficiency of isolated bacterial strains using Pikovskaya’s medium

| Isolate | Bacterial genera | Phosphate solubilization on Agar (%) | Phosphate solubilization in Broth (µg.ml⁻¹) |
|---------|------------------|-------------------------------------|------------------------------------------|
| PB1     | Ps. Putida       | 65.30a                              | 87.20a                                   |
| PB2     | B. megaterium    | 38.83b                              | 47.51b                                   |
| PB3     | B. megaterium    | 55.50c                              | 76.00c                                   |
| PB4     | Ps. Putida       | 78.50f                              | 96.15d                                   |
| PB5     | B. megaterium    | 39.45f<sup>st</sup>                 | 45.00<sup>hn</sup>                       |
| PB6     | B. megaterium    | 41.95<sup>st</sup>                 | 60.50<sup>e</sup>                       |
| PB7     | B. megaterium    | 29.00<sup>st</sup>                 | 31.00<sup>f</sup>                       |
| PB8     | Ps. Putida       | 72.90<sup>g</sup>                  | 93.32<sup>g</sup>                       |
| PB9     | Ps. Putida       | 81.80<sup>g</sup>                  | 95.65<sup>g</sup>                       |
| PB10    | Ps. Putida       | 69.93<sup>h</sup>                  | 70.54<sup>hl</sup>                      |
| PB11    | B. megaterium    | 44.50<sup>g</sup>                  | 61.29<sup>g</sup>                       |
| PB12    | Ps. Putida       | 59.71<sup>h</sup>                  | 68.20<sup>h</sup>                       |
| PB13    | B. megaterium    | 46.00<sup>hm</sup>                 | 59.04<sup>g</sup>                       |
| PB14    | Ps. Putida       | 75.20<sup>h</sup>                  | 88.70<sup>g</sup>                       |
| PB15    | Ps. Putida       | 87.93<sup>h</sup>                  | 100.2<sup>h</sup>                       |
| PB16    | Ps. Putida       | 91.20<sup>g</sup>                  | 115.2<sup>g</sup>                       |
| PB17    | B. megaterium    | 50.32<sup>g</sup>                  | 71.58<sup>k</sup>                       |
| PB18    | B. megaterium    | 26.91<sup>g</sup>                  | 31.00<sup>l</sup>                       |
| PB19    | Ps. Putida       | 42.80<sup>g</sup>                  | 64.22<sup>m</sup>                       |
| PB20    | Ps. Putida       | 32.50<sup>g</sup>                  | 44.85<sup>n</sup>                       |

Values are means of triplicate samples, within each vertical column; values followed by the same letter are not significantly different at P≤0.05
3.3. Effectiveness of PSB isolates on growth of chick pea

The performance of PSB isolates on the growth of chick pea at greenhouse experiment is shown in (Table 2), all treatments showed significant effects on various growth parameters (shoot height, root length, shoot and root dry weight, and P-uptake) compared to non-inoculated chick pea plant. Growth was found to be further enhanced when seeds inoculated with Ps.putida and B. megaterium or both train with TCP.

The inoculated plants with different inoculum or P-source (TCP) alone showed root length ranging from (13.08 cm) up to (27.34 cm). All the isolates used as inoculant exhibited significant increase in root length of chick pea over control, the maximum increase (27.34 cm) was shown by co-inoculation of Ps.p +B.m.+TCP, while the plants which treated by TCP alone was report the lowest value of root length(13.90 cm). Similar to these results, increasing of plant root length by PSB strain have also been reported by (Chaihan and Lumyone 2011, Silini-Cherif et al. 2012). It is likely that phosphate solubilizing strains might have helped in plant root proliferation, elongation and production of plant growth regulators by the bacteria at root interface which resulted in better water absorption and nutrient such as P by host plant (Gupta et al. 2002, Barea et al. 2005). Increase in root length of testes plants may be attributed to the production of promoting substances (Karpagam and Nagalakshmi, 2014). The PSB strains produce ACC deaminase which stimulates plant root elongation through lowering the ethylene concentration in plant (Poonguzhali et al., 2008).

The effect of the different treatments on shoot height of chick pea plant showed significant variations compared with control (Table 2), the highest shoot height (28.30 cm) was recorded from co-inoculation Ps.p +B.m.+TCP, followed by Ps.p +B.m treatment (25.96 cm), which were significantly different from other treatment. The least value of shoot height was found by TCP treatment(14.92 cm). Significant (P≤0.05) increased in shoot height after inoculation of PSB strains Ps. Putida or B. megaterium or co-inoculation is in agreement with the study of Karpagam and Nagalakshmi (2014).

As shown in (Table 2), shoot and root dry weight showed similar trend to other growth parameters. Increase in root and shoot dry weight was observed in inoculated plants, a significant difference in root and shoot dry weight was found among single inoculated plants, co-inoculated plants and non-inoculated plants. The best performing treatments were the co-inoculation of (Ps.p +B.m.+TCP) and inoculation of Ps.p +B.m. followed by Ps.p alone treatments, while treated plant with TCP showed non-significant increase in root and shoot dry weight as compared to control plants. The inoculation of PSB in some research was known to increase plant dry weight. Our results were found to be similar to Rodríguez and Fraga (1999), Shwetha and Lakshman (2013), who also noted that the PSB inoculation increase shoot and root dry weight significantly. Although a recent study reported that inoculation of coffee plant with PSB showed significant increase in stem dry matter over phosphate fertilized and non-inoculated controls (Shwetha and Lakshman, 2013).

In this study, inoculation of bacterial strain to chick pea had effect on P-content of plant and soil. The P-content of plants was higher in PSB inoculated plants than non-inoculated chick pea plants regardless the single or co-inoculation. Maximum P-content was reported when co-inoculation of two PSB strains with TCP (0.640%), and statically analysis of data showed that there was significant difference among P-content of inoculated plants with PSB isolates together and with non-inoculated control. This is in agreement with the report of similar increases in P-uptake of plants due to inoculation of PSB strains was observed by Ghanem and Abbas (2009).
Increased growth and P-uptake of several crop plants as the result of phosphate-solubilizing bacteria inoculations have also been reported by (Liu et al 2011, Parasanna et al 2011, Shwetha and Lakshman 2013, Walpola and Yoon 2012). Inoculation with two strains of *Ps. putida* selected for their P-solubilization ability has been shown to improve root colonization and growth promotion and to increase significantly the P concentration in lettuce and maize (Lifshitz et al 2008, Poonguzhali et al 2008). On the other hand, a strain of *Ps. putida* also stimulated the growth of roots and shoots and increased 32P-labeled P-uptake in canola (Maliha et al., 2004). Simultaneous increases in P-uptake and crop yields have also been observed after inoculation with *Bacillus sp.* (Banish and Dupta, 2008). Walpola and Yoon, (2012), concluded that the phosphate-solubilization effect of Rhizobia and other mineral phosphate-solubilizing microorganisms seems to be the most important mechanism of plant growth promotion in moderately fertile and very fertile soils. Inoculation of rice seeds with PSB strain increased the phosphate ion content and resulted in significant improvement of root length and fresh and dry shoot weights and P-content (Parasanna et al., 2011).

Table 2: Effect of single and co-inoculation of (*Pseudomonas putida* and *Bacillus megaterium*) on the growth and P-uptake of Chickpea (*Cicer arietinum*) plant

| Treatment | Shoot height (cm) | Root length (cm) | Shoot and Root dry weight (g/plant) | P-uptake (%) |
|-----------|------------------|------------------|------------------------------------|--------------|
| Soil+ Ps.p | 21.10<sup>a</sup> | 19.98<sup>a</sup> | 10.40<sup>ad</sup> | 0.460<sup>a</sup> |
| Soil + Ps.p+ TCP | 23.80<sup>b</sup> | 22.05<sup>b</sup> | 11.03<sup>b</sup> | 0.540<sup>b</sup> |
| Soil + B.m. | 18.34<sup>c</sup> | 18.00<sup>c</sup> | 8.00<sup>bc</sup> | 0.358<sup>c</sup> |
| Soil + B.m. +TCP | 19.95<sup>c</sup> | 18.94<sup>ad</sup> | 9.16<sup>ad</sup> | 0.390<sup>c</sup> |
| Soil + Ps.p + B.m. | 25.96<sup>d</sup> | 24.04<sup>c</sup> | 13.43<sup>c</sup> | 0.580<sup>d</sup> |
| Soil + Ps.p.+B.m.+TCP Soil+ | 28.30<sup>e</sup> | 27.34<sup>de</sup> | 15.65<sup>e</sup> | 0.640<sup>e</sup> |
| TCP | 14.92<sup>f</sup> | 13.90<sup>e</sup> | 5.23<sup>e</sup> | 0.213<sup>e</sup> |
| Control Soil without PSB and TCP | 13.89<sup>f</sup> | 13.08<sup>e</sup> | 5.01<sup>f</sup> | 0.209<sup>f</sup> |

Values are means of triplicate samples, within each vertical column; values followed by the same letter are not significantly different at P≤0.05.

The increases in shoot length, root length, shoot dry weight, root dry weight, and P-uptake of plants inoculated with PSB strains could be attributed to a greater absorption of nutrients, especially phosphorous. Co-inoculation treatments resulted in higher growth performances and phosphorous uptake than those from single inoculation, suggesting that both strains acted synergistically in promoting plant growth. However, phosphate solubilization is not the only way PSB promote plant growth, because they facilitate the growth of plants by stimulating the efficiency of producing plant hormones such as auxins, cytokinins, gibberellins as well as some volatile compounds (Podile and Kishor, 2006). The production by PSB strains of other metabolites beneficial to the plant, such as phytohormones, antibiotics, or siderophores, among others, has created confusion about the specific role of phosphate solubilization in plant growth and yield stimulation (Walpola and Yoon, 2012). Therefore, enhanced plant growth after inoculation of PSB strains may be attributed to the ability of strains to make phosphorous available and to simultaneously produce plant growth promoting substances (Khalid et al., 2004; Linu et al., 2009). Ghanem and Abbas (2009), observed an increase in plant height, number of branches, number of pods, grain
weight, and eventually, higher seed and straw yields in green gram plants after inoculation of *B. megaterium* in salt affected soils. Increased growth and phosphorous uptake have been reported in wheat from *Azotobacter chroococcum* (Kumar *et al.*, 2001), in peanut from *Ps. fluorescens* (Dey *et al.*, 2004), in walnut from *B. cereus* and *Pseudomonas* *sp.* (Liu *et al.*, 2011), in tomato from *B. polymyxa* and *B. Megaterium* (Walpole and Yoon, 2012), and in soybean plants from *Burkholderia* *sp.* (Fernandez *et al.*, 2007).

Results in (Table 3) showed that available phosphorous content of rhizosphere soil inoculated with either a single PSB or both strains was found to be significantly (p ≤ 0.05) higher than those in non-inoculated soil. This was further enhanced by the addition of TCP. The highest level of available phosphorus content (5.39 µg. g⁻¹ soil) in co-inoculated (Ps.p +B.m) soil with TCP, while the least increase of available-P was recorded by TCP treatment (3.0µg.g⁻¹ soil) Other researchers found similar results that inoculation of soil with PSB strains due to the increasing of available-P concentration in soil (Wani *et al.*, 2007; Woo *et al.*, 2010).

### Table 3: Effect of single and co-inoculation of (*Pseudomonas putida* and *Bacillus megaterium*) and TCP addition on soil available phosphorus content

| Treatment                      | Soil available phosphorus content (µg. g⁻¹ soil) |
|-------------------------------|-----------------------------------------------|
| Soil+ Ps.p                    | 4.11ᵃ                                         |
| Soil + Ps.p+ TCP              | 4.53ᵇ                                         |
| Soil + B.m.                   | 3.13ᶜ                                         |
| Soil + B.m. +TCP              | 3.67ᵈ                                         |
| Soil + Ps.p + B.m.            | 4.95ᵉ                                         |
| Soil + Ps.p.+B.m.+TCP         | 5.39ᶠ                                         |
| Soil+ TCP                     | 3.00ᵍ                                         |
| Control Soil without PSB and TCP | 2.90ʰ                                       |

Values are means of triplicate samples, within each vertical column; values followed by the same letter are not significantly different at P≤0.05.

### 4. CONCLUSIONS

The current study shows that the inoculation of plants with PSB strain (*Ps. Putida* and *B. megaterium*) tend to enhance the growth of chickpea and responsible for the increase in several growth parameters (shoot height, root length, shoot and root dry weight). The strains also improved the uptake of phosphorous by chickpea plants and the available phosphorous content in the soil compared to the control. Combined inoculation resulted in higher growth performances and phosphorous uptake than those from single inoculation. Based on the results, it could be concluded that PSB isolates (*B. megaterium* and *Ps. putida*) possess great potential to be developed as biofertilizer to enhance soil fertility and plant growth.
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