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# Integrative Application of Soil P-Solubilizing Bacteria and Foliar Nano-P Improves Antioxidant, Hormonal, And Nutrient Contents and Phosphatase Activity in *Phaseolus Vulgaris* Plants Grown Under Calcareous Soil Conditions

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## Abstract

Two pot experiments were conducted in fall season of 2018 and summer season of 2019 in a greenhouse, with climatic conditions of 20.2 ± 3.0 °C as average day/night temperatures and 65.7 ± 8.8% as average relative humidity, at the Experimental Farm of the Faculty of Agriculture, Fayoum, Egypt. Healthy, uniform seeds of *Phaseolus vulgaris*, cv. Bronko were planted in plastic pots filled in equal quantities (12 kg) with calcareous soil (22% $CaCO_3$). Soil enzyme activities (e.g., phosphatase and phytase) were significantly increased by inoculating the tested soil with phosphate-solubilizing bacteria (PSB) in both seasons of study. Inoculation of the calcareous soil with PSB and/or foliar application of *Phaseolus vulgaris* plants with MAP or NP resulted in significant increases in the activities of superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase, and acid phosphatase, the contents of osmoprotectants (soluble sugars, glycine betaine, and proline), phytohormones (indole-3-acetic acid, gibberellic acid, and cytokinins), antioxidants (ascorbate and glutathione), N, P, Mn, while the contents of abscisic acid, Fe, Zn, and Cu, were decreased significantly compared to the control. PSB+NP was the best treatment in both growing seasons. Based on the study results, it can be concluded that inoculation of calcareous soil with PSB in integration with foliar spray with NP significantly improved nutrient contents of *Phaseolus vulgaris* plant grown under high carbonate ($CaCO_3$; calcareous state) stress by up-regulation of antioxidant and phytohormone metabolisms and osmoprotectant accumulations.

**Keywords:** Phaseolus vulgaris, calcareous soil; phosphate-solubilizing bacteria; antioxidative defense system; phytohormones; nutrient elements; soil enzymes

## Introduction:

Globally, especially in developing countries, the demand for food is growing rapidly, where croplands and resources scarcely contribute to the effective production of the strategic crops, which are needed to meet this pressing demand for food. There is an urgent need to maximize agricultural productions in sustainable techniques in defected soils such as calcareous soils. Among these technologies, the use of effective agricultural bio-systems that take into account the biochemical diversity of entire agricultural ecosystems and their capacity to mitigate the adverse effects of low soil fertility and abiotic stresses, including high carbonate content in soils; calcareous state (Timmusk *et al.*, 2017; Bargaz *et al.*, 2018; Belal *et al.*, 2019). In this context, the issue of global food security will promote dependence on innovation, development, and delivery of technologies that elevate food production, while confirming sustainable intensification of agriculture. One of the adopted innovative and effective technologies is the integrated bio- (e.g., phosphate solubilizing bacteria; PSB) and chemical fertilization (e.g., phosphorus; P) strategy that provide highly valuable information for monitoring and securing crop productivity (Salih *et al.*, 1989; Sundara *et al.*, 2002; Shi *et al.*, 2017).

High carbonate content (e.g., calcareous soils) is a factor that limits the availability of mineral nutrients, especially phosphorus (P) and agricultural productivity (Belal *et al.*, 2019). Calcareous soil contains a large amount of calcium carbonate ($CaCO_3$), which

predominates in problems of agricultural land use (FAO, 2016). Leytem and Mikkelsen (2005) have defined calcareous soils as they contain large amounts of free excess lime (e.g., $CaCO_3$ or $MgCO_3$). They have also defined calcareous soils as soils containing more than 14–17% $CaCO_3$ or more than 4–7% active $CaCO_3$ with reference to the hydraulic properties of the entire soil. These soils are very widespread in Mediterranean regions and represent the dominant type of soil in many dry (e.g., arid and semi-arid) climates (Leytem and Mikkelsen, 2005). In addition, the occurrence of these soils have been verified in arid (arid and semi-arid) and humid (humid and sub-humid) areas (Brady and Weil, 2008). Calcareous soils are evaluated as having a few–95% $CaCO_3$ and covering more than 30% of the Earth's surface (Marschner, 1995). High carbonates control the chemistry of these soils, which have alkaline reactions. In most calcareous soils, carbonates negatively affect the pH value to be around 7.5–8.5, making nutrients unavailable to plants, adversely influence the physical properties (e.g., availability of soil water to plants and crust of soil surface), and detrimentally affect, directly or indirectly, the chemical properties (e.g., availability of macro- and micro-nutrients; N, P, K, Mg, Zn, Cu, and Fe) (Marschner, 1995). All these harmful effects of high carbonates lead to detrimental effects on soil structure and fertility associated with plant growth (FAO, 2016). In addition, soils with high $CaCO_3$ and pH, and low organic matter, enzymatic activity and available nutrients. These undesirable properties make the soil defective and less productive. Therefore, to cultivate these soils, many challenges should be addressed. Among these challenges, low CEC, low water-holding capacity, low organic matter (OM) and clay contents, poor structure, low available nutrients, especially P and micronutrients, nutritional imbalances, nutrient loss by leaching or deep percolation, N fertilizer loss, surface crusting and cracking, serious compaction, high pH, and high infiltration rate (El-Hady and Abo-sedera, 2006). However, in the presence of phosphate solubilizing micro-organisms such as phosphate solubilizing bacteria (PSB) and the availability of P, high carbonates content conditions (calcareous state) tend to repair.

PSB play a pivotal role in solubilizing soil P and increasing its bioavailability for plants through transforming insoluble P to available P in the soil, improving fertilizer use efficiency and crop productivity (Hu *et al.*, 2012; Shi *et al.*, 2017). Application of PSB in combination with chemical P fertilizer into defected soil (e.g., calcareous) is an integrated biotechnology practice for comprehensive management and improvement of soil fertility (Sundara *et al.*, 2002; Shi *et al.*, 2017). This practice can promote soil fertility status and increase its content of available P and other nutrients, thus shortening the period of defected soil repair (Shi *et al.*, 2017). Previous investigations concerning the application of PSB to disordered soils have focused mostly on increasing availability of soil P and biological activity. For example, the application of PSB biofertilizer considerably promoted the biochemical capacity and enzymatic activities in calcareous soil (Li *et al.*, 2014). In addition, the integrated application of bio-fertilizer PSB+chemical fertilizer+organic fertilizer was more useful for defected soil repair (Liang *et al.*, 2010; Shi *et al.*, 2017). As one of the essential nutrients necessary for plant growth and development, P plays a pivotal role as a key ingredient in DNA, RNA, ATP, and phospholipids (Schachtman *et al.*, 1998; Rodríguez and Fraga, 1999). Availability of soil P is one of the most important determinants of soil fertility (Shi *et al.*, 2017). Therefore, it is necessary to apply biotechnology practice (e.g., integrative soil PSB + foliar chemical P fertilizer source) to effectively improve the availability of soil P for plants. To increase its use efficiency (PUE), P can be used in nanoparticles form (the so-called "smart fertilizer"), especially as foliar application. Nano-fertilizers are defined as materials with a single-unit ranging in size from 1 to 100 nm in at least one dimension. These nanoparticles have a positive and negative charge on the same particle that improves the uptake of other nutrients by retaining those nutrients in the soil against various losses (Liu and Lal, 2014).

Food legumes are an important constituent in promoting sustainable agriculture and human nutrition worldwide. Legumes are a rich source of protein, especially common bean (*Phaseolus vulgaris* L.), which represents 50% of the total grain legumes consumed globally (Broughton *et al.*, 2003). The cultivation of legumes is beneficial to non-legume crops through numerous agro-ecological contributions such as biological fixation of N, enhancement of soil fertility and production of N-rich green manure (Isaac *et al.*, 2011). However, the nutritional, ecological and economic contributions of legumes are often compromised by their sensitivity to environmental stresses that reduce crop growth and productivity (Scheelbeek *et al.*, 2018). Among these environmental stresses, the damaging biotic and abiotic constraints of the calcareous soil such as limited availability of water, scarcity of nutrients (especially P), increased compaction of soil, increase of carbonates, and decreased fertility and defected structure of soil (Belal *et al.*, 2019).

Although many investigations have used different strategies to recycle P after being added to the soil (Cabeza *et al.*, 2019; Khan *et al.*, 2019), few investigations have evaluated the effect of PSB on recycling P after being added to reclaimed calcareous soils. Therefore, this study was planned to examine the effect of inoculation of calcareous soil (22% $CaCO_3$) with PSB biofertilizer and foliar treatment of *Phaseolus vulgaris* plants with some P forms (e.g., mono-ammonium phosphate; MAP and P in nanoparticles) on biochemical attributes, components of antioxidative defense system, and hormonal and nutrient contents. In addition, *Phaseolus vulgaris* crop was selected for this study because it is one of the most sensitive crops to different types of environmental stressors (Sultana *et al.*, 2014; Bargaz *et al.*, 2016).

## Materials and Methods:

**Growing conditions of plant material, treatments, and experimental layout:**

Two pot experiments were conducted in two different growing seasons; fall, 2018 and summer, 2019 using an open greenhouse at the experimental farm of the Faculty of Agriculture, Fayoum (29°17′06"N 30°54′55"E), Egypt. The climatic conditions were 12.3 to 28.1°C as daily temperatures with an average of 20.2 ± 3.0°C, and 52.4 to 79.0% as daily relative humidity with an average of 65.7 ± 8.8%.

Healthy and uniform seeds of common bean (*Phaseolus vulgaris*) cv. Bronko were purchased from the Horticulture Research Institute, Agricultural Research Center, Ministry of Agriculture, Giza, Egypt. The seeds were surface sterilized with 1% (v/v) NaOCl for 5 min and then thoroughly washed several times with double-distilled water. The seeds were left to air dry for 1 h and

then prepared for sowing. Plastic pots of 35 cm in inner diameter and 32 cm depth were filled in equal quantities (12 kg) with soil that characterized as calcareous (21.8 – 22.2% with an average of 22% $CaCO_3$ for all growing seasons). Based on the physicochemical analyses (Page *et al.*, 1982; Klute and Dirksen, 1986) of this calcareous soil for all preliminary and main studies, it was clay in texture. The physicochemical analyses of this tested soil are shown in Table 1.

**Table 1:** Physical and chemical properties of the experimental soil used for two different seasons before beginning the experiments

| Parameter | Fall season of 2018 | Summer season of 2019 |
|---|---|---|
| Clay | 49.8 | 50.2 |
| Silt | 30.2 | 30.5 |
| Sand | 20.0 | 19.3 |
| Soil texture | Clay | |
| pH | 8.18 | 8.11 |
| EC (dS $m^{-1}$) | 2.28 | 2.19 |
| Organic matter (%) | 0.74 | 0.71 |
| $CaCO_3$ (%) | 21.8 | 22.2 |
| CEC ($cmol_c$ $kg^{-1}$) | 5.79 | 5.66 |
| Available macro- and micronutrients (mg $kg^{-1}$ soil) | | |
| Available N | 12.4 | 12.8 |
| Available P | 5.41 | 5.60 |
| Available K | 24.5 | 26.4 |
| Available Fe | 5.91 | 6.22 |
| Available Mn | 5.04 | 5.12 |
| Available Zn | 3.50 | 3.34 |

"dS $m^{-1}$" means decisiemens per meter, "CEC" means cation exchange capacity, "$cmol_c$ $kg^{-1}$" means centimole of cation exchange capacity per kilogram soil, and "mg $kg^{-1}$" means milligram per kilogram.

A total number of 120 pots were used for six treatments for each growing season. Each treatment needed to 20 pots as four replicates, 5 pots for each. The calcareous soil of 60 pots (3 treatments) was inoculated by phosphate solubilizing bacteria (PSB; a mixture of *Pseudomonas mallei* and *Pseudomonas cepaceae*) and the soil of the other 60 pots (3 treatments) was not inoculated, forming 6 treatments as follows: (1) control (without any treatments), (2) soil inoculated with PSB, (3) soil without inoculation + spraying plants with 1.0 g $L^{-1}$ MAP, (4) soil without inoculation + spraying plants with 0.1 g $L^{-1}$ NP, (5) soil inoculated with PSB + spraying plants with 0.5 g $L^{-1}$ MAP, and (6) soil inoculated with PSB + spraying plants with 0.05 g $L^{-1}$ NP. The MAP fertilizer (Great Neck, NY 11021, USA) used contains N, P, and K at a ratio of 12, 61, and 0 %, respectively. It is 100% water soluble with low pH. The amount of N found in MAP was calculated and added (as foliar spray) to plants in all treatments that did not receive MAP to offset the effect of N in all treatments. NP was prepared in the laboratory using ball-milling (Photon Company, Egypt) following Eleyan *et al.* (2018). Transmission Electron Microscopy (TEM) was used to investigate and measure NP particle size (4.92–8.62 nm) using JEOL transmission electron microscope (JEM-1400 TEM, Japan) following Wang *et al.* (2014). The soil in all pots received the full recommended dose of NPK and organic manures. Each pot (12 kg soil) received 3.6 g of ammonium sulfate (20% N) + 2.4 g of calcium superphosphate (15% $P_2O_5$) + 1.2 g of potassium sulfate (48% $K_2O$).

In both experimental seasons, experiments was repeated 3 times in a layout itemized depending on the completely randomized design (CRD) with 20 pots for each treatment. Pots of all treatments were rotated (from place to place) every 2 days to ensure fairness in the distribution of light and sunlight intensity for all plants. In each pot, 10 homogenous seeds were sown and after full emergence, thinning was attained to maintain 3 uniform seedlings per pot. All pots were irrigated day by day. The types of phosphorus (MAP and NP) were sprayed for plants two times at 25 and 40 days after sowing (DAS). A handheld manual sprayer (model 0417.02.00; Guarany Ind. & Com. Ltd) was used to spray the different solutions of MAP and NP on the upper leaf surface until run-off (approximately 120 ml per pot), and few drops of Tween-20 were added to the spray solutions as a surfactant. In addition, all agricultural practices were applied as recommended for commercial common bean production.

At 50 DAS, common bean plants ($n$ = 9) were harvested to assess plant biochemical attributes, different components of the antioxidant defense system, hormonal and nutrient contents, and phosphatase activity.

**Isolation, identification, and application of phosphate-solubilizing bacteria (PSB) inoculants:**

The PSB (a mixture of *Pseudomonas mallei* and *Pseudomonas cepaceae*) were produced using the Nutrient Broth (NB) medium. This PSB inoculant was isolated from wheat rhizosphere in the Microbiology Laboratory, Faculty of Agriculture, Fayoum University. The isolates were molecularly-identified in a specialized laboratory, National Research Center, Cairo, Egypt. The oligonucleotide primers used for specific PCR were as follows:

| Target species | Primer | 23S rDNA helices containing target position | Sequence | Size of PCR product (bp) | Annealing temp (°C) |
|---|---|---|---|---|---|
| *P. mallei* | M 23-2 | 78ab | 5'-CAC CGA AAC TAG CA-3' | 526 | 47 |
| *P. Cepaceae* | CVP 23-2 | 78ab | 5'-CAC CGA AAC TAG CG-3' | 526 | 47 |

The bacteria (*P. mallei* and *P. cepaceae*) were tested for its ability to solubilize P and to reduce pH in culture conditions and microcosms, and also identified and reported as PSB and plant growth-promoting rhizobacteria (PGPR). The two isolates exhibited no antagonistic activity against each other.

Subsequently, the obtained PSB inoculant was added to a carrier material, which was a mixture of compost and peat at a ratio of 1: 1. This carrier material was encapsulated using aluminum foil and sterilized using an autoclave. Thereafter, the PSB inoculant was added at a ratio of 10% to the carrier material (e.g., 1 L of inoculant for each 10 kg of carrier material). The PSB inoculant was packed and maintained until use. At 48 h prior to sowing, the treatment with the PSB inoculant was applied to the calcareous soil at 1 g (0.1 mL of net PSB) $kg^{-1}$ soil.

**Assaying of soil enzymatic activities:**

After harvest of *Phaseolus vulgaris*, soil samples were collected from pots in which soil was inoculated with PSB in addition to soil samples taken prior to inoculation with PSB. Replicates of each soil sample were well mixed and passed through a < 2-mm sieve to discard pebbles and plant stubbles. Soil samples were stored at 4 °C in a refrigerator until use to determine soil enzymatic activities. Soil phosphatase activity was assayed colorimetrically using disodium phenyl phosphate (Guan, 1986). Assaying phytase activity in soil solutions and suspensions was performed using a sample: buffer ratio of 1:1. Assays were conducted against an InsP6 substrate for 60 min at 37 °C at 2 mM as a final concentration, pH 5.5, with 15 mM of 2-morpholinoethanesulfonic acid (MES). Prior to use, the stock solution (InsP6; 20 mM) was acidified to pH 5.5 with 10 M HCl, and the filtrate was sterilized (0.22 mm) (George *et al.*, 2005; Giaveno *et al.*, 2010). The reactions were stopped with an equal volume of 10% TCA (trichloroacetic acid). Samples were then centrifuged at 3,800 × g for 5 min. Thereafter, P concentration was determined in the supernatant using malachite green (Irving and McLaughlin, 1990). As P released during 60 min assay, phytase activity (nKat $g^{-1}$ soil) was calculated as follows:

Phytase activity (nKat $g^{-1}$ soil) = (P × D × V × 16.67) ÷ (T × 31), where P is the P concentration (mg $L^{-1}$), D is the divide ratio, V is the volume (mL), and T is the incubation time (60 min).

**Determination of osmoprotectant and antioxidant contents:**

To determine the content of potassium ($K^+$), a weight of 0.2 g of dried leaves was digested with 96% $H_2SO_4$ in the presence of $H_2O_2$ (Wolf, 1982). The digestion solution was diluted with distilled water up to 100 mL. The content of $K^+$ was determined using Flame photometer (Lachica *et al.*, 1973).

The Irigoyen *et al.* (1992) method was applied to extract (in 96% ethyl alcohol) and determine total soluble sugars content (mg $g^{-1}$ DW). A volume of 100 µL of the extract was permitted to react with anthrone reagent (150 mg freshly prepared anthrone in 100 mL of 72% $H_2SO_4$ in a final volume of 3 mL). Thereafter, using a water bath, boiling was performed for 10 min. Sample absorbance was read after cooling spectrophotometrically at 625 nm.

The Grieve and Grattan (1983) method was applied to estimate the content of glycine betaine (GB). The periodide crystals formed was observed at 365 nm after reaction of the mixture with a cold reagent (KI–$I_2$) under an acidic state.

The content of free proline was determined as outlined in Bates *et al.* (1973). Due to the interferences between P5C and free proline during reading the absorbance of free proline, free proline values were subtracted from P5C values, which were obtained with applying a standard (e.g., DL-Δ1-pyrroline-5-carboxylate acid; Miller *et al.*, 2009).

The fresh top fully (third and fourth)-expanded leaves were utilized to determine the content (µmol $g^{-1}$ FW) of ascorbate (AsA) as outlined in the method of Kampfenkel and Van Montagu (1995). The extract was added to a mixture of a 30 mM buffer (K-phosphate, pH 7.4), 2.5% TCA, 8.4% $H_3PO_4$, 0.8% bipyridyl, and 0.3% $FeCl_3$. After conducting the reaction for 30 min on 40 °C, the absorbance was read at 525 nm.

The fresh top fully (third and fourth)-expanded leaves were utilized to determine the content (µmol $g^{-1}$ FW) of the reduced GSH and the total GSH (reduced GSH + oxidized GSSG) as outlined in the method of Griffth (1980). To determine the GSH, the reaction mixture containing the extract, 0.13 M and 7 mM of buffers (Na-phosphate, pH 7.4 and 6.8, respectively), and 6 mM of DTNB was heated at 30 °C for 10 min. The absorbance was then read at 412 nm.

**Assaying of antioxidant enzyme activities:**

A weight of 0.5 g of fresh tissue of upper fully-expanded leaves was used to extract the antioxidant enzymes. Samples were macerated using an ice-cold buffer (100 mM K-phosphate, pH 7.0), containing 1% PVP with a pre-chilled clean pestle and mortar. At 4 °C for ¼ h, the obtained homogenates were centrifuged at 12,000 × *g*. Supernatants were used as a source of enzymes to assay the activities of superoxide dismutase (SOD) according to the method detailed in Dhindsa and Matowe (1981), catalase (CAT) according to the method detailed in Aebi (1984), ascorbate peroxidase (APX) according to the method detailed in Nakano and Asada (1981), glutathione reductase (GR) according to the method detailed in Foster and Hess (1980).

Assaying of the activity (U $mg^{-1}$ protein) of SOD (EC: 1.15.1.1) was performed using a spectrophotometer apparatus at 560 nm. To assay the ability of the enzyme to inhibit the NBT photochemical reduction, a mixture consisting of a P-buffer (100 mM, pH 7.4), 10 mM of methionine, 1.0 mM of EDTA, 50 µM of riboflavin, 75 µM of NBT, and the enzymatic extract (100 µL) was prepared to incubate under a fluorescent light for ¼ h.

Assaying of the activity (U $mg^{-1}$ protein) of CAT (EC: 1.11.1.6) was performed using a spectrophotometer apparatus at 240 nm. To assay the ability of the enzyme to decompose the $H_2O_2$ for 2 min, 2 mL of reaction mixture of a P-buffer (50 mM, pH 6.0), 0.1 mM of EDTA, 0.02 M of $H_2O_2$, and 0.1 mL of the enzymatic extract was applied, and the extinction coefficient 39.4 $mM^{-1}$ $cm^{-1}$ was also applied.

Assay the activity (U $mg^{-1}$ protein) of APX (EC: 1.11.1.1) was performed by using 2 mL of a reaction mixture consisting of a P-buffer (50 mM, pH 7.5), 100 µM of EDTA, 300 µM of AsA, 0.1 mL of $H_2O_2$, and 0.1 mL of the enzymatic extract was observed spectrophotometrically at 290 nm for 2 min, and the extinction coefficient 2.8 $mM^{-1}$ $cm^{-1}$ was applied.

Assay the activity (U $mg^{-1}$ protein) of GR (EC: 1.6.4.2) was performed by observing the changes occurred in the absorbance of the reaction mixture (100 mM of K-phosphate buffer; pH 7.0, 100 µM of EDTA, 0.5 mM of NADPH, 0.1 mM of oxidized glutathione, and 100 µL of the enzymatic extract in 3 mL as a final volume) at 340 nm for 3 min.

**Determination of phytohormone contents:**

Extraction and purification of ABA, IAA, $GA_3$ and cytokinins were according to Yurekli *et al.* (2001). With some modifications, analysis of the plant hormones was according to the methods

reported by Nefedieva (2003). Extracts were dissolved in a small volume of methanol and used for HPLC analysis. Samples (20 µL) were injected to a reversed-phase $LC_{18}$ column (250 × 4.6 mm, 5 µ) (Supelco) connected to an HPLC pump (Cecil 1100, Cambridge, UK), and the column was eluted with a linear gradient using 20–80% methanol in 1% (w/v) aqueous acetic acid at a flow rate of 1.2 mL $min^{-1}$ for ABA, with 20–75% methanol in 0.4% acetic acid at a flow rate of 1.0 mL $min^{-1}$ for $GA_3$ and IAA and with 10% acetonitrile at a flow rate of 2.0 mL $min^{-1}$ for cytokinins.

**Determination of macro- and micro-nutrients contents:**

Nitrogen (N) was determined according to the method outlined in Hafez and Mikkelsen (1981) as follows: an Orange-G dye solution was prepared by dissolving 1.0 g of 96% (w/w) assay-dye in 1 l of distilled water with 21.0 g citric acid, which acted as a buffer to maintain the correct pH, and 2.5 ml 10% (v/v) thymol in 10% (v/v) ethanol as an inhibitor of microbial growth. Milled plant material (0.2 g) was placed in a centrifuge tube and 20 ml of the dye reagent solution was added. The contents of each tube were shaken for 15 min. After filtration, the solution was diluted to 100 mL with distilled water and its absorbance was measured at 482 nm. N contents were calculated using the formulae:

N (%) = 0.39 + 0. 954 × Dye absorbed (g /100 g) and Dye absorbed (g /100 g) = (a – b / a) (cfv / w) × 100 where, *a* was the absorbance of the dye reagent solution at 482 nm without plant material (blank), *b* was the absorbance of the dye reagent solution at 482 nm with plant material, *c* was the concentration of the dye reagent (1.0 g l–1 distilled water), *f* was the purity factor of the dye reagent (96%), *v* was the volume of the dye reagent solution used per sample (20 ml), and *w* was the weight of ground dry material in g (0.2).

To determine P content, a weight of 0.2 g of dried leaves was digested with 96% $H_2SO_4$ in the presence of $H_2O_2$ (Wolf, 1982). The digestion solution was diluted with distilled water up to 100 mL. The content of P was determined colorimetrically using ascorbic acid method of Watanabe and Olsen (1965).

Leaf contents (in mg $g^{-1}$ DW) of micro-nutrients (Fe, Mn, Zn, and Cu) were determined using a Model 3300 Perkin-Elmer Atomic Absorption Spectrophotometer (Chapman and Pratt, 1961).

**Assaying of acid phosphatase enzyme activity in plant leaf and root:**

Enzyme extraction was performed by grinding 1.0 g of fresh leaf material using a cooled pestle and mortar. A volume of 20 ml of 100 mM sodium acetate-acetic acid buffer (pH 5.8) was used for 4 min at 2 °C. The homogenate was centrifuged at 30,000 ×g for 10 min at 2 °C. The supernatant was then assayed for the activity of acid phosphatase (Besford, 1979).

Based on p-nitrophenyl phosphate hydrolysis, the leaf enzyme activity was assayed. Absorbance read was recorded at 405 nm using a UV=VIS Spectrophotometer. The acid phosphatase activity was determined by reference to standard curve of p-nitrophenol (Clark, 1975). Standards ranging from 0–16 mM were prepared using deionized water. To assess the activity of acid phosphatase in roots, plant root was put in beaker, in which 250 ml of nutrient solution was added. To obtain a concentration of 0.1 mM, a standard volume of P-nitrophenyl-phosphate was also added to the beaker and the pH was adjusted to 4.0 using HCl. An aerated solution without roots was control reactions. After 30 min, 3.0 ml samples was drawn from the beaker and added to a test tube containing 1.0 ml of NaOH (2 N). The tube was shaken and centrifuged at 3000 ×g for 2 min and absorbance read was recorded at 405 nm using a spectrophotometer. The concentration of p-nitrophenol formed by phosphatase enzyme-mediated hydrolysis of p-nitrophenyl phosphate was determined by reference to standard curve of p-nitrophenol (Clark, 1975). Standards ranging from 0–16 mM were prepared using deionized water.

**Statistical analysis:**

Data are presented in terms of means (± SE; standard error). The completely randomized design (CRD) was the layout of the preliminary and main studies. ANOVA was followed to statistically analyses of all data. Tukey's HSD test (SPSS 14.0; SPSS Chicago, IL, USA) was then applied and $P \leq 0.05$ was used to analyze the significant differences among treatments.

## Results:

**Soil enzymatic activities:**

Soil enzyme activities (e.g., phosphatase and phytase) have been increased by inoculating the tested calcareous soil with phosphate-solubilizing bacteria (PSB) both in the fall season of 2018 and summer season of 2019 (Table 1). The increases were 153 and 158% for phosphatase activity, and 143 and 134% for phytase activity in both seasons, respectively.

**Table 2:** Physical and chemical properties of the experimental soil used for two different seasons before and after its inoculation with phosphorus-solubilizing bacteria (PSB)

| Parameter | Prior to soil inoculation with PSB | | After soil inoculation with PSB | |
|---|---|---|---|---|
| | Fall season of 2018 | Summer season of 2019 | Fall season of 2018 | Summer season of 2019 |
| Clay | 49.8 | 50.2 | 49.9 | 50.4 |
| Silt | 30.2 | 30.5 | 30.5 | 30.6 |
| Sand | 20.0 | 19.3 | 19.6 | 19.0 |
| Soil texture | Clay | | | |
| pH | 8.18 | 8.11 | 7.91 | 7.86 |
| EC (dS $m^{-1}$) | 2.28 | 2.19 | 2.31 | 2.24 |
| Organic matter | 0.74 | 0.71 | 0.80 | 0.82 |
| $CaCO_3$ (%) | 21.8 | 22.2 | 19.7 | 19.9 |
| CEC ($cmol_c$ | 5.79 | 5.66 | 6.82 | 6.80 |
| Available macro- and micronutrients (mg $kg^{-1}$ soil) | | | | |
| Available N | 12.4 | 12.8 | 14.2 | 14.6 |
| Available P | 5.41 | 5.60 | 9.74 | 9.86 |
| Available K | 24.5 | 26.4 | 27.2 | 28.9 |
| Available Fe | 5.91 | 6.22 | 6.21 | 6.31 |
| Available Mn | 5.04 | 5.12 | 5.18 | 5.23 |
| Available Zn | 3.50 | 3.34 | 3.62 | 3.56 |
| Soil enzymatic activities | | | | |
| Phosphatase (mg | 0.53 | 0.55 | 1.34 | 1.42 |
| Phytase (nKat $g^{-1}$ soil) | 5.71 | 6.03 | 13.9 | 14.1 |

Meaning of abbreviations: "dS $m^{-1}$" means decisiemens per meter, "CEC" means cation exchange capacity, "$cmol_c$ $kg^{-1}$" means centimole of cation exchange capacity per kilogram soil, "mg $kg^{-1}$" means milligram per kilogram, and "mg $P_2O_5$ 100 $g^{-1}$ $h^{-1}$" means milligram of phosphorus pentoxide per 100 gram soil per hour.

**Osmoprotectant and antioxidant contents:**

Soil fertilization by PSB and/or plant treatment by MAP or NP led to significant increases in soluble sugars and glycine betaine contents, and proline content was significantly decreased, while $K^+$ content was not affected in *Phaseolus vulgaris* plants compared to control (Table 3). PSB+MAP and PSB+NP were more effective as integrative treatments than individual ones. Compared to PSB+MAP, PSB+NP was better, increasing soluble sugars and glycine betaine contents by 202 and 154%, and 109 and 130% in both seasons, respectively.

Soil fertilization by PSB and/or plant spraying by MAP or NP significantly increased AsA and GSH contents in *Phaseolus vulgaris* plants compared to control (Table 3). PSB+MAP and PSB+NP were more effective as integrative treatments than individual ones. Compared to PSB+MAP, PSB+NP was better, increasing AsA and GSH contents by 102 and 85%, and 128 and 125% in both seasons, respectively.

**Table 3:** Effect of soil application with phosphorus-solubilizing bacteria and foliar application with traditional (MAP; mono-ammonium phosphate) or nano phosphorus (NP) on osmoprotectants and antioxidants contents in common bean plants (cv. Bronco) grown under calcareous soil conditions

| Treatments | Parameters | | | | | |
|---|---|---|---|---|---|---|
| | $K^+$ (mg $g^{-1}$ DW) | Soluble sugars (mg $g^{-1}$ DW) | Glycine betaine (µg $g^{-1}$ DW) | Proline (µmol $g^{-1}$ DW) | AsA content (µmol $g^{-1}$ FW) | GSH content (µmol $g^{-1}$ FW) |
| Fall season of 2018 | | | | | | |
| Control | 21.4±0.4a | 12.3±0.3e | 29.2±0.7e | 36.3±0.8a | 1.62±0.02e | 1.22±0.01e |
| Soil PSB | 20.9±0.4a | 21.4±0.5d | 42.1±0.9d | 28.8±0.6b | 2.04±0.03d | 1.54±0.01d |
| Foliar $MAP_{1.0}$ | 21.4±0.5a | 21.9±0.5d | 42.4±0.9d | 28.9±0.6b | 2.08±0.03d | 1.55±0.01d |
| Foliar $NP_{0.1}$ | 21.5±0.5a | 26.2±0.6c | 48.7±1.1c | 26.7±0.5c | 2.46±0.04c | 1.94±0.02c |
| Foliar $MAP_{0.5}$+Soil PSB | 21.5±0.5a | 33.1±0.8b | 54.2±1.3b | 23.7±0.5d | 2.98±0.04b | 2.31±0.03b |
| Foliar $NP_{0.05}$+Soil PSB | 21.6±0.5a | 37.2±0.8a | 61.1±1.4a | 21.6±0.3e | 3.27±0.05a | 2.78±0.04a |
| Summer season of 2019 | | | | | | |
| Control | 22.8±0.5a | 13.8±0.4e | 25.1±0.6e | 37.9±1.0a | 1.70±0.03e | 1.18±0.01e |
| Soil PSB | 22.1±0.4a | 19.9±0.6d | 39.8±0.9d | 31.1±0.8b | 1.98±0.03d | 1.49±0.01d |
| Foliar $MAP_{1.0}$ | 22.4±0.5a | 20.1±0.6d | 40.1±1.1d | 31.0±0.7b | 2.06±0.03d | 1.49±0.01d |
| Foliar $NP_{0.1}$ | 22.6±0.5a | 24.8±0.7c | 45.6±1.3c | 27.8±0.7c | 2.30±0.04c | 1.91±0.02c |
| Foliar $MAP_{0.5}$+Soil PSB | 22.6±0.6a | 30.2±0.9b | 52.1±1.6b | 25.0±0.5d | 2.74±0.04b | 2.32±0.02b |
| Foliar $NP_{0.05}$+Soil PSB | 22.7±0.6a | 35.1±1.0a | 57.8±1.8a | 22.1±0.4e | 3.14±0.04a | 2.66±0.03a |

Data presented are means ± SE (n = 9). Different letters next to mean values indicate significant differences at $P \leq 0.05$. All pots of all treatments, including the **control**, received full recommended doses of NPK fertilizers for common bean production on calcareous soils.

**Activity of antioxidant enzymes:**

Soil inoculation by PSB and/or plant spraying by MAP or NP significantly increased the activities of SOD, CAT, GST, APX, GR, MDHAR, and DHAR in *Phaseolus vulgaris* plants compared to control (Table 4). Compared to individual treatments, PSB+MAP and PSB+NP were more effective, from which PSB+NP was better, increasing SOD, CAT, APX and GR activity by 146 and 116%, 61 and 59%, 84 and 85%, and 97 and 103%, in both seasons, respectively.

**Table 4:** Effect of soil application with phosphorus-solubilizing bacteria and foliar application with traditional (MAP; mono-ammonium phosphate) or nano phosphorus (NP) on activities of antioxidant enzymes and enzymes of ascorbate–glutathione cycle of common bean plants (cv. Bronco) grown under calcareous soil conditions

| Treatments | Parameters | | | |
|---|---|---|---|---|
| | SOD activity (EU $mg^{-1}$ protein) | CAT activity (EU $mg^{-1}$ protein) | APX activity (EU $mg^{-1}$ protein) | GR activity (EU $mg^{-1}$ protein) |
| Fall season of 2018 | | | | |
| Control | 102±2e | 41.3±0.8e | 17.2±0.2e | 15.8±0.2e |
| Soil PSB | 164±3d | 50.4±0.9d | 19.6±0.3d | 19.8±0.2d |
| Foliar $MAP_{1.0}$ | 166±3d | 50.2±0.9d | 19.9±0.3d | 20.4±0.3d |
| Foliar $NP_{0.1}$ | 197±3c | 54.8±0.9c | 22.8±0.3c | 23.2±0.3c |
| Foliar $MAP_{0.5}$+Soil PSB | 228±4b | 60.1±1.0b | 26.1±0.4b | 26.7±0.3b |
| Foliar $NP_{0.05}$+Soil PSB | 251±4a | 66.6±1.2a | 31.7±0.4a | 31.2±0.4a |

| | | | | |
|---|---|---|---|---|
| Summer season of 2019 | | | | |
| Control | 112±2e | 39.7±0.6e | 18.1±0.3e | 16.7±0.2e |
| Soil PSB | 158±2d | 47.2±0.8d | 21.4±0.3d | 19.7±0.3d |
| Foliar $MAP_{1.0}$ | 164±3d | 48.0±0.8d | 21.2±0.4d | 19.6±0.2d |
| Foliar $NP_{0.1}$ | 189±3c | 53.1±0.9c | 24.3±0.4c | 24.2±0.4c |
| Foliar $MAP_{0.5}$+Soil PSB | 218±4b | 58.4±0.9b | 27.9±0.4b | 29.0±0.4b |
| Foliar $NP_{0.05}$+Soil PSB | 242±5a | 63.2±1.0a | 33.5±0.5a | 33.9±0.5a |

Data presented are means ± SE (n = 9). Different letters next to mean values indicate significant differences at $P \leq 0.05$. All pots of all treatments, including the **control**, received full recommended doses of NPK fertilizers for common bean production on calcareous soils.

**Phytohormones contents:**

Soil fertilization by PSB and/or plant treatment by MAP or NP led to significant increases in the contents of indole-3-acetic acid (IAA), gibberellic acid ($GA_3$), and cytokinins (CKs), and led to significant decrease in abscisic acid (ABA) content in *Phaseolus vulgaris* plants compared to control (Table 5). PSB+MAP and PSB+NP were more effective as integrative treatments than individual ones. Compared to PSB+MAP, PSB+NP was better, increasing IAA, $GA_3$, and CKs by 74 and 67%, 81 and 68%, and 50 and 53%, and reduced ABA content by 43 and 40% in both seasons, respectively.

**Table 5:** Effect of soil application with phosphorus-solubilizing bacteria and foliar application with traditional (MAP; mono-ammonium phosphate) or nano phosphorus (NP) on contents of phytohormones contents of common bean plants (cv. Bronco) grown under calcareous soil conditions

| Treatments | Parameters | | | |
|---|---|---|---|---|
| | IAA (µg $g^{-1}$ FW) | $GA_3$ (µg $g^{-1}$ FW) | Cytokinins (µg $g^{-1}$ FW) | ABA (µg $g^{-1}$ FW) |
| Fall season of 2018 | | | | |
| Control | 1.44±0.02e | 1.18±0.02e | 2.14±0.03e | 3.34±0.04a |
| Soil PSB | 1.63±0.02d | 1.36±0.02d | 2.34±0.03d | 2.91±0.03b |
| Foliar $MAP_{1.0}$ | 1.64±0.02d | 1.40±0.02d | 2.35±0.03d | 2.88±0.03b |
| Foliar $NP_{0.1}$ | 1.91±0.03c | 1.59±0.03c | 2.59±0.04c | 2.65±0.03c |
| Foliar $MAP_{0.5}$+Soil PSB | 2.22±0.03b | 1.85±0.03b | 2.80±0.04b | 2.23±0.02d |
| Foliar $NP_{0.05}$+Soil PSB | 2.51±0.04a | 2.14±0.04a | 3.21±0.05a | 1.91±0.02e |
| Summer season of 2019 | | | | |
| Control | 1.61±0.02e | 1.32±0.02e | 2.24±0.03e | 3.06±0.03a |
| Soil PSB | 1.89±0.03d | 1.51±0.02d | 2.48±0.04d | 2.70±0.03b |
| Foliar $MAP_{1.0}$ | 1.91±0.03d | 1.56±0.02d | 2.50±0.04d | 2.71±0.03b |
| Foliar $NP_{0.1}$ | 2.18±0.03c | 1.74±0.03c | 2.78±0.04c | 2.39±0.02c |
| Foliar $MAP_{0.5}$+Soil PSB | 2.44±0.04b | 1.98±0.03b | 3.15±0.05b | 2.09±0.02d |
| Foliar $NP_{0.05}$+Soil PSB | 2.69±0.04a | 2.22±0.04a | 3.43±0.05a | 1.84±0.02e |

Data presented are means ± SE (n = 9). Different letters next to mean values indicate significant differences at $P \leq 0.05$. All pots of all treatments, including the **control**, received full recommended doses of NPK fertilizers for common bean production on calcareous soils.

**Macro- and micro-nutrients contents:**

Soil fertilization by PSB and/or plant treatment by MAP or NP led to significant increases in the contents of nitrogen (N), phosphorus (P), and manganese (Mn), and led to significant decrease in iron (Fe), zinc (Zn), and copper (Cu) contents of *Phaseolus vulgaris* plants compared to control (Table 6). PSB+MAP and PSB+NP were more effective as integrative treatments than individual ones. Compared to PSB+MAP, PSB+NP was better, increasing N, P, and Mn contents by 43 and 37%, 248 and 243%, and 59 and 58%, and reduced Fe, Zn, and Cu contents by 9 and 14%, 50 and 50%, and 36 and 42% in both seasons, respectively.

**Table 6:** Effect of soil application with phosphorus-solubilizing bacteria (PSB) and foliar application with traditional (MAP; mono-ammonium phosphate) or nano phosphorus (NP) on contents of nitrogen (N), phosphorus (P), and micronutrients in common bean plants (cv. Bronco) grown under calcareous soil conditions

| Treatments | Parameters | | | | | |
|---|---|---|---|---|---|---|
| | N (mg $g^{-1}$ DW) | P (mg $g^{-1}$ DW) | Fe (mg $g^{-1}$ DW) | Mn (mg $g^{-1}$ DW) | Zn (mg $g^{-1}$ DW) | Cu (mg $g^{-1}$ DW) |
| Fall season of 2018 | | | | | | |
| Control | 16.4±0.3b | 0.85±0.02e | 0.34±0.01a | 0.17±0.00e | 0.14±0.01a | 0.11±0.00a |
| Soil PSB | 22.7±0.4a | 2.14±0.04d | 0.30±0.01b | 0.21±0.00d | 0.11±0.00c | 0.09±0.00b |
| Foliar $MAP_{1.0}$ | 22.6±0.4a | 2.22±0.05d | 0.30±0.01b | 0.20±0.00d | 0.12±0.00b | 0.09±0.00b |
| Foliar $NP_{0.1}$ | 23.0±0.4a | 2.46±0.05c | 0.31±0.01b | 0.23±0.01c | 0.09±0.00d | 0.08±0.00c |
| Foliar $MAP_{0.5}$+Soil PSB | 23.3±0.5a | 2.67±0.06b | 0.30±0.01b | 0.25±0.01b | 0.07±0.00e | 0.08±0.00c |
| Foliar $NP_{0.05}$+Soil PSB | 23.4±0.5a | 2.96±0.07a | 0.31±0.01b | 0.27±0.01a | 0.07±0.00e | 0.07±0.00d |
| Summer season of 2019 | | | | | | |
| Control | 17.8±0.3b | 0.89±0.02e | 0.37±0.01a | 0.19±0.00e | 0.16±0.01a | 0.12±0.00a |
| Soil PSB | 23.4±0.4a | 2.22±0.04d | 0.31±0.01b | 0.23±0.01d | 0.13±0.01b | 0.10±0.00b |
| Foliar $MAP_{1.0}$ | 23.7±0.4a | 2.28±0.04d | 0.31±0.01b | 0.22±0.01d | 0.14±0.01b | 0.10±0.00b |
| Foliar $NP_{0.1}$ | 23.9±0.5a | 2.62±0.06c | 0.31±0.01b | 0.25±0.01c | 0.11±0.00c | 0.09±0.00c |
| Foliar $MAP_{0.5}$+Soil PSB | 24.1±0.5a | 2.84±0.07b | 0.32±0.01b | 0.28±0.01b | 0.09±0.00d | 0.07±0.00d |
| Foliar $NP_{0.05}$+Soil PSB | 24.3±0.5a | 3.05±0.08a | 0.32±0.01b | 0.30±0.01a | 0.08±0.00d | 0.07±0.00d |

Data presented are means ± SE (n = 9). Different letters next to mean values indicate significant differences at $P \leq 0.05$. All pots of all treatments, including the **control**, received full recommended doses of NPK fertilizers for common bean production on calcareous soils.

**Activity of acid phosphatase enzyme in leaves and roots:**

Soil inoculation by PSB and/or plant treatment by MAP, or foliar NP significantly decreased the activity of acid phosphatase in both leaves and roots of *Phaseolus vulgaris* plants by compared to control (Table 7). Compared to individual treatments, PSB+MAP and PSB+NP were more effective, from which PSB+NP was better, decreasing acid phosphatase activity in leaves and roots by 68.5 and 69.8%, and 64.6 and 72.3%, in both seasons, respectively.

**Table 7.** Effect of soil application with phosphorus-solubilizing bacteria (PSB) and foliar application with traditional (MAP; mono-ammonium phosphate) or nano phosphorus (NP) on the activity of acid phosphatase enzyme in leaves and roots of common bean plants (cv. Bronco) grown under calcareous soil conditions

| Treatments | Parameters | |
|---|---|---|
| | Phosphatase activity in leaves (µM P-nitrophenol $g^{-1}$ leaf $h^{-1}$) | Phosphatase activity in roots (µM P-nitrophenol $g^{-1}$ root $h^{-1}$) |
| Fall season of 2018 | | |
| Control | 33.7±1.8a | 98.8±3.7a |
| Soil PSB | 21.6±1.2b | 61.4±2.8b |
| Foliar $MAP_{1.0}$ | 20.7±1.2b | 61.5±2.8b |
| Foliar $NP_{0.1}$ | 17.4±0.8c | 60.0±2.4b |
| $MAP_{0.5}$+PSB | 14.2±0.6d | 44.1±2.0c |
| $NP_{0.05}$+PSB | 10.6±0.4e | 29.8±1.1d |
| Summer season of 2019 | | |
| Control | 31.6±1.7a | 96.3±3.3a |
| Soil PSB | 22.2±1.3b | 64.8±2.6b |
| Foliar $MAP_{1.0}$ | 19.0±1.0c | 63.9±2.6b |
| Foliar $NP_{0.1}$ | 18.8±0.8c | 54.6±2.1c |
| $MAP_{0.5}$+PSB | 15.3±0.7d | 39.9±1.6d |
| $NP_{0.05}$+PSB | 11.2±0.5e | 26.7±1.2e |

Data presented are means ± SE (n = 9). Different letters next to mean values indicate significant differences at $P \leq 0.05$. All pots of all treatments, including the **control**, received full recommended doses of NPK fertilizers for common bean production on calcareous soils.

## Discussion

The calcareous soil used in this study has poor structure and undesirable properties such as high pH and calcium carbonate ($CaCO_3$) content. It also contains a low content of organic matter (OM), available nutrients, especially P, and low enzymatic activities (Table 1). These undesirable properties indicate a low fertility with nutritional imbalance that makes the soil defective and less productive (Rady *et al.*, 2020). These results are consistent with those obtained by El-Hady and Abo-Sedera (2006), Aboukila *et al.* (2018), and Rady *et al.* (2020). Under these harsh conditions, it is difficult to obtain a satisfactory level of yield, especially for *Phaseolus vulgaris*, a crop sensitive to various types of environmental stressors (Sultana *et al.*, 2014; Bargaz *et al.*, 2016), including high carbonate content (e.g., calcareous). Therefore, effective tools should be used to repair such harsh conditions of the tested calcareous soil.

Among a number of bacterial genera, *Pseudomonas sp*. are able to solubilize the metallic P-complex to release bioavailable P in orthophosphate form through specific mechanisms. These mechanisms mainly include organic acids and the production of siderophore and enzymes (e.g., phosphatase and phytase) that play a key role in hydrolyzing organic P forms (Table 1) into an absorbable form by the roots of plants (Rady *et al.*, 2020).

Inoculation of the calcareous soil, used in this study, by phosphate-solubilizing bacteria (PSB) helped release of P from the fixation state to be available to plant roots (Rady et al., 2020). In addition, PSB effectively decreased $CaCO_3$ and pH and increased OM, CEC, available nutrient, and enzymatic activity (e.g., phosphatase, and phytase) in the tested soil (Table 1). These improved properties by PSB make this soil productive, especially when PSB applied in integration with foliar application with P (mono-ammonium phosphate; MAP or nano-phosphorus; NP) for *Phaseolus vulgaris* plants (Tables 2–7).

In this study, PSB (a mixture of *Pseudomonas mallei* and *Pseudomonas cepaceae*) facilitated the transformation of insoluble P to soluble/available P in the tested soil. This mechanism elevated soil P availability to roots of *Phaseolus vulgaris* plants, contributing to the increase in P content, growth, and productivity of plants (Rady *et al.*, 2020). These results are consistent with those obtained by Hu *et al.* (2012) and Shi *et al.* (2017). The superior effect of the integrative PSB+NP treatment is due to the efficacious capacity of PSB strains to solubilize P through the increase in the soil enzymes (e.g., phosphatase and phytase) as an effective mechanism, which increased the inorganic form of soil P to be available to plant roots (Table 2). In addition, NP could be an effective source of P nutrient as a soluble P fertilizer and plants can effectively take up P in nanoparticle formulation applied as foliar spray. It has been proved that P is important for the development and growth of plant cells, roots, flowers, fruits, and seeds. It also improves plant quality and strengthens plants against easily fall and diseases (Elfiati, 2005). In addition, P plays a pivotal role as a key ingredient in DNA, RNA, ATP, and phospholipids for healthy cell membranes (Schachtman *et al.*, 1998; Rodríguez and Fraga, 1999).

Availability of soil P by PSB is one of the most important determinants of soil fertility in terms of increased contents of available nutrients and OM, and reduced content of $CaCO_3$ (Table 2). Soil inoculation using PSB in integration with foliar spraying of P, especially NP, supports each other in supplying plants with nutrients, especially P for their life (Table 6). *Pseudomonas sp.* work synergistically to produce phosphatases (Table 2) through mineralization and immobilization processes to transform organic P into inorganic form, so that the growth of *Pseudomonas sp*. can still be optimal from vegetative to harvest stage of plants (Fitriatin *et al.*, 2014; Rady *et al.*, 2020). As an efficient mechanism, the PSB strains secrete, quantitatively and qualitatively, organic acids (mainly as a gene-dependent; Zhen *et al.*, 2016) in the soil to compete with P ions for the P adsorption sites, increasing P release in the soil for plants. PSB can promote the productivity of calcareous soil and elevate its biological activity (biochemical capacity of soil microorganisms and relevant enzyme; phosphatase and phytase activities) and available P content and other nutrients in such soil (Table 2). PSB enhance P use efficiency directly through exudation of organic acids and P-hydrolyzing phosphatase enzymes to improve P pool bioavailability, or indirectly through production of phytohormones, antifungal and toxin-resistance compounds, and other high value bioactive molecules which can help build a vigorous shoot/root system, especially under abiotic and biotic constraints (Shi *et al.*, 2017) such as the problem under study; calcareous state. The influence of organic acids in solubilizing P is often attributed to reduced pH (from 8.11–8.18 to 7.86–7.91) and cation chelating properties (Table 2), which were obtained due to PSB inoculation of the tested soil. Acidification of microbial cells perimeter results in the release of P anion by replacing $H^+$ and $Ca^{2+}$ (Behera *et al.*, 2017) as a potential mechanism. Other potential mechanisms for solubilization of P in calcareous soil, the release of protons after $NH_4$ assimilation by microbial cells, the production of inorganic acids (i.e., $H_2SO_4$ and $HNO_3$), and the production of specific enzymes (Table 2) acting on amphiphilic fatty substances (Alori *et al.*, 2017). In addition to microbial solubilization of mineral P, mineralization of organic P through microorganisms action also plays a pivotal role in P cycling, giving that organic P content in soil (often in inositol polyphosphates form) can account for between 30 and 50% of total P. Mineralization process of P is extensively controlled by specialized P-hydrolyzing enzymes produced by PSB such as phosphatases and phytases, which are a non-specific exo-enzymes produced mainly by bacteria (Alori *et al.*, 2017). In addition to their positive contribution to the enhancement of P bioavailability, PSB-mediating soil P availability possess other worthy attributes of agronomic interests, including production of plant hormones, enhancing the ability to resist biotic and abiotic stresses through producing specific (e.g., antifungal) compounds, and the regulation of key metabolic pathways (Sharma *et al.*, 2013).

Rady *et al.* (2020) reported that the integrative application of soil PSB and foliar NP significantly reduced of $H_2O_2$ and $O_2^{\bullet-}$ accumulations, lipid peroxidation (MDA content), and electrolyte leakage (EL) in *Phaseolus vulgaris* plants grown under high $CaCO_3$ stress. This result can be attributed to the positive effect of P in maintenance of antioxidant system components (Tables 3 and 4) and phytohormones contents (Table 5). Supplying plants with P significantly improved antioxidant enzyme activities (Tables 4 and 7) and elevated ascorbate (AsA) and glutathione (GSH) contents (Table 3), thereby protecting *Phaseolus vulgaris* plants against high $CaCO_3$-induced oxidative stress ($H_2O_2$ and $O_2^{\bullet-}$; Rady *et al.*, 2020).

Through the ROS dismutation process, SOD removes the radicals of $O_2^{\bullet-}$ in association with both CAT and APX, which carry more dismutation. P-stimulated up-regulation of SOD may modulate the substrates $O_2^{\bullet-}$ and $H_2O_2$. This mechanism leads to the reduction in the formation of more toxic radicals of hydroxyl ($OH^-$) (Singh and Prasad, 2014). P-induced accumulations in AsA and GSH levels can protect high $CaCO_3$-stressed *Phaseolus vulgaris* plant from ROS-stimulated injuries. Enzymatic and non-enzymatic antioxidants such as GR, APX, GSH, and AsA (Tables 3 and 4) are from the components of the ROS scavenging pathway (ascorbate-glutathione cycle; Rady *et al.*, 2020) and P-stimulated up-regulation of these components boosts the tolerance strategies of plant against any potential oxidative damage (Rady *et al.*, 2020). For example, in this report, authors concluded that high $CaCO_3$-stressed *Phaseolus vulgaris* plants provided with P showed reduction in the accumulation of ROS ($H_2O_2$ and $O_2^{\bullet-}$) and elevated protection to photosynthetic pathways leading to better plant

growth and yield productivity.

Mittler (2002) reported that the $H_2O_2$ produced as a result of $O_2^{\bullet-}$ elimination by SOD activity can be dismantled in the cytoplasm by CAT or in the ascorbate–glutathione cycle by APX. This cycle includes a series of reactions of redox, including the bioactive participation of AsA, GSH, and NADPH. The enzyme APX plays a pivotal role in scavenging of $H_2O_2$ in the chloroplasts and cytosol, thus preventing the diffusion of $H_2O_2$ to other organelles to avoid any damage. The optimal functioning of the pathway of AsA-GSH cycle due to supplying plants with P (Rady *et al.*, 2020) effectively preserved the components of redox, including the AsA and GSH, therefore, decreasing the oxidative stress impacts of high $CaCO_3$. The elevated activity of enzymatic and non-enzymatic antioxidants is associated with the improved other stress tolerance in plants (Semida and Rady, 2014; Ahanger *et al.*, 2018; Rehman *et al.*, 2018; Alzahrani and Rady, 2019).

In the present study, supplying bean plants with P encouraged osmoprotectant accumulations (e.g., soluble sugars, proline, and glycine betaine; GB) (Table 3) to increase plant water content to cope with high $CaCO_3$ stress. Proline accumulation is limited in this study due to the up-regulation of proline synthesizing enzymes with down-regulation of catabolizing enzymes (Rady *et al.*, 2020). This is due to the increase in other factors (antioxidant system components, soluble sugars, and GB) (Tables 3 and 4) enabling plants to cope with stress. In this case, proline is incorporated into proteins (Ahmad, 2010). P-induced improvement in the accumulation of soluble sugars and GB possibly helped common bean plant to avoid the high $CaCO_3$ effects. Soluble sugars and GB maintain plant water balance, minimizing the injurious effects of stresses on its metabolism (Ahanger *et al.*, 2014), especially by protection of protein turnover, expression of stress-protective proteins, and enzyme activities (Thakur and Sharma, 2005; Ahanger and Agarwal, 2017). P is one of the most important nutrients involved in plant growth and metabolism. Cellular inorganic orthophosphate (Pi) regulates enzyme activity, phytohormone contents and metabolic pathways as well as the transport processes, affecting various photosynthetic aspects (Terry and Rao, 1991, Mohamed *et al*.,2006, Ghallab *et al*.,2007,Rady *et al.*,2019).

Supplying *Phaseolus vulgaris* plants with P (especially with the integrative PSB + NP treatment) significantly increased phytohormones; indole-3-acetic acid (IAA), gibberellic acid ($GA_3$), and cytokinins (CKs) contents, while the content of absiscic acid (ABA) was significantly reduced (Table 5). This positive result may be attributed to the improvements in nutrients contents (Table 6), which are considered one of very important factors that improve plant hormonal status in plants.

The roles of phytohormones, such as ABA, cytokinins and auxins, in the growth responses induced by P availability have been frequently addressed (Lopez-Bucio *et al.*, 2002). Availability of P in soil nutrient solution and uptake by plants awarded some positive effects on phytohormone contents, suggesting an involvement of these plant hormones in growth responses of plants to availability of phosphorus (Ribot *et al.*, 2008). The levels of endogenous phytohormones (e.g., IAA, $GA_3$, CKs, and ABA) in plants were also changed correspondingly with availability of nutrients, including P (Lei, and Ya-qing, 2015).

Differences in nutrient contents, in this study (Table 6), have revealed clear biochemical differences in common bean plant response to the stress of high soil carbonate ($CaCO_3$) content and P availability occurred by P treatments, especially the integrative PSB + NP treatment. Availability of P significantly increased the nutrients N, P, and Mn, while Fe, Zn and Cu contents were reduced. This reduction in Fe, Zn and Cu contents may be attributed to that the plants required these micro-nutrients in small quantities (Bargaz *et al.*, 2016). Availability of P failed to increase K content, which unchanged by P treatments, and this may be due to that plant not need more K due to the increase occurred in other osmoprotectants (Rady *et al.*, 2020). On the other hand, Malik *et al.* (1999); El-Ganaini *et al*.,2005 and Bargaz *et al.* (2016) reported that synergistic relationship between P and other beneficial elements like P, N and Mn might have initiated an osmotic effect and thus can be held responsible for plant tolerance to some degree of calcareous state. Results of the current study confirmed this result where P availability through P treatments (especially PSB + NP) increased P, N and Mn contents, while reduced Fe, Zn and Cu contents.

Supplying *Phaseolus vulgaris* plants with P significantly decreased acid phosphatase activity in leaves and roots under high $CaCO_3$ under study (Table 7). This may be related to the increased content of P more than the plant needs (Table 6). This result agreed with Rady *et al.* (2018) & (2020), who indicated that increased P content lead to decrease in acid phosphatase activity, while Wassaki *et al.* (1997) reported that P deficiency induces acid phosphatase synthesis in lupin roots. In addition, Romer and Fahning (1998) noted that the activity of root phosphatase increased with the reduction of shoot P status of *Lolium multiflorum* inbred lines. Kaya *et al.* (2002) reported also that acid phosphatase activity was increased in the leaves and roots of tomato plants grown at high zinc induced P deficiency. They attributed this result to that the application of inorganic P to soil supplies adequate amount of available P to plants, which restricts the activity of phosphatase and helps mineralization of total P present in the soil.

Supplying with P (especially by the integrative PSB+NP treatment) enabled *Phaseolus vulgaris* plants to develop/adopt some potential mechanisms to increase their tolerance to high $CaCO_3$ stress. For example, the increased accumulation of osmoprotectant compounds awarded a potential mechanism to prevent water loss from leaves for maintaining membrane stability and healthy metabolic processes under high $CaCO_3$ stress. The increased activities of various (enzymatic and non-enzymatic) antioxidants conferred another potential mechanism to strengthen the antioxidant defense system to increase plant resistance to high $CaCO_3$ stress. These mechanisms along with others led to stay greenness and delay senescence of plant leaves, and improved chlorophyll content and photosynthesis efficiency to maintain healthy growth of plants under stress (Rady *et al.*, 2020). Taken together, these helps limiting the oxidative damage induced by high $CaCO_3$ stress by the improvement in antioxidant defense components (e.g., all antioxidant system components, including ascorbate-glutathione cycle).

## Conclusions:

Based on the study results, it can be concluded that soil inoculation

with phosphate-solubilizing bacteria in integration with foliar spray using phosphorus in nano-particles has improved nutrient contents, especially P of *Phaseolus vulgaris* plant under high carbonate ($CaCO_3$; calcareous state) stress by up-regulation of antioxidant and phytohormones metabolisms and osmoprotectant accumulations. The increase in nutrient and hormonal contents coincided with a decrease in acid phosphatase activity in phosphorus-provided plants led to maintenance of cellular functioning and higher photoprotection. All these observations point to the appropriateness of the integrative phosphate-solubilizing bacteria + phosphorus in nano-particles to exploit the genetic potential of *Phaseolus vulgaris* plant under high carbonate stress. However, more systematic studies are needed to explain the mechanisms of plants taking up phosphorus in nano-particles as a nutrient source and why phosphorus in nano-particles performed better over the conventional phosphate fertilizer; mono-ammonium phosphate or calcium superphosphate in improving plant growth and yield. Therefore, future investigations in this tendency can be helpful.

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