Effectiveness of endophytic bacteria combined with micronutrients on growth characteristics and productivity of faba bean

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ABSTRACT

Field experiment was carried out to evaluate the interaction among biostimulant strains (*Pseudomonas* sp KY648982.1+ *Klebsiella* sp KY662036.1 + *Pseudomonas* sp KY662037.1), chemical fertilization (NPK) treatments and microelements as a foliar application during growing season of 2016-2017 on faba bean yield. Results emphasized that inoculation with endophytic bacteria combined with micronutrients significantly increased DHA, P-ase and N_2-ase compared with uninoculated ones. Also, nitrogen fixation activities, growth characteristics, N, P and K available, uptake and protein of seeds and yield and its components were significantly increased in faba bean inoculated treatments with plant growth promoting endophytic bacteria (PGPE) as a biostimulant combined with half dose of nitrogen fertilizers. Also, foliar spraying of faba bean plants with micronutrients gave significant higher records of abovementioned parameters than non-sprayed plants.

INTRODUCTION

Faba bean (*Vicia faba* L.) is considered one of the most important legume crops in Egypt. It is used for human consumption as a good source of vegetarian protein. However, the cultivated area from faba bean in 2005 reached to 198,000 faddan. This area gives total production 1.82 million ardab. Egypt's self-sufficiency in faba bean production improved in 2013-2014 and is on the increase. Faba bean production has been increased from 139,000 tons in 2011-2012 to 182,000 in 2013-2014, thus improving food security. It is very important to increase the productivity of unit area of faba bean to face the increasing demand by using different agricultural practices. (El Habbasha *et al.*, 2007). Accordingly, in both managed and natural ecosystem, beneficial plant associated bacteria play a key role in supporting and/or increasing plant health and growth (Compant *et al.,* 2010). In general, the beneficial effects of endophytes are greater than those of many rhizobacteria and these might be aggravated when the plant is growing under either biotic or abiotic stress

conditions (Barka *et al.,* 2006 and Hardoim *et al.,* 2008). Microelements are essential and important elements for plant growth and development that uses at lower values than macro-elements. These elements include iron, zinc, copper, molybdenum, boron and manganese (Amirani and Kasraei, 2015).This research amied to study the effect of endophytic bacterial strains in combination with microelements as foliar application on microbial activities in soil and its impact on growth performance and productivity of faba bean plants.

MATERIALS AND METHODS

Field experiment

Field experiment was carried out during October 2016 to April 2017 in Fac. Agric. at Moshtohor, El-Qalubia Governorate, Egypt to evaluate the efficiency of the endophytic bacterial consortium in presence of foliar application with micronutrients on growth and yield of fababean plant. Experimental soil was subjected to mechanical and chemical analyses according to the method described by Page *et al.* (1982).

Table 1. Physical and chemical analyses of field experimental soil.

Parameters	Unit	Values	Parameters	Unit	Values
Particle size distribution			Chemical analysis		
Coarse sand	(%)	3.91	Organic matter	(%)	1.52
Fine sand	(%)	24.04	$CaCO_3$	(%)	0.55
Silt	(%)	25.22	Total nitrogen	(%)	0.23
Clay	(%)	44.14	Total phosphorus	(%)	0.12
Textural class	(%)	Clay loam	Total potassium	(%)	0.27
			pH		7.5

Endophytic bacteria

Consortium of the endophytic bacteria biostimulant contains three bacterial strains namely *Pseudomonas* sp KY648982.1, *Klebsiella* sp KY662036.1 and *Pseudomonas* sp KY662037.1

Bacterial inocula preparation

The inocula of the endophytic bacterial strains were prepared separately in nutrient broth medium and incubated at 30°C for 2 days to reach each cell suspension 10^7 CFU/ml. Equal dose from each cell suspension were mixed and used as biostimulant.

Rhizobium leguminosarum. bv. vicieae

This strain was obtained from biofertilization unit, Fac. Agric. Ain Shams Univ for faba bean. Cell suspension containing about 10^9 cells ml^{-1} was used as standard inoculum and added with the irrigation.

Experimental design

Treatments were distributed in a randomized complete block design with three replicates. The experimental area was 10.5 m^2 (3 m x 3.5 m). Every plot consists of five rows (each was 3.5 m length and 60 cm width) and the distance between plants was 15 cm. This experiment included the following treatments:

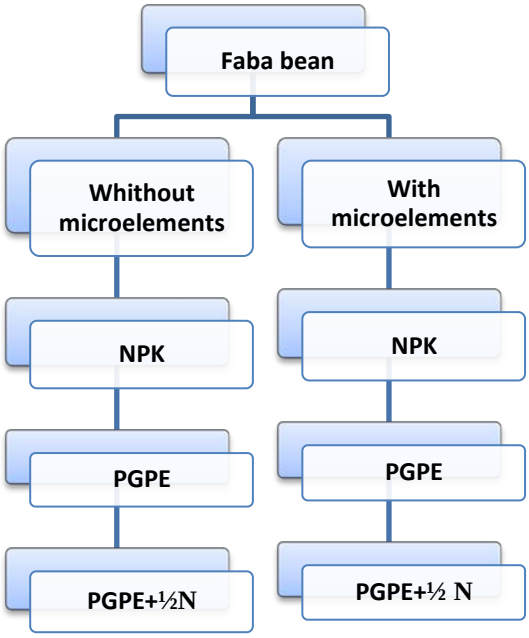


Fig1. Flow chart of experimental treatments of faba bean in field experiment

Micronutrients solution

Micronutrients solution was obtained from Sphinx for International Trade Company, Cairo, Egypt. Micronutrients solution contains 5.1% N, 6% Fe, 0.5% Zn, 0.5% Mn, 0.5% Cu, 0.0005% Mo, 4% S, $0.0.02\%$ B and 10% citric acid was prepared at a rate of (2 ml/L) and then used as foliar spray three times at a rate of 3L/fed. The first one after 30 days from sowing, the second was added after one month from the first, the third was added after one month from the second.

Cultivation process

Before sowing, seeds of faba bean were soaked for one hour in the prepared inocula biostimulants. Sucrose solution (30%) was added as an adhesive agent prior to inoculation, spread in plates and allowed to air drying before sowing. The control seeds were soaked by the same manner, but in the corresponding media without inocula. Chemical fertilizers (NPK) were used as recommended full dose 100% NPK (40 kg N_2 as ammonium sulfate, 30 kg P_2O_5 as calcium superphosphate and 50 kg K_2O as potassium sulfate). Endophytic bacteria treatments received half dose of nitrogen fertilizer (20 kg). The normal cultural practices i.e. irrigation, fertilizer and pest control for the faba bean production were followed.

Microbiological determination

Dehydrogenase activity (DHA)

Dehydrogenase activity was assayed in soil according to Glathe' and Thalmann (1970).

Phosphatase activity

The alkaline phosphatase activity was measured according to the method described by Tabatabai (1982).

Nitrogen fixation activities

Nitrogenase activity (N_2-ase)

N_2-ase activity was estimated in nodules of faba bean, the measurement based on the reduction of acetylene to ethylene as quantities by gas chromatography. Acetylene reduction was performed by a protocol modified from (Silvester, 1983).

Nodulation status

Five plants from each plot were taken at random after 50 days from sowing to evaluate nodulation i.e. number of nodules/plant, fresh dry weight of nodules g/ plant and legheamoglobin content.

Determination of legheamoglobin content

The legheamoglobin content was determined according to the method described by Wilson and Reisenauer (1963).

Plant growth characteristics

Five plants from each plot were taken at random after 50 days from sowing to evaluate vegetative characters i.e. plant height (cm), number of branches/plant, number of leaves/plant and number of flowers/plant.

Macro-elements content

Plant samples were taken after 60 days from sowing to determine total nitrogen, phosphorus and potassium contents according to the methods described by (A.O.A.C., 2005); A.P.H.A. (1992) and Dewis and Freitas (1970), respectively. While, rhizosphere soil samples were taken for available nitrogen and phosphorus contents according to the method described by Black *et al*. (1982).

Nitrogen content in seeds

Five plants from each plot were chosen at random after 120 days from sowing, mature green pods were continuously harvested at suitable maturity stages and then estimate nitrogen content in seeds according to (A.O.A.C., 2005). Total crude protein percentage was calculated by multiplying N-values by 6.25 (A.O.A.C. 2005).

Scanning Electron Microscopy (SEM)

Faba bean roots after 60 days from sowing were washed twice with sterile water, fixed with 2.5% (v/v) glutaraldehyde and 4% (v/v) paraformaldehyde in 0.1M sodium cacodylate buffer, pH 7.2-7.4, for 2 h at 28°C. Samples were subsequently mounted on aluminum stubs with double coated carbon conductive tape and coated by gold sputter coater (SPI-Module, USA).Then, observed by scanning electron microscope (Model:JSM-5500 LV; JEOL Ltd-Japan) by using high vacuum mode as described by Phukon *et al*. (2011). This examination was carried out at the Regional Center of Mycology and Biotechnology, Cairo, Egypt to examine the endophytic bacteria in the roots and comparison with control.

Yield and yield components

Five faba bean plants from each plot were taken at random after 130 days from sowing to evaluate number of seeds/pod, dry weight of pods (g), number of pods/plants, average weight of 100 seeds (g) and seed yield (kg/fed)

Statistical analysis

Statistical analysis was carried out according to Snedecor and Cochran (1989). The differences between the means value of various treatments were compared by Duncan's multiple range test (Duncan's, 1955).

RESULTS AND DISCUSSION

Changes in enzymatic activities in faba bean rhizosphere

Data in Table (2) emphasized that rhizosphere of faba bean plants treated with full dose of chemical fertilizer (NPK) only gave the lowest DHA and phosphatase. These results are in harmony with those observed by Macci *et al.* (2012) who reported that chemical fertilization could affect the population of soil microorganisms and consequently dehydrogenase activity. It is often assumed that inorganic fertilizers had relatively less effect on dehydrogenase activity than organic or biofertilizers.

In addition, rhizosphere of faba bean plants inoculated with plant growth promoting endophytic bacteria (PGPE) as a biostimulant combined with half dose of nitrogen fertilizers gave the highest significant values of DHA and phosphatase followed by plants inoculated with PGPE solely. This might be due to the role of PGPE combined with NPK in enhancing soil biological activity. These results are in agreement with Siczek and Lipiec (2016) who reported that the inoculation with microorganism induced a significant increase in a majority of DHA in the rhizosphere throughout the vegetative and flowering period of faba bean. Also, Vijayalakshmi *et al.* (2016) used phosphate solubilizing bacteria for the agricultural fields which are poor in phosphate and showed that the highest amounts of phosphate release from insoluble phosphate (tricalcium phosphate) and apply it to commercial crops gave an increase of plant growth and yield.

Generally, foliar spraying of faba bean plants with microelements gave significant higher records of dehydrogenase and phosphatase than non-sprayed plants. This trend of results was observed with all fertilization treatments. Obtained results are in agreement with those obtained by Sarkar *et al.* (2007) who proved that microelements particularly Zn, Fe and Mn applied by foliar spraying have an important role as a metal component of microbial enzymes. Summing up, determined soil enzymes activity values were gradually increased through growing season to reach maximum at 60 days then decreased thereafter. These results are in logic and agreement with Lipiec and Nski (2011) who reported that the rhizosphere is a narrow zone of soil that is influenced by root exudates and inhabited by most microorganisms (bacteria and fungi), including those beneficially affecting soil health and ecosystem functioning.

Table 2. Interaction among PGPE, fertilization and microelements on enzymes activity in fababean rhizosphere.

Treatments		Dehydrogenase activity (µgTPF/g dry soil/day)			Phosphatase activity (µg p-nitrophenol .g^{-1}.dm^{-1}.h^{-1})		
		Periods (day)					
		30	60	90	30	60	90
NPK	Without microelements	26.30^{d}	39.33^{e}	35.00^{e}	19.66^{e}	42.11^{f}	40.66^{e}
PGPE		33.33^{c}	55.33^{d}	55.10^{c}	25.66^{d}	49.33^{d}	49.00^{c}
PGPE +½ N		49.61^{a}	66.66^{b}	61.00^{b}	30.00^{b}	53.00^{b}	51.33^{b}
NPK	With microelements	27.00^{d}	40.10^{e}	40.00^{d}	20.00^{e}	44.00^{e}	43.00^{d}
PGPE		41.61^{b}	60.90^{c}	55.20^{c}	27.00^{c}	50.91^{c}	50.66^{b}
PGPE +½ N		50.00^{a}	70.00^{a}	69.33^{a}	32.33^{a}	60.90^{a}	57.30^{a}

NPK: full dose (100%) of chemical fertilizers

PGPE+½ N: Endophytes strains combined to half dose (50%) of nitrogen fertilizers

Plant growth promoting endophytic bacteria strains: (*Pseudomonas* sp KY648982.1+ *Klebsiella* sp KY662036.1 + *Pseudomonas* sp KY662037.1)

Nitrogen fixation activities

Data in Table (3) indicated that all nodules parameters i.e. nod. number/plant and nod. fresh and dry weight, nitrogenase activity and legheamoglobin were lower in plants fertilized with full dose of chemical fertilizers than plants inoculated with PGPE. These results are in agreement with those obtained by Anne-Sophie *et al.* (2002) who demonstrated that the addition of chemical fertilizers decreased the nitrogenase activity. Also, Ferguson *et al.* (2010) who reported that nitrogen is the main component of plant acquires in the legume-rhizobia symbiosis, it seems highly plausible that a mechanism has evolved to prevent the plant from forming nodules when nitrogen levels in the rhizosphere are

already sufficient Also, the highest significant values of all estimated nodules parameters were observed in faba bean root

Table 3. Nitrogen fixation activities in faba bean nodules treated with PGPE, chemical fertilizers and sprayed with microelement after 50 days from sowing .

Treatments		Nodules parameters			Nitrogenase activity	Legheamoglobin
		Nod. number/ plant	Nod. FW (g/ plant)	Nod. DW (g/ plant)	(µl C_2H_4/g dry nodules)	(O.D)
NPK	Without microelements	19.00^{e}	0.30^{c}	0.100^{c}	37.00^{e}	0.076^{f}
PGPE		30.00^{c}	2.00^{ab}	0.210^{b}	86.00^{c}	0.217^{d}
PGPE +½ N		50.00^{b}	2.10^{a}	0.550^{a}	90.33^{b}	0.328^{c}
NPK	With microelements	21.00^{d}	1.00^{bc}	0.200^{bc}	47.33^{d}	0.090^{e}
PGPE		50.00^{b}	2.30^{a}	0.480^{a}	87.00^{c}	0.370^{b}
PGPE +½ N		55.00^{a}	2.40^{a}	0.530^{a}	91.66^{a}	0.540^{a}

Abbreviations: as those stated for Table (2)

O.D: optical density

inoculated with PGPE combined with half dose of nitrogen fertilizers and sprayed with microelements. Similar trend of results was obtained by Barea *et al.* (2005) who said that the co-operative interaction between rhizobia and other plant root colonizing bacteria is of relevance in improvement of nodulation and N_2 fixation in legume plants. Also, Weisany *et al.* (2013) who reported that small amounts of microelements are essential for root nodule growth.

Growth characteristics

Data in Table (4) emphasized that the lowest records of all estimated growth characteristics i.e (plant height, dry weight of root and shoot, number of branches, leaves and flowers) were observed in faba bean plants inoculated with PGPE without foliar spraying with microelements. These results are in agreement with those obtained by Awad (1998) who found that using microorganisms alone without stimulative rates from mineral fertilizers was less effective than the recommended rates of chemical fertilizers. The highest records of plant height, dry weight of root,

number of branches and number of flowers of faba bean were observed in faba bean inoculated with PGPE in combination with half dose of nitrogen fertilizers.

The beneficial effect of the tested endophytic bacteria as biostimulant on growth characteristics may be attributed to their ability to produce IAA, solubilization of phosphate and potassium as well as their ability to fix atmospheric nitrogen. These results are in agreement with obtained by Mishara *et al.,* (2014) who reported that growth parameters like number of branches, and number of leaves increased with inoculated with microorganisms. So combined application of NPK as chemical fertilizers along with microorganisms can increase the growth significantly than control (no-inoculation). This might be due to the fact that N_2-fixer and P-solubilizing microorganism secrete certain organic acids and some biochemical which are plant growth promoting on nature. Moreover, faba bean sprayed with microelement gave significant higher values of above mentioned parameters than non-sprayed with microelements. This trend of results was observed at all treatments. These results are in agreement with those obtained by Sarkar *et al.* (2007) who reported that the addition of a small amount of micronutrients, particularly Zn, Fe and Mn applied by foliar spraying was significantly increase the vegetative growth of cultivated plants.

Table 4. Interaction among PGPE, fertilization and microelements on growth characteristics of faba bean.

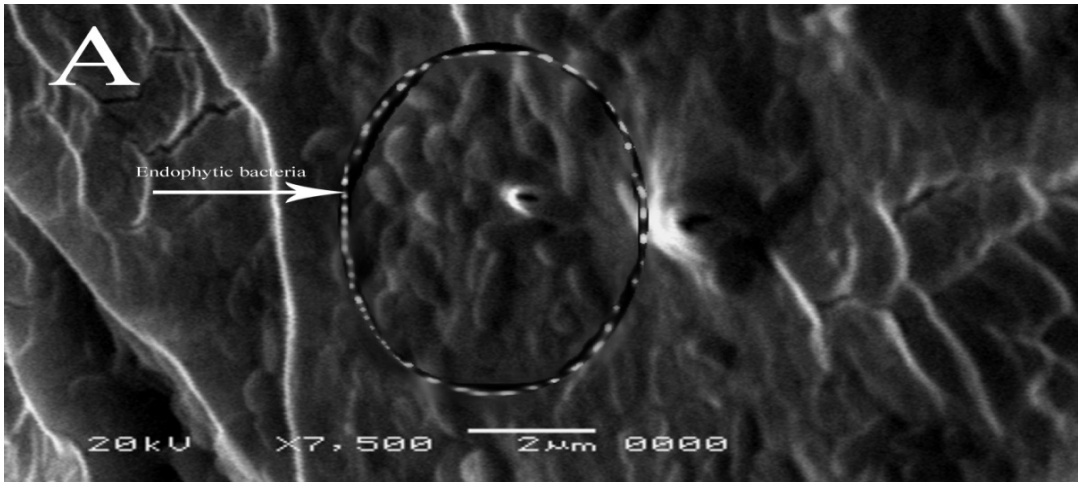
Treatments		Plant height (cm)	Dry weight of root (g)	Dry weight of shoot (g)	Number of branches	Number of leaves	Number of flowers
NPK	Without microelements	13.73^{d}	67.50^{e}	29.40^{b}	5.00^{c}	47.99^{b}	91.32^{d}
PGPE		10.36^{f}	57.00^{f}	19.20^{f}	4.50^{d}	40.20^{d}	80.00^{e}
PGPE +½ N		19.26^{b}	70.50^{d}	21.30^{d}	5.50^{bc}	41.04^{b}	101.32^{c}
NPK	With microelements	17.34^{c}	78.00^{b}	30.78^{a}	6.50^{b}	53.00^{a}	110.66^{b}
PGPE		12.49^{e}	72.00^{c}	20.28^{e}	6.00^{b}	45.88^{c}	80.10^{e}
PGPE +½ N		24.92^{a}	90.00^{a}	21.48^{c}	7.50^{a}	49.00^{b}	140.66^{a}

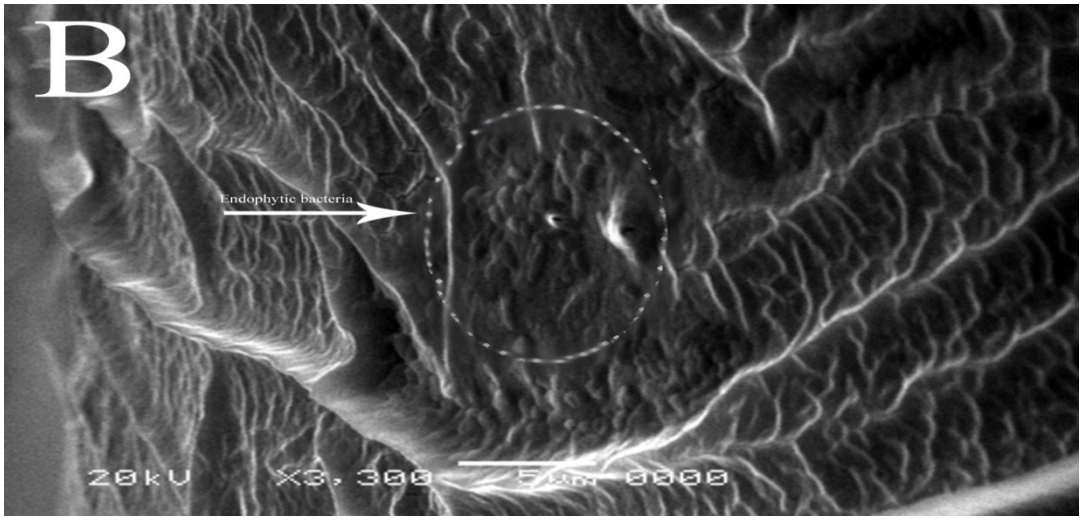
Abbreviations: as those stated for Table (2)

Scanning electron microscopy

Figs (2 A & B) showed that the colonization intensity of endophytic bacteria associated with plants is very high in faba bean inoculated with PGPE. On the other hand, Fig (2 C) showed that the colonization intensity of endophytic bacteria associated with plants is very low in faba bean treated with chemical fertilizers only. Although in chemical fertilization treatment faba bean plants don't received any endophytic bacteria, there were endophytes in their roots under scanning microscopy. This may be due to the presence of native soil endophytes.

Coombs and Franco (2003) reported that endophytic bacteria used in this experiment have shown to be excellent plant colonizers in side roots of faba bean. The colonization intensity of plants by putative endophytes has been visualized by using laser scanning confocal microscope.





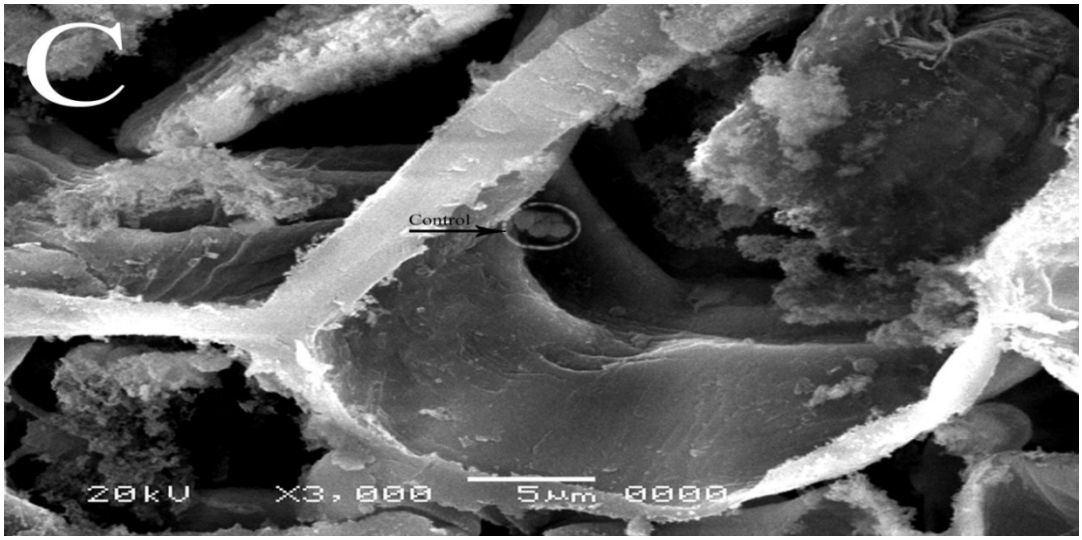


Fig (2) Scanning microscopy (SEM) images of colonization of faba bean roots with PGPE. The endophytic bacterial cells colonized on the roots of faba bean (A &B), untreated roots of faba bean with PGPE(C).

Available N, P and K in faba bean rhizosphere

Data in Table (5) showed that that the lowest significant values of available N, P and K were observed in the rhizosphere of faba bean fertilized with full dose of chemical fertilizers only. Whereas, faba bean inoculated with PGPE solely gave higher values of available N, P and K in rhizosphere compared to plants treated with full dose of chemical fertilizers solely. Additionally, the highest significant values of above mentioned parameters were observed in the rhizosphere of faba bean plants inoculated with PGPE combined with half dose of nitrogen fertilizers and sprayed with microelements, this may be due to the beneficial effects of endophytic bacteria on changing soil pH by secreting organic acids (e.g., acetic, propionic, fumaric and succinic) which leading to change nutrients to available forms ready for uptake by plants.

Table 5. Interaction among PGPE, fertilization and microelements on available N, P and K in faba bean rhizospheare.

Treatments		N	P	K
		ppm		
NPK	Without microelements	160.0^{d}	119.0^{d}	16.80^{c}
PGPE		180.0^{bc}	134.0^{bc}	16.90^{c}
PGPE +½ N		190.0^{b}	138.0^{b}	19.00^{b}
NPK	With microelements	170.0^{b}c	127.0^{c}	18.70^{bc}
PGPE		200.0^{ab}	170.0^{ab}	19.00^{b}
PGPE +½ N		220.0^{a}	190.0^{a}	21.80^{a}

Microorganisms are known to play an important role in increasing availability of nitrogen and phosphorus besides improving biological fixation of atmospheric nitrogen and enhance phosphorus availability to crop (Bhat *et al.,* 2013).

Generally, foliar spraying of faba bean with microelements at all treatments gave significant higher values of available N, P and K in rhizosphere compared to non-spraying with microelements.

N, P and K uptake and protein of seeds

Data in Table (6) showed that the significant lowest values of N, P and K uptake, nitrogen and protein content were recorded in faba bean treated with full dose of chemical fertilizers without foliar application. In addition, the significant highest values were obtained in faba bean inoculated with PGPE combined with half dose of nitrogen fertilizers and sprayed with microelements. It could be concluded that the high uptake of N, P and K which recorded in plants inoculated with biostimulant may be due to the increased N, P and K availability in soil by these endophytic bacteria. These results are in agreement with those obtained by Gabr *et al.* (2007) who revealed that the highest values for nitrogen, potassium and phosphor contents of leaves were recorded in pea inoculated with PGPE. Also, the protein percentage in the seeds of faba bean was increased by applying chemical

with microorganisms as one treatment in comparison to the un-inoculated plants or other treatments Mekki (2016).

Yield and its components of faba bean

Data in Table (7) indicated that faba bean inoculated with PGPE individually gave the significant lowest records of (number of pods/plant, number of seed/pod, dry weight of pods, dry weight of 100 seed (g) and protein yield/ kg /fed with spraying and non-spraying of microelements. The highest significant values of yield components were observed in faba bean inoculated with PGPE combined with half dose of nitrogen fertilizers and sprayed with microelements. These results are in agreement with obtained by Mekki (2016) who reported that the applied chemical with biostimulant or chemical + biostimulant + organic increased number of pods per plant, pods and seed yield per plant, number of seeds per plant,100-seed weight and biological yield compared to other treatments or control plants.

It is important to mention that the lowest significant records of seed yield were observed in faba bean inoculated with biostimulant being 724 kg/fed .On the other hand, faba bean inoculated with biostimulant combined with half dose of nitrogen fertilizers and sprayed with microelements gave the highest significant values of seed yield they being 1284 kg/fed followed by faba bean inoculated with biostimulant combined with half dose of nitrogen fertilizers without foliar application.

Table 6. Interaction among PGPE, fertilization and microelements on N, P and K uptake mg/plant and protein of faba bean seeds.

Treatments		Uptake (mg/plant)			Seeds constituents %	
		N	P	K	N	protein
NPK	Without microelements	130.52^{f}	11.85^{e}	55.67^{f}	3.99^{f}	24.93^{f}
PGPE		149.64^{d}	13.93^{d}	63.31^{d}	4.29^{d}	26.81^{d}
PGPE +½ N		163.97^{b}	18.50^{b}	79.11^{b}	4.54^{b}	28.37^{b}
NPK	With microelements	131.68^{e}	14.00^{d}	58.23^{e}	4.01^{e}	25.06^{e}
PGPE		152.74^{c}	16.32^{c}	63.94^{c}	4.35^{c}	27.19^{c}
PGPE +½ N		166.08^{a}	22.36^{a}	82.91^{a}	4.91^{a}	30.69^{a}

Abbreviations: as those stated for Table (2)

Table 7. Interaction among PGPE, fertilization and microelements on yield components of faba bean.

Treatments		Number of pods/plant	Number of seeds/pod	Dry weight of pods (g)	Dry weight of 100 seed (g)	Protein yield/ kg /fed	Seed yield (kg/fed)
NPK	Without microelements	20.00^{c}	5.00^{bc}	120.00^{c}	44.59^{e}	160.55^{e}	644^{e}
PGPE		19.90^{c}	4.30^{c}	100.00^{d}	42.22^{f}	139.41^{f}	520^{f}
PGPE +½ N		24.66^{b}	6.00^{ab}	120.66^{c}	46.11^{d}	278.02^{b}	980^{b}
NPK	With microelements	25.55^{ab}	5.30^{abc}	130.00^{b}	50.66^{b}	234.57^{c}	936^{c}
PGPE		20.00^{c}	5.00^{bc}	100.33^{d}	50.35^{c}	196.86^{d}	724^{d}
PGPE +½ N		25.77^{a}	6.60^{a}	150.00^{a}	54.97^{a}	394.06^{a}	1284^{a}

Abbreviations: as those stated for Table (2)

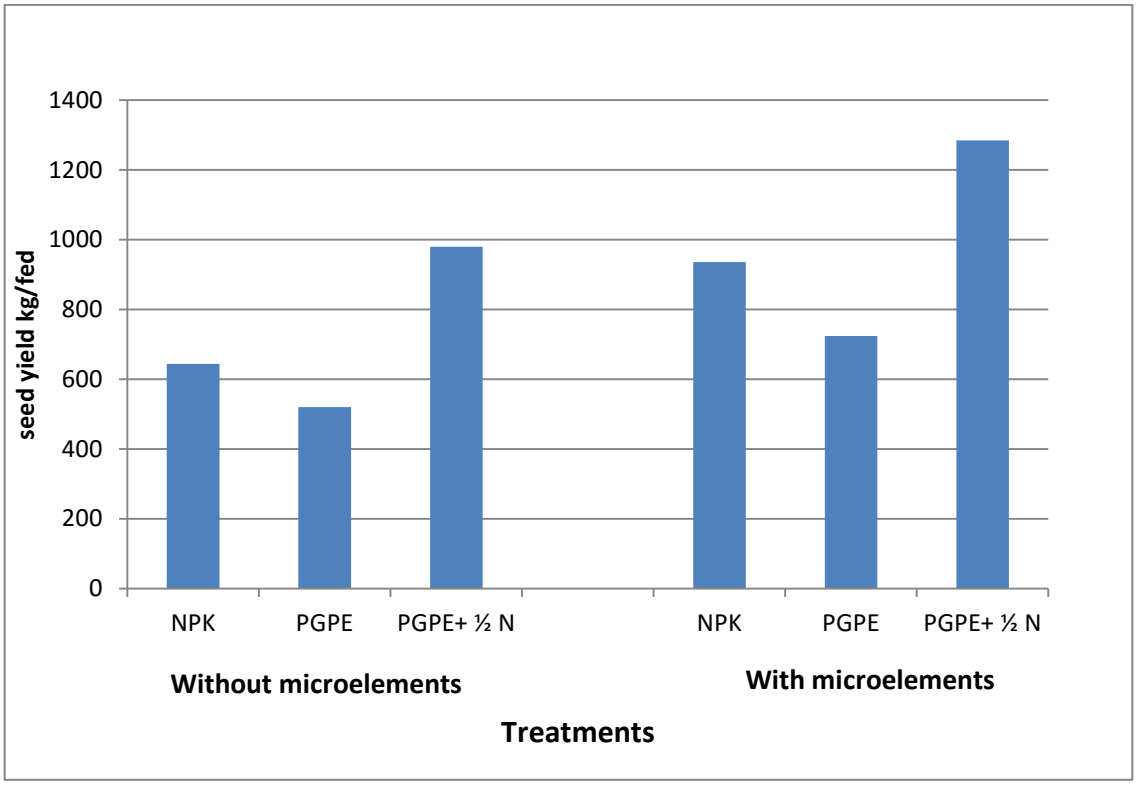


Fig 3.Interaction among PGPE, fertilization and microelements on seed yield of faba bean.

These results are in harmony with those obtained by Abdallah (2014) indicated that application of 70% NP recommended combined with microorganisms in faba bean plants gave seed yield reached to 1340 (kg /fed) compared to application of 70% NP recommended with compost reached to 1200 (kg /fed).

Summing up, spraying of faba bean plants with microelements gave significant higher values of yield and its components than non-sprayed plants. This trend was observed at all treatments. These results are in agreement with those obtained by El Bassiouny *et al.* (2010) who found that foliar application significantly increased plant of weight, weight of 100 seed/gm, seed yield /ton and straw yield of faba bean. Also, Savitha (2008) showed that, the application of some minerals as foliar spray caused an enhancement in plant growth, yield and its physical and chemical properties.

CONCLUSION

In conclusion, this research carried out to evaluate the interaction among biostimulant strains, chemical fertilization (NPK) treatments and microelements as a foliar application on faba bean yield. Results showed that growth performance and yield and its components of faba bean were significantly affected by application of plant growth promoting endophytic bacteria as a biostimulant in combination with microelements. In addition, results proved that using of biostimulant reduced the amount of chemical fertilizers by 50% and reduced the production coast and maintain the environment from pollution resultant from the excessive using of chemical fertilizers. Also, spraying with PGPE can be an alternative method for plant spraying with synthetic industrial growth phytohormones to avoid their adverse effects on human health.

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