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Effect of plant growth-promoting rhizobacteria on growth and symbiotic nitrogen fixation of *vicia faba* plants under salt stress.

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The effect of three bacterial strains as inoculant (*Rhizobium leguminosarum, Azospirillum brasilense* and *Paenibacillus polymyxa*) on plant growth promotion of *Vicia faba* (Giza 843) plants was investigated under two levels of salinity stress (50 and 100 mM NaCl). The results indicated that increasing salt concentration up to 100 mM NaCl, decreased all of plant growth parameters, plant chlorophyll, and plant soluble proteins, while plant proline was increased, comparing to the control (uninoculated). Inoculation with the three bacterial species (PGPR) improved most morphological parameters of broad bean under salt stress. The plants treated with *Rhizobium* plus *Paenibacillus* significantly reduced the negative impact of salinity stress on shoot growth, nitrogen and phosphorus content, leaf chlorophyll content and antioxidant enzyme activity. Inoculation with *Azospirillum* plus *Rhizobium* resulted in increasing nodule numbers (41%), plant soluble protein, nitrogen content and appearance of additional new protein bands of broad bean plants, compared to that inoculated with *Rhizobium* alone.

Keywords: salt stress, Vicia faba, PGPR, rhizobia

INTRODUCTION

Plant growth promoting-rhizobacteria (PGPR) are a group of bacteria that colonize roots of plant actively and increase plant growth and development (Viveros *et al*., 2010, Bhattacharyya and Jha 2012, Gupta *et al*., 2015). Different bacteria that have been reported as PGPR belong to the genera *Pseudomonas*, *Agrobacterium, Bacillus*, *Phyllobacterium, Azospirillum*, *Azotobacter*, *Enterobacter* and *Rhizobium* PGPR include increases in germination rates, root growth, yield, leaf area, chlorophyll content, magnesium, nitrogen and protein content, hydraulic activity, tolerance to drought and salt stress, shoot and root weights and delayed leaf senescence (Lucas-García *et al*., 2004).PGPR facilitate plant growth indirectly by their biocontrol properties such as antibiotic production, production of lytic enzymes, competition with phytopathogens for nutrients and colonizing sites, and induced systemic resistance, or directly by nitrogen fixation, phosphate solubilization, facilitating the nutrient uptake through phytohormone production (e.g. auxin, cytokinin and gibberellins), by enzymatic lowering of plant ethylene levels and/or by production of siderophores (Kohler *et al*., 2006, Akhtar *et al*.,

2011, Glick 2012, Ahmad and Kibret 2014, Desai *et al*., 2016, Karthik *et al*., 2016, Omara *et al*., 2017. The use of this environmentally friendly approach could be among the most efficient methods for minimizing the use of chemicals, which can adversely impact human health directly and indirectly.

PGPR inoculation improves plant growth and yield of various crops under normal and stress conditions (Arruda *et al*., 2013, Barnawal *et al*. 2014, Martínez *et al*., 2015, Mahmood *et al*., 2016, Omara *et al*, 2017). PGPR promoting effect under stress conditions has also been suggested to be beneficial for crop production,the plant height, root length, dry weight of shoot and root. These parameters were significantly increased due to *Pseudomonas* strain treatment (Ahmad *et al*., 2013, El-Nahrawy and Omara 2017, Khalid *et al*., 2017). Nitrogen-fixing rhizobia-legume symbioses represent a great potential to improve crop yields and to reduce the use of fertilizers in agriculture (Zahran 2009, Zahran 2017). In addition, some rhizobia, together with *Pseudomonas* and *Bacillus*, were reported to have phosphate-solubilizing capacity (Demissie *et al*., 2013, Bouizgarne *et al*., 2015).

Inoculation of *V. faba* by *Rhizobium* and *Azotobacter* or *Rhizobium* and *Azospirillum combinations* led to change in the total content, concentration and distribution of macro and micronutrients (K, P, Mn, and Zn), when compared with plant inoculated with *Rhizobium* alone (Rodelas *et al*., 1999). Co-inoculation of rhizobia with phosphate-solubilizing bacteria revealed a synergistic effect on symbiotic parameters such as increasing nodule number and plant biomass, which resulted in grain yield of legumes (Saharan and Nehra 2011).

El-Nahrawy and Omara(2017) found that co-inoculation of common bean plant with *R. leguminosarum*plus *Pseudomonas koreensis*exhibited the highest values of growth, symbiosis, photosynthesis and nitrogen content. Inoculation with *Rhizobium* sp. into saline soils alleviated the salinity effects on the antioxidant enzymes ascorbate, peroxidase and glutathione reducates, along with those on photosynthesize, minerals content and growth (Han and Lee 2005, Roy *et al*., 2014).

Broad bean (*V. faba*L.) is one of the most important food crops in Egypt. It is considered as one of the main sources of plant proteins for human nutrition in Egypt. *V. faba* covers a considerable part of protein needs, although its production is insufficient to meet the total demand in the country. High yield production of faba bean is urgently needed to meet the increasing population and growing demand for protein food in Egypt.

Broad bean (*Vicia faba* L.) plant contains high protein content which ranges from 270 to 320 g/Kg of dry seeds (Crepon *et al*., 2010) and appreciable amount of minerals and vitamins (Yetneberk and Wondimu 1994). Broad bean (*V. faba*), a common food in the Mediterranean region, is an important winter legume crop worldwide. Broad bean is beneficial to soil health because of its fixing nitrogen capacities (fixing nitrogen with root nodule bacteria known as *Rhizobium leguminosarum* bv. *viciae*). It is the most efficient nitrogen fixer of all cool season pulse crops (McVicar *et al*., 2005). Broad bean is also used as green manure, to provide large quantities of N to spring-sowing species such as maize or vegetables (FAOSTAT 2016).

MATERIALS AND METHODS

Microrganisms and culture condition

The PGPR strains used in this study are strain (RV1) isolated from root-nodules of *V. faba* plant and identified as *Rhizobium leguminosarum* bv *viciae* by 16SrRNA analysis.Pure cultures of *R. leguminosarum* were routinely maintained on yeast extract mannitol agar (YEMA) medium and pure culture of *A.brasilense*, and *P. polymyxa*, which were obtained from Agriculture Research Center (ARC)**,** maintained on nutrient agar (NA) medium

Green house Experiment

Plant growth promotion was evaluated under axenic conditions using broad bean (*V. faba*) as a test plant. The experiment was performed at Side Agriculture Research Station under completely controlled conditions in a greenhouse. For seed inoculation, inoculum was prepared without agar. For this purpose, 250-ml Erlenmeyer flasks containing 60 ml broth were inoculated with the selected strains and placed on a shaking (100 rev min^{-1}) incubator at 28 °C for 72 h. Surface-sterilized seeds were inoculated with each respective bacterial strain (dipping in broth inoculated with bacterial strains for 5 min), and then, five seeds were sown in plastic pots containing 4 kilograms sterilized sand soil. The details of treatments used for growth chamber are T1: Non-inoculated with no salt, T2: Inoculation with RV1 with no salt, T3: Inoculation with RV1 +*A. brasilense*with no salt, T4: Inoculation with RV1 + *P. polymyxa* with no salt, T5: Non-inoculated with 50 mM NaCl, T6: Inoculation with RV1 with 50 mM NaCl, T7:

Inoculation with RV1+ *A. brasilense* with 50 mM NaCl. T8: Inoculation with RV1 + *P. polymyxa* with 50 mM NaCl, T9: Non-inoculated with 100 mM NaCl, T10: Inoculation with RV1 with 100 mM NaCl, T11: Inoculation with RV1 + *A. brasilense* with 100 mM NaCl, T12: Inoculation with RV1 + *P. polymyxa* with 100 mM NaCl. After germination, the plants were thinned to three plants per pot. Pots were supplemented with about 1.5 mM KNO_3 which equivalent to half of the field dose (7.5 Kg KNO_3 for feddan of broad bean). KNO_3 was omitted from inoculated plants after two weeks. Plants were fed with nutrient solution. The composition of the nutrient solution is as the following (g/l): K_2SO_4 17.4, KH_2PO_4 6.8, K_2HPO_4 4.4, $MgSO_4.7H_2O$ 12.3, $CaSO_4$ 0.12, H_3BO_3 1, $ZnSO_4.7H_2O$ 0.1, $CuSO_4.5H_2O$ 0.05, $MnCl_2.4H_2O$ 0.05, $Na_2MO_4.2H_2O$ 0.01, $FeCl_3$ 0.1, ferric citrate 0.025 and pH at 6.8. Desired levels of salinity in soil were obtained as the following, control without salt, 50 mM and 100 mM NaCl, the required amount of NaCl was dissolved in distilled water and added to each pot to soil saturation percentage before sowing.

Growth and yield parameters

Plants were harvested after 6 weeks of growth, data on plant growth variables such as shoot FW, root FW, shoot DW, and root DW per plant were determined. The plant material for dry weight was dried at 80 ˚C for 48 h.

Nitrogen fixation (acetylene reduction) assay

Nitrogenase activity was determined by acetylene reduction for nodules on entire root system. Nodulated root portions of each plant were placed in separate vials. Ten percent of internal atmosphere was replaced with acetylene and vials were incubated at 28 °C for one hour, then 1 ml aliquots were taken and analyzed for ethylene production in a Hewlett Packard model 6890 gas chromatograph equipped with a HP-plot Al_2O_3cappillary column (Ligero *et al*., 1986).

Chlorophyll estimation

Chlorophyll estimation was performed according to a modified method of Wellburn (1994). Leaf tissues (1 g) were soaked in 10 ml 80 % acetone and incubated for 24 h in the dark. After incubation, the absorbance of the green solution was read at 662 and 645 nm. Total chlorophyll content was calculated using the following formula (Lichtenthaler and Wellburn1985). The amount of pigment was expressed as µg/g f.w. Chlorophyll a (Chl a) = [(ABS at 662 x 11.75)– (ABS at 645 x 2.35)]. Chlorophyll b (Chl b) = [(ABS at 645 x 18.61)– (ABS at 662 x 3.96)]. The amount of pigment was expressed as mg/g f.w.

Determination of nitrogen and phosphorus content

For the determination of nitrogen and phosphorus content, oven-dried shoots and roots were separately powdered and then roots and shoots of each plant were combined, and three replicate measurements were done for each treatment. The powder was digested with 98 % H_2SO_4 and 30 % H_2O_2. The total nitrogen content in plant tissues was determined following the Kjeldahl procedure. The phosphorus content of plant samples was determined spectrophotometrically using molybdenum blue method, developed by Murphy and Riley (1962).

Extraction of enzymes and estimation of enzymatic activities

A known weight (0.25 g) from fresh plant sample (green leaves) which was frozen, was extracted with 2.5 ml of 67 mM cold phosphate buffer (pH 7.0) as described by Shann and Blum (1987). The homogenates were centrifuged at 10.000 g for 15 min at 4 °C. The clear supernatant was used as a raw extract material for enzymatic assay.

Catalase and peroxidase assay

Catalase and peroxidase activity was assayed following the method of Kar and Mishra (1976)**.** Five ml of the assay mixture contained 30 µM of phosphate buffer (pH 6.8), 100 µM of hydrogen peroxide and 1 ml of crude enzyme extract. After incubation at 28 °C for 15 min, the reaction was stopped with the addition of 10 ml of 2% sulphuric acid and the residual hydrogen peroxide was titrated against 0.01 N $KMnO_4$ until a faint purple colour persisted for at least 15 seconds. A control was run at the same time in which the enzyme activity was stopped at zero time. Catalase activity was expressed as µM of hydrogen peroxide destroyed per gram leaf fresh weight per hour. The absorbance was measure at 430 nm, and the peroxidase activity was expressed as change in optical density per gram fresh weight per hour

Analytical method for estimation of free proline

The free proline content was estimated using the acid ninhydrin method as described by Bates *et al*., (1973). Five grams of plant leaves were grounded in a mortar and pestle with 3% (w/v) sulfosalicylic acid aqueous solution and the

homogenate was filtered through Whatman No. 1 filter paper, then 2 ml of filtered extract was taken for the analysis to which 2 ml acid ninhydrin and 2 ml glacial acetic acid were added. The reaction mixture was incubated in a boiling water bath for 1 h and the reaction was finished in an ice bath. Four ml of toluene was added to the reaction mixture and the organic phase was extracted, in read at 520 nm using toluene as blank by UV-visible spectrophotometer. Proline concentration was determined using calibration curve and expressed as µg g/FW.

Leaf relative water content (LRWC)

LRWC is a useful measure of the physiological water status of plants (Gonzalez and Gonzalez-Vilar 2001). Two leaves were collected from the young fully expanded leaves of two plants per replicate. Individual leaves were first detached from the stem and then weighed to determine the fresh weight (FW). To determine the turgid weight (TW), leaves were floated in distilled water inside a closed Petri dish. Leaf samples were weighed periodically after gently wiping the water from the leaf surface with tissue paper until a steady state was achieved. At the end of the imbibition period, leaf samples were placed in a preheated oven at 80 °C for 48 h to determine the dry weight (DW). Values of FW, TW, and DW were used to calculate LRWC using the equation. LRWC % = (FW-DW/TW-DW) x 100 (Kaya *et al*., 2003).

Extraction and estimation of proteins

Tissue samples (1 g fresh wt) were placed into liquid nitrogen and then homogenized with a pre-chilled mortar and pestle under ice cold-conditions in 4 ml 50 mM potassium phosphate buffer, pH 7.0, with the addition of 1 mM EDTA. The homogenate was centrifuged at 1200 rpm, at 4 °C for 30 min. The supernatant was used for soluble protein assay and protein electrophoresis. Protein content was determined according to the method described by (Bradford 1976) using Coomassie dye reagent (100 mg of Coomassie brilliant blue G250was dissolved in 250 ml of 95% ethanol and then mixed with 100 ml of 85% phosphoric acid). The mixture was diluted to 1000 ml using distilled water, left overnight at 4 °C in dark bottle and then filtered through Whatman No. 1 filter paper.

Estimation of soluble protein

A 0.1 ml of soluble protein extract was added to 5 ml of Coomassie dye reagent, mixed well and left to stand for 5 min. Optical density of the produced colour was read using spectrophotometer at 595 nm wave length against water reagent blank. Soluble protein content was calculated as mg protein per gram fresh weight.

SDS-PAGE protein electrophoresis

The method that described by (Laemmli 1970) was used for electrophoretic analysis of protein using SDS-PAGE 10%, with a slight modification.

Statistical analysis.

The collected data was subjected to analysis of variance (ANOVA) and treatment means were compared by Duncan's New Multiple Range Test (DMRT) at $P < 0.05$ using a computer-based statistical software package (IBM SPSS Statistics 21).

RESULTS

Strains used in this study were tested. RV1 (*Rhizobium leguminosarum* bv *viciae*) and another two bacterial species (*Azospirillum brasilense* and *Paenibacillus polymyxa*) were found to be positively influence plant growth. These strains were also screened for their salinity stress tolerance. The PGPR strains were able to produce IAA, siderophore, and EPS exhibited phosphate solubilization activities, and inhibition of pathogenic fungi (Table 1).

Effect of PGPR on growth parameters of *V. faba* under salt treatment

Results of the greenhouse experiments showed that *V. faba* growth was significantly (p<0.05) reduced with increasing salinity levels. As observed for shoot length, root length and shoot and root dry weights, were decreased in non-inoculated plants when subjected to 50 and 100 mM NaCl salinity. The present study demonstrates that PGPR inoculations alleviated the deleterious effects of salt stress on growth of *V. faba* plants. *A. brasilense,* or *P. polymyxa* combined with R. *leguminosarum* significantly improved root growth of *V. faba* plants as compared to those inoculated with *R. leguminosarum* alone, under normal as well as salt-stressed conditions (Fig. 1). At 50 and 100 mM NaCl, the shoot length was increased 92 and 19 %, respectively. Inoculation of plants with *A. brasilense* and *P. polymyxa* increased root length, shoot and root dry weight at saline conditions as compared to unstressed control. The benefit of PGPR inoculation was most evident for plants grown at 50 mM NaCl, since inoculation increased the total root lengths by 63% for plants inoculated with *P. polymyxa* plus *R. leguminosarum*.

Table- 1. Morphological, biochemical and physiological characteristics of PGPR isolates

Characters	*A. brasilense*	*P. polymyxa*	*R. leguminosarum* bv.*viciae*
Shape	Rod	Rod	Rod
Gram stain	-ve	+ve	-ve
Nitrogenase	+	+	+
Maximum growth at NaCl	5%	6%	6%
Catalase	+	+	+
Gelatinase	+	+	+
IAA(µg/ml)	160	90	134
Siderophore production	+	+	+
Phosphate solubilization	+	+	+
EPS production	+	+	+
Growth inhibition of:			
F. oxysporum	+	+	+
R. solani	+	+	+

Figure. (1): Effect of PGPR on root growth of *V. faba* plants a (normal condition), b (at 100 mM NaCl). A: plants non-inoculated, B: plant inoculated with *R. leguminosarum*, C: plant inoculated with *R. leguminosarum* (RV1) + *A. brasilense,* D: plant inoculated with *R. leguminosarum* (RV1) + *P. polymyxa*.

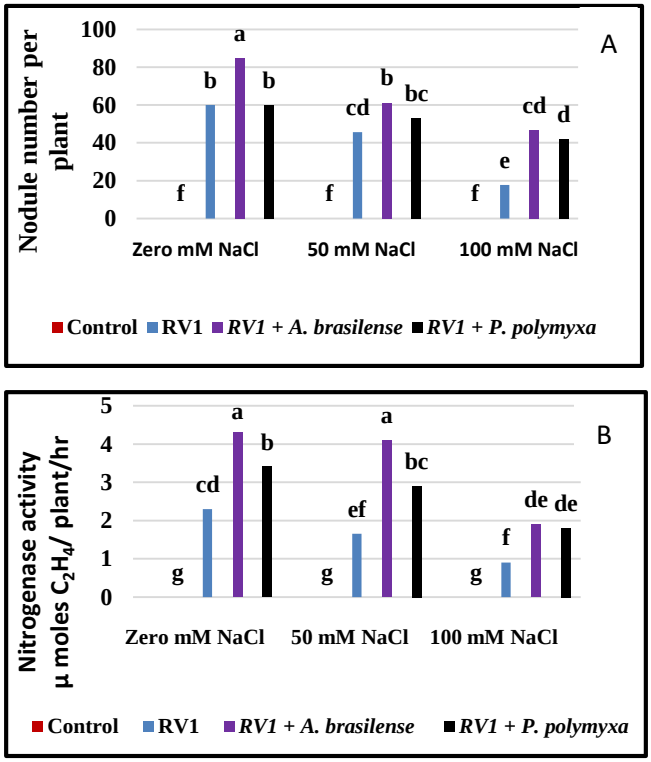

Figure. (2): Effect of different treatments on A (nodule number), B (nitrogenase activity) and C (nodule dry weight), means sharing the same letter (s) are statically non-significant (p < 5) according to Duncan's multiple Rang Test. Means sharing the same letter (s) are statically non-significant according to Duncan's multiple Rang test.

since inoculation increased the total root lengths by 63 % for plants inoculated with *P. polymyxa*plus *R. leguminosarum*. The results showed better performance of *P. polymyxa* for enhancing root growth at the higher level of salinity (Table 2). As expected and based on the results of shoot and root lengths, the effects of inoculation with PGPR strains were also manifested by improved shoot and root biomass. Plants inoculated with *P. polymyxa* plus *R. leguminosarum* had the highest shoot and root dry weights as compared to those inoculated with *A. brasilense* and *R. leguminosarum* at all salinity levels. Inoculation with *P. polymyxa* resulted in 55, 33 and 121 %an increase in shoot fresh weight at control, 50 and 100 mM NaCl salinity levels, respectively. The increase in root dry weight due to *P. polymyxa* inoculation over the non-inoculated control was 36 and 126 % at control (non- saline) and50 mM NaCl salinity. Inoculation with *P. polymyxa* resulted in 18, 63 and 150 % increase in root length when grown at control, 50 and 100 mM NaCl salinity levels, respectively (Table 2).

Effect of PGPR on nodule number, and nitrogenase activity of *V.faba* plants under salt stress

Nodule number, and nitrogenase activity increased after inoculation of plants by PGPR. The best results of nodule number, and nitrogenase activity, for plants grown under salinity stress, were obtained as plant inoculated with *A. brasilense*plus RV1 strain of R. *leguminosarum* (Fig. 2). Inoculation with *A. brasilense* increased nodule number by 41% compared to that inoculated with *R. leguminosarum*(RV1) alone, grown at control. Under salt stress (50 mM NaCl) co-inoculation with *A. brasilense* and *R. leguminosarum*(RV1) or with *P. polymyxa* plus RV1 resulted in increased nodule number, and nitrogenase activity, as compared to inoculation with *R. leguminosarum* (RV1) alone. At 100 mM NaCL nodule number decreased, but inoculation with PGPR strains led to significantly increased (p<0.05) nodule number, and nitrogenase activity, as compared with inoculation with RV1 alone.

Table-2. Effect of inoculation / co-inoculation of *R. leguminosarum* (RV1) and *A. brasilense* or *P. polymyxa* on growth parameters.

Treatments	Root length (cm)	Root fresh weight(g)	Root dry weight(g)	Shoot length (cm)	Shoot fresh weight(g)	Shoot dry weight(g)
Control 0mM NaCl						
T1: Non-inoculated	18.3ab	6.0ab	0.66b-e	24.0cd	15.0cd	2.1ef
T2 Inoculation (RV1	20.0a	6.7a	0.82bc	26.3bc	16.9bc	2.8d
T3Inoculation (RV1 +*A. brasilense)*	20.7a	7.0a	0.80bc	27.6b	19.0b	3.2cd
T4Inoculation (RV1 + *P. polymyxa*	21.7a	7.3a	0.90b	30.6a	23.3a	4.1ab
50mM NaCl						
T5Non-inoculated	13.3c	3.8b-d	0.38ef	14.6f	12.3d	1.8f
T6Inoculation (RV1)	20.7a	6.0a	0.47d-f	24.3bc	13.2d	2.7de
T7Inoculation (RV1+ *A. brasilense)*	20.3a	6.2a	0.73b-d	25.6bc	16.4bc	3.1cd
T8Inoculation (RV1 + *P. polymyxa)*	21.7a	6.6a	0.86b	27.0bc	16.4cd	4.3a
100 mM NaCl						
T9Non-inoculated	7.7d	2.03d	0.23f	7.56e	4.60e	0.2h
T10Inoculation (RV1)	12.7c	3.00cd	0.42ef	13.0e	6.30e	1.0g
T11Inoculation (RV1 + *A. brasilense)*	15.3b	5.10ab	0.53c-e	21.6cd	9.20cd	3.0cd
T12Inoculation (RV1 + *P. polymyxa)*	19.3a	5.90ab	0.9a	22.3d	10.2d	3.6bc
LSD AT p <0.05	3.2	2.07	0.26	3.2	2.73	0.62

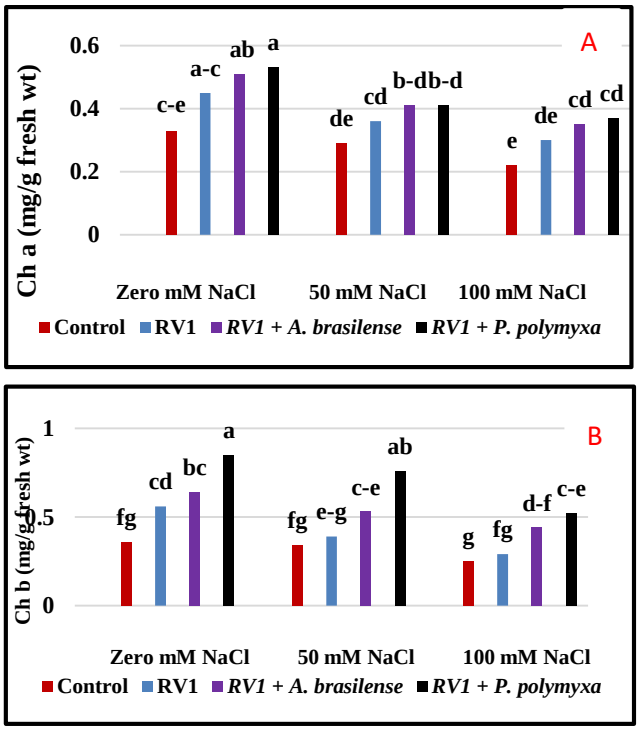

Figure. (3): Effect of different treatments on A (chl a) and B (chl b), means sharing the same letter (s) are statically non-significant (p < 5) according to Duncan's multiple Rang Test. Means sharing the same letter(s) are statically non-significant according to Duncan's multiple Rang test.

The co-inoculation of RV1 and *A. brasilense* showed the best results under non-saline and saline conditions, for the nodule number, and nitrogenase activity (Fig 2).

Effect of PGPR on *V. faba* leaf chlorophyll content under salt stress.

Both salinity stress levels showed negative effect on plant chlorophyll a which was decreased at the salinity levels (50 and 100 mM NaCl), when compared to the control (0mM NaCl). At non-saline conditions, the treatment of *P. polymyxa* plus *R. leguminosarum* (RV1) significantly (p<0.05) increased plant chlorophyll a by 60% compared to the control values (Fig. 3).

At 100 mM NaCl, cholorophyll a significantly increased when plants inoculated with *P. polymyxa plus RV1 compared to that inoculated with RV1 alone. Inoculation of V. faba plants with A. brasilense plus R. leguminosarum (RV1) or P. polymaxa plus RV1 caused significant increase in chlorophyll b,* as compared to the control plants or plant inoculated with RV1 alone at salt stress (50 and 100 mM NaCl) (Fig 3b).

Effect of PGPR on *V. faba*plants nitrogen (N) and phosphorus (P) contents under salt stress.

The nitrogen content in *V. faba* plants increased in response to bacterial treatments. Under non-stressed conditions, *R. leguminosarum* (RV1) inoculation increased N content in plants by 35 %, and the co-inoculation (*A. brasilense* plus *R. leguminosarum*) by 81 % (Fig. 4a). When *V. faba* plants were inoculated either with *R. leguminosarum* alone or combined with *A. brasilense* or *P. polymyxa*, the nitrogen content increased in salt-treated plants by 45, 129 and 95 % in those grown under 50 mM NaCl, respectively, compared to non- salt-treated plants. Inoculation of *V. faba* plants with *R. leguminosarum* plus *A. brasilense*was the best treatment that give the best value of nitrogen content in two the salt treatments as compared to other bacterial treatments. The phosphorus content in *V. faba* plants decreased in response to salt treatment. The P content was significantly increased when non-salt-treated *V. faba* plants were inoculated either with *R. leguminosarum* alone or combined with *P. polymyxa* or *A. brasilense* (Fig. 4b).

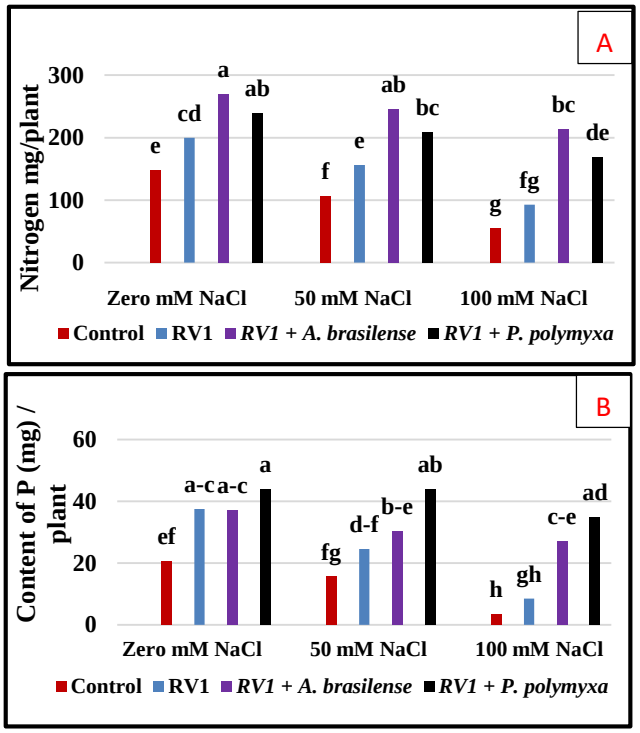

Figure. (4): Effect of different treatments on A (nitrogen content) and B (phosphorus content), means sharing the same letter (s) are statically non-significant (p < 5) according to Duncaṅs multiple Rang Test.Means sharing the same letter (s) are statically

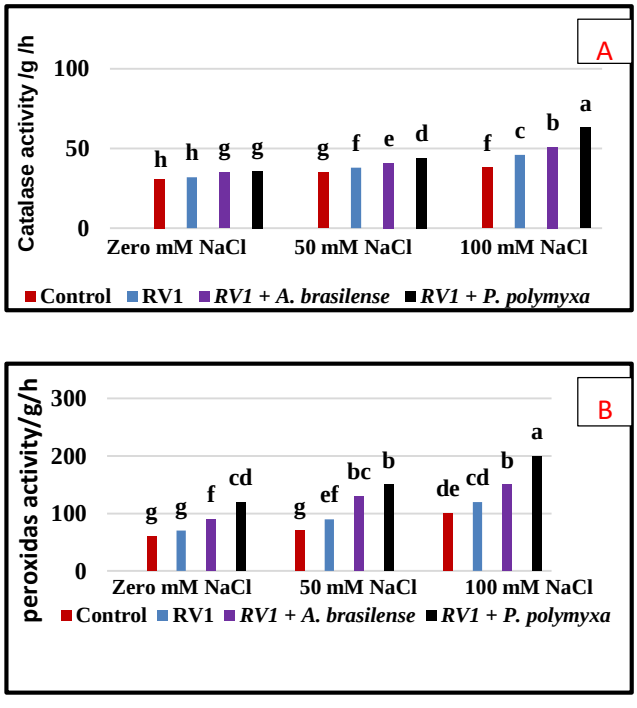

Figure. (5): Effect of different treatments on A (catalase activity) and B (peroxidase activity). Means sharing the same letter (s) are statically non-significant (p < 5) according to Duncaṅs multiple Rang Test.

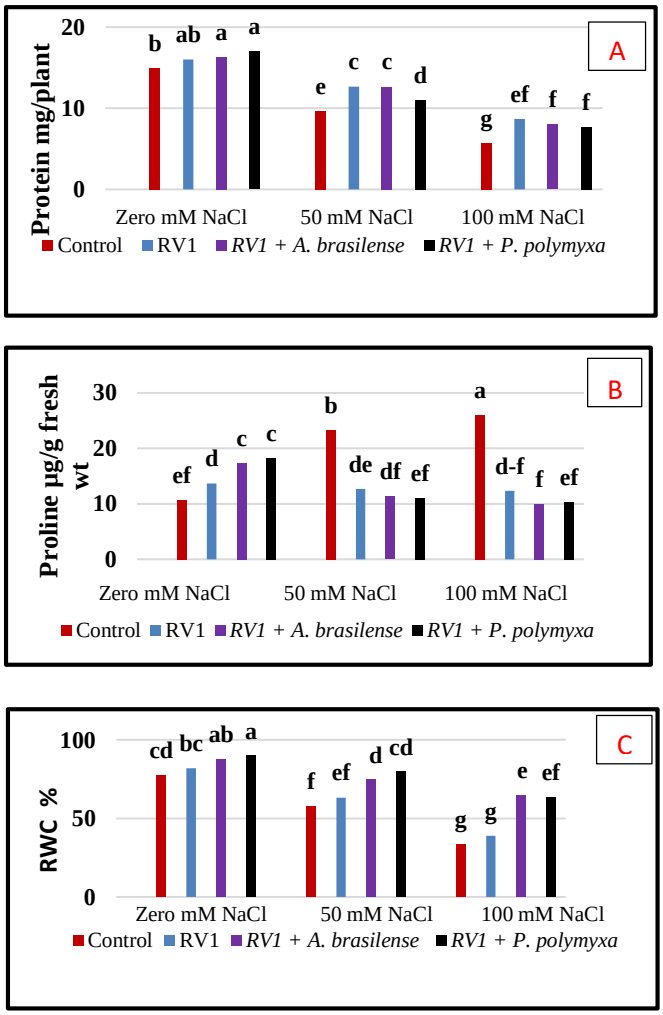

Fig. (6): Effect of different treatments on A (protein), B (Proline) and C (RWC %). Means sharing the same letter (s) are statically non-significant (p < 5) according to Duncan's multiple Rang Test

V. faba plants contained 67 % more P in plants treated with PGPR than plants which were inoculated with *Rhizobium* alone and grown at 100 mM NaCl solution. The increase in plant P content was significant for *V. faba* plants grown at 50 mM NaCl solution and co-inoculated with *R. leguminosarum* and *P. polymyxa* by 63 %, compared with plants inoculated with *Rhizobium* alone.

Effect of PGPR on antioxidant enzyme activities of *V. faba* under salt stress

Salinity increased the specific total CAT and POX activities in the non-inoculated and inoculated plants (Fig. 5). The inoculation with *P. polymaxa* plus *R. leguminosarum* (RV1), as well as the treatment with *A. brasilense* plus *R. leguminosarum* (RV1), significantly (P≤ 0.05) increased CAT and POX activities in plant leaves as compared to those inoculated with *R. leguminosarum* alone, at all salt stress levels as shown in (Fig.5). At the high salinity level (100 mM NaCl), CAT and POX activities were increased by 65% and 100% higher than the control plant, respectively (Fig. 5).

Effect of PGPR on the contents of protein and proline, and % RWC of *V. faba* plants under salt stress.

V. faba plants contained 67 % more P in plants treated with PGPR than plants which were inoculated with *Rhizobium* alone and grown at 100 mM NaCl solution. The increase in plant P content was significant for *V. faba* plants grown at 50 mM NaCl solution and co-inoculated with *R. leguminosarum* and *P. polymyxa* by 63 %, compared with plants inoculated with *Rhizobium* alone.

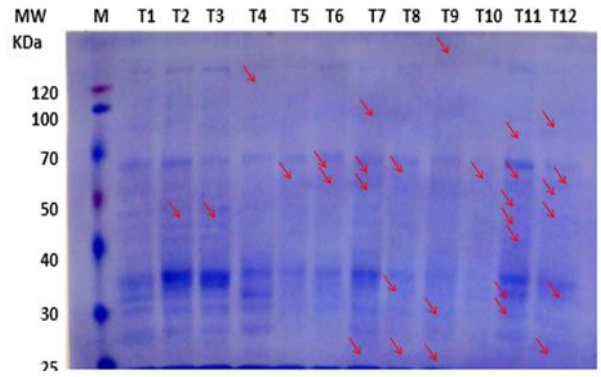

Figure. (7): Effect of PGPR on SDS-PAGE protein patterns of leaves of faba bean plants under salt stress. (M = Marker protein, T1: Non-inoculated without salt, T2: Inoculation with RV1 with no salt, T3: Inoculation with RV1 + *A. brasilense* no salt. T4: Inoculation with RV1 + *P. polymyxa* no salt, T5: Non-Inoculated with 50 mM NaCl, T6: Inoculation with RV1 with 50 mM NaCl, T7: Inoculation with RV1+ *A. brasilense* with 50 mM NaCl, T8: Inoculation with RV1 + *P. polymyxa* with 50 mM NaCl, T9: Non-inoculated with 100 mM NaCl, T10: Inoculation with RV1 with 100 mM NaCl, T11: Inoculation with RV1 + *P. polymyxa* with 100 mM NaCl and T12: Inoculation with RV1 + *A. brasilense* with 100 mM NaCl)

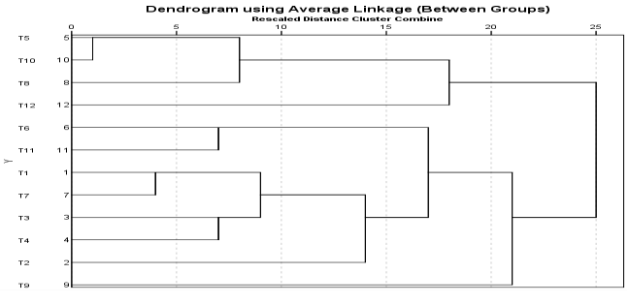

Figure. (8) Dendrogram illustrating variation in faba bean (Giza 843) due to different salt concentration stresses and inoculation with PGPR generated by UPGMA cluster analysis-based protein electrophoresis data analysis.

The interaction between salinity stress and PGPR caused a slight change in plant proline content in *V. faba* plants. The highest values for proline content were found in inoculated plants at 100 mM NaCl (26 µg/g fresh wt), but the lowest values of proline were observed at plant treated with RV1 plus *P. polymyxa* (10.3 µg/g fresh wt) (Fig. 6b). The leaf relative water content showed different pattern compared to protein and proline contents (Fig. 6c). Leaf relative content showed different pattern compared to protein and proline contents. Leaf relative water content was significantly (at P< 0.05) decreased as salt increased in the case of inoculated plant with PGPR. Inoculated plants with PGPR significantly increased LRWC. Dual inoculation with RV1 and *P. polymaxa* gave the best value of LRWC in all salt values. (Fig. 6c). Effect of PGPR on SDS-PAGE protein patterns of leaves of faba bean plants under salt stress. The growth in the presence of NaCl altered the whole-cell protein patterns in SDS-PAGE of faba bean cultivar (Giza-843), some bands disappeared, and new bands appeared after treatment by salt, as shown in Fig. (8). The synthesis of polypeptide bands with molecular masses of 46.06 KDa in faba bean grown under different saline conditions 50 and 100 mM NaCl which was undetected in the control faba bean grown under non-saline conditions. The appearance of polypeptide bands with molecular masses of 60.23 and 37.69 kDa were recorded in faba bean plants grown under saline and non-saline conditions. Bacterial inoculation caused an induction or inhibition in the synthesis of some polypeptides in faba bean cultivar (Giza-834) such as polypeptide bands with molecular masses of 112.53 KDa (due to inoculation with RV1), 63.33 KDa (due tothe interaction of inoculation with RV1

and *P. polymyxa*) and 67.57 and 63.33 KDa (due to the interaction of inoculation with RV1 and *A. brasilense*) in SDS-PAGE protein extracted from faba bean inoculated with bacteria which was undetected in the control plants, whereas the appearance of polypeptide bands with molecular masses of 60.23 and 37.69 kDa were recorded in the inoculated and un-inoculated control faba bean plants. The response of expressed proteins to the interaction between salt stress (NaCl) and the bacterial inoculation resulted in a remarkable change in the protein pattern and characterized by the synthesis of 98.26, 67.57, 50.77 and 25.24 KDa (in T7) and 63.33 KDa (in T10). The formation of the polypeptide bands with molecular masses of 112.53 KDa (in T7 and T10) were inhibited in faba bean inoculated plants with RV1 and grown under saline conditions when it was compared with the inoculated plants and grown under non-saline conditions. Concerning to inoculation with RV1 and *P. polymyxa,* the response of expressed proteins to the interaction between salt stress (NaCl) and the bacterial inoculation was varied and characterized by the synthesis of 67.57 and 31.40 KDa (in T8 and T11) and 25.24 KDa (in T8) and 55.62, 46.06, 43.30 and 27.90 KDa (in T11). The formation of the polypeptide bands with molecular masses of 72.53 KDa (in T11) were inhibited in faba bean inoculated plants with RV1 and *P. polymyxa* and grown under saline conditions when it was compared with the inoculated plants and grown under non-saline conditions. Regards to inoculation with RV1 and *A. Brasilense* the response of expressed proteins to the interaction between salt stress (NaCl) and the bacterial inoculation was varied and characterized by the synthesis of polypeptide bands with molecular masses of 25.24 KDa (in T9 and T12), 27.90 KDa (in T9) and 83.55 KDa (in T12). The formation of the polypeptide bands with molecular masses of 67.57 KDa (in T9 and T12), 121.85 and 55.62 KDa (in T9) and 43.30 and 34.00 KDa (in T12) were inhibited in faba bean inoculated plants with RV1 and *A. brasilense* and grown under saline conditions if compared with the inoculated plants and grown under non-saline conditions. PGPR-inoculated *V. faba* plants showed higher root and shoot biomass than non-inoculated plants under salinity stress (Figs. 1-2). Plant-growth-promoting-rhizobacteria have been documented to facilitate the growth of a variety of plants under high salinity conditions (Fasciglione *et al*., 2015, Egamberdieva *et al*., 2017, Khalid *et al.*, 2017).

DISCUSSION

Effect of PGPR on growth and symbiotic N_2 fixation of *V. faba*

The bacteria *A. brasilense* and *P. polymyxa* had the most positive effect on shoot and root weight, and plant height, in all salinity levels (50 and 100 mM NaCl) (Table 1). In our study co-inoculation *V. faba* plants with *R. leguminosarum* (RV1) plus *A. brasilense* or RV1plus*P. polymyxa* increased nodule number under salt stress, compared to the control (without salt). Inoculation of plants with PGPR improved yield of legumes, and nodulation compared to inoculation with *Rhizobium* alone (Yadegari and Rahmani 2010). Inoculation of chickpea with *Pantoeadispersa* significantly improved plant biomass, pod number, pod weight, seed number, seed weight and relative leaf water content, chlorophyll content, and K uptake in salt- (150 mM NaCl) affected plants (Panwar *et al*. 2016). Figueiredo *et al*., (2008) observed that common bean plants co-inoculated with both *Rhizobium tropici* and *P. polymyxa* showed improved plant growth, shoot dry matter, nodule dry matter, and N uptake as well as higher nodule numbers than those inoculated with *R. tropici*alone. Metwali *et al*., (2015) found that faba bean plants, treated with *P. fluorescens*, showed significant increase in growth traits such as plant length, plant shoot fresh weight and plant leaf area. El-Nahrawy and Omara (2017) reported that co-inoculation of common bean plant with *Pseudomonas* and *Rhizobium* exhibited the highest values of growth, symbiosis, photosynthetic and nitrogen content compared to single inoculation or control due to the amounts of ACC deaminase, synthesized by *Pseudomonas* and *R. leguminosarum*. Dual-inoculation of faba bean with *Azospirillum* and *Rhizobium* increased nodule number, and nitrogenase activity (Fig. 2). Our results agreed with previous studies that have demonstrated that PGPR co-inoculation can enhance early nodule initiation and development on soybean (Omara *et al*., 2017), mung bean (Noreen *et al*., 2012), alfalfa (Younesi *et al*., 2013), pea (Sanchez *et al*., 2014, Talaat *et al*., 2015), common bean (El-Nahrawy and Omara 2017), and Lucerne (Le *et al*., 2016). Their reports have also shown positive results of inoculation of PGPR on nodulation, growth and productivity of legumes. Growth and nodulation improvement could be due to production of phytohormones such as auxin, gibberellins and cytokinins that has been reported as a mechanism used to enhance nodule formation

(Sanchez *et al*., 2014).The positive effects of PGPR treatments on the yield and growth of plants can be attributed to the production of phytohormones such as indole-3-acetic acid and cytokinins, N_2-fixation ability, phosphate solubilizing capacity, and antimicrobial substance production, organic acid, amino acid, enzyme, and hormone production of PGPR **(**Karlidag *et al*., 2013, Gunes *et al*., 2015, Hingole *et al*., 2016 Turan *et al*., 2017), siderophores, HCN (hydrogen cyanide), ammonia, exopolysaccharides, heavy metal mobilization, nitrogenase activity, ACC (1-aminocyclopropane-1-carboxylate) deaminase, biocontrol potentials, antifungal activity, N_2 fixation, induced systemic resistance, Zn solubilization, Zn resistance, Pb and Cd resistance, antibiotic resistance, gibberellin, kinetin, metal resistance, and cytokinin (Bhattacharyya and Jha 2012, Hingole *et al*., 2016, Ilangumatan and smith 2017). These findings explain the improvement in growth and symbiotic activity of *V. faba* plants after co-inoculation by *R. leguminosarum*and *A. brasilense* in this study.

Effect of PGPR on cholorophyll content

Total chlorophyll identified the plants with the greenest leaves, a feature strongly correlated with photosynthetic efficiency (Kirschbaum2011). Chl a and Chl b contents in leaves of *V. faba* plants were significantly decreased with increasing NaCl concentrations, while co-inoculation with PGPR increased Chl a, Chl b, in leaves of *V. faba* plants under salt stress (Fig. 3). These results are in accordance with Mohamed and Gomaa (2012), Khalid *et al*., 2017). *Rhizobium, Azospirillium* and *Bacillus* caused chlorophyll accumulation (Fig. 3). These results may be due to the effect of *Rhizobium, Azospirillium* and *Bacillus* as a biofertilizer, or due to the increase in the ACC-deaminase activity in PGPR-treated plants, which slowed Chl degradation or probably due to the increase in the photosynthetic rate or the role of N nutrition in producing growth-promoting substances resulting in more efficient absorption of nutrients, which are main components of photosynthetic pigments and consequently the Chl content was increased (Mayak *et al*., 2004, Kumar *et al*., 2015, El-Nahrawy and Omara 2017). Inoculated plants have higher chlorophyll content compared with control plants (Hassine and lutts 2010, Abbas *et al*., 2013). In salt-treated and non-inoculated plants, chlorophyll is destroyed due to excessive amount of salts, ions (Na^+ and Cl^-), or reactive oxygen species (ROS), which disturb the cellular metabolism and result in the degeneration of cell organelles in the leaf tissue (Hassine and lutts 2010, Abbas *et al*., 2013). El-Nahrawy and Omara (2017) found that inoculation of common bean with *Rhizobium* and *Pseudomonas* spp. increased chlorophyll content as compared to those inoculated with *Rhizobium* alone. *Khalid et al*., *(2017)* reported that inoculation of *A. brasilence* considerably improved the leaf chlorophyll content in white clover plant, cultivated under both non-saline as well as under variable salt concentrations (40, 80, and 120 mM NaCl).

Effect of PGPR on mineral up-take

Bacterial inoculation increased uptake of elements such as nitrogen and phosphorous. Inoculation of *V. faba* with PGPR had significant effects on nutrient uptake under salinity levels (Fig. 4). Plant inoculation with PGPR facilitated elements uptake, this may be related to the ability of PGPR to produce sidrophores. Sidrophore production has been demonstrated by PGPR such as *P suedomonas*, *Azospirillum* sp. and *Azotobacter* (Yildirim *et al*., 2011, Karlidag *et al*., 2013). Increased N contents have been observed in *V. faba* plants grown under salt stress conditions (Fig.4a). When plant growth inhibited by salt stress, the consumption of nitrogen decreases, therefore, nitrogen starts to accumulate inside plant tissues (Hiz *et al*., 2014). The combined inoculation of *R. leguminosarum* plus *A. brasilense* plus *R. leguminosarum* plus *P. polymyxa* resulted in long roots and good nodulation; high N, and P content in *V. faba* in both non-saline and saline conditions (Fig. 4). An explanation for the improved nutrient uptake could be that *A. brasilense* and *P. polymyxa*, plus *R. leguminos*arum, facilitated the absorption of more nutrients through increased root system, and formation of nitrogen-fixing nodules. Our results agreed with the results of Qureshi *et al*. (2011), who observed that nutrient concentration in grain and different parts of mung bean plants increased by co-inoculation of *P. polymyxa* and *R. leguminosarum*in a pot experiment. PGPR inoculations have been reported to reduce the Na content or increase K and Ca contents compared with the non-inoculated plants (Karlidage *et al*., 2013). Nutrient analysis in plants showed that N, P and K concentration was more pronounced in inoculated and coinoculated plants (El-Nahrawy and Omara 2017).

Effect of PGPR on antioxidant enzymes

Salinity stress leads to the formation of ROS which cause severe damage to cell structures by exerting oxidation of cell membranes in a process

known as oxidative stress (Hussain *et al*., 2013). However, a defensive system called the antioxidant enzyme system is also activated under stress conditions. This system consists of several ROS-scavenging enzymes such as superoxide dismutase (SOD), peroxidase (POD), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), ascorbate peroxidase (APX), and catalase (CAT). These antioxidant enzymes can remove the free radicals produced during abiotic stress conditions in the cell (Abbas *et al*., 2013, Baniaghil *et al*., 2013). The broad bean plants inoculated with PGPR (*Rhizobium*, *Azospirillium* and *Paenibacillus*) exhibited significant elevation of antioxidant enzyme activities (APX, CAT) under saline conditions (Fig. 5). The level of enzymes significantly increased with inoculation by bacteria. Inoculation with *Azospirillum* increased leaf catalase enzyme of *V. faba* plants (Fig. 5). The results of this study showed that increase of salinity caused a significant increase in catalase and peroxidase enzymes activity in leaf in comparison to control. These results agreed with the results of Dai *et al*., (2009), who showed that the level of superoxid dismutase, catalase and peroxidase were increased in *Brassica napus* L. under salt stress. Also, Baniaghil *et al*., (2013), and Habib *et al*., (2016) found thatcatalase, superoxide dismutase, peroxidase activity increased as salt increased in okra plants.

Effect of PGPR on RWC

Bacterial inoculations significantly ($P<0.05$) increased RWC, which is a useful measure of the physiological water status of plants. Inoculation of plants with *P. polymyxa*plus *R. leguminosarum* increased RWC values than other combinations (Fig. 6c). Increased RWC by PGPR have been reported for lettuce (Yildirim *et al*., 2011), and strawberry (Karlidag *et al*., 2013) grown under salt stress. Mayak *et al*., (2004) reported that PGPR could facilitate the rooting and growth of plants grown under salt stress by improving the water use efficiency. Relative water content was improved in inoculated and co-inoculated plants that might be due to longer roots which ultimately resulted in increased water uptake from the deeper soil (Aamir *et al*., 2013). Ahmad *et al*., (2013) noted increased RWC of wheat and mung bean, due to bacterial inoculation under saline conditions. Bacterial inoculation may reduce the inhibitory effect of salt stress on roots and aid in the development of more effective root systems, which could help plants to absorb relatively more water from deeper soil under stress conditions (Mahmood *et al*., 2016)

Improvement of protein and proline content by PGPR treatment

Inoculation of *V. faba* plants with PGPR (*R. leguminosarum* and *A. brasilense* and *P. polymyxa*) significantly increased protein content of plants that might be attributed to more availability of N which is an important constituent of protein (Fig. 6 a) This agrees with Metwali *et al*., (2015) and Aslam *et al*., (2010) who found that grain protein content improved in chickpea by *Rhizobium* inoculum. PGPR could help the growth of plants by mitigating deleterious effects of salinity conditions by promoting the accumulation of proline and glutamate (Karlidag *et al*., 2013). In the current study, salinity stress caused a significant decrease in soluble proteins, while proline was increased in salt-treated plants compared to non-salinized control plants (Fig. 6b). Proline was reduced in PGPR-salt treated plants (Fig. 6b). The reduction of protein was previously recorded by Sadak *et al*., (2010), and Metwali*et al*. (2015). They concluded that, the reduction of protein under salinity stress was suppressed by the accumulation of total amino N and proline. The above conclusions agreed with our results. The increased proline content in faba bean suggests an excellent mechanism to mitigate the effect of osmotic potential in this plant. This supports the presumption that proline accumulation is a part of physiological response of plants to intense stress (Rabie and Almadini 2005, Metwalil *et al*., 2015).Several PGPR strains, such as *Burkholderia* (Barka *et al*., 2006), *Arthrobacter,* and *Bacillus* (Sziderics *et al*., 2007), enhanced proline synthesis in stressed plants, which helps in maintaining the cell water status; thereby helping the plant to cope with the salinity stress.

The different concentrations of salt stress and PGPR inoculation on broad bean plants casused the synthesis and increased of the intensity of the original protein bands, which caused the appearance and disappearance of bands. Inoculation of broad bean plants with RV1 (*R. leguminosarum*) and *P. polymyxa* caused appearance of new band of 112.53 KDa. Salt stress caused the disappearance of 55.62 KDa band. (Fig. 7). In response to environmental stress, increased gene expression has been noticed to provoke the synthesis of proteins, collectively called stress proteins, having putative role in stress protection (El-Mashed and Kamel 2001). It has been reported that salinity adversely affects protein metabolism due to decrease in protein synthesis, accelerated proteolysis, decreased availability of

amino acid denaturation of enzymes associated with protein synthesis (Abdelraouf *et al*., 2016).

It is concluded that the bacteria examined (*Rhizobium leguminosarum*, *Azospirillun brasilense* and *Paenibacillus polymyxa*) in this study are good plant growth-promoting rhizobacteria (PGPR) and is suggested to be a good candidate as a biofertilizer in sustainable agriculture.

CONCLUSION

In the present investigation, PGPR application reduces some deleterious effect of salt on the shoot and root growth of *V. fabavia* significantly improved the growth parameter, photosynthetic capacity, protein content and moisture content. Furthermore, PGPR improves nutrient absorption capacity of the plant under salt stress. Further researches are needed to find out more PGPR strains which have positive consequences on management of this crop at planting and germination under salt stress to replace the chemical fertilizer and pesticide.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

AM and ZHH chose the research point. RRI and OMN designed, performed the experiments carried out the practical experiment and the manuscript writing. CRR helped data analysis, write up of manuscript. ESA design and conducts research reviewing manuscript and submit manuscript. All authors read and approved the final version.

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