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Integrative Application of Phosphorein and Microbein Improves *Vicia faba* (L.) Performance and Controls Soil-borne Diseases

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A B S T R A C T

Plants are constantly exposed to biotic stresses, which cause changes in plant metabolism including physiological damages, leading to crop productivity losses. This study was conducted to investigate bio-fertilizer applications (i.e., phosphorein, microbein, and combination of them at a ratio of 1:1 for seed before sowing) influences on two varieties (i.e., Giza 429 and Giza 40) of *Vicia faba* (L.) plant performances on borne diseases-infected soil. Growth, yield and its quality, physio-biochemical attributes, nutrient contents and disease assessment were investigated. Combined phosphorein and microbein treatment significantly increased all plant growth characteristics, leaf photosynthetic pigments, all physio-biochemical attributes, and nutrients contents compared to individual phosphorein or microbein application, which in turn significantly exceeded the control (seed without bio-fertilizers). All these improved parameters significantly reflected in highest yield and its components with the phosphorein+microbe in application. In contrast, Na^+ content along with percentages of damping-off and root-rot incidence, as well as disease severity were significantly decreased compared to individual treatments and the control. Data of the present study also show that, variety of Giza 429 recorded better results than variety of Giza 40, concluding that Giza 429 was more soil borne disease-tolerant. Results of the current study are important as the potential of combined phosphorein+microbein application to suppress soil-borne diseases and enhance faba bean performance under this biotic stress conditions.

Introduction

Faba bean (*Vicia faba* L.) is a popular legume consumed worldwide as an important protein source for human and animal nutrition. Its seeds are also rich in carbohydrates, minerals, and fibers. Where, faba beans are still failed under stress conditions, they need external supports such as selecting tolerant varieties, using antioxidants or bio-fertilizers (Wood and Myers 1997; Rady *et al.*, 2017). Soil-borne diseases are considered one of the serious biotic stresses that challenge both horizontal and vertical expansion of faba beans. Fungi such as *Rhizoctonia solani*, *Fusarium solani*, *Macrophomina phaseolina*, *Alternaria alternata*, and *F. moniliforme* are considered as the most serious biotic stress, restricting faba bean productions (Abd El-Ati and El-Hadidy 2013). In Egypt, soil-borne diseases, specifically damping-off and root-rot diseases are increased due to the continuous cultivation in the same soil areas for long periods, shortage of high yielding disease resistant-varieties, lack of proper development or technology for growing and harvesting of faba beans, and scarcity of researchers concerned to present situation and practical strategies for disease management. Use of resistant faba bean varieties has been suggested for the disease management (Habtegebriel and Boydom 2016). Therefore, it is necessary to assess the differences among crop varieties for their resistance mechanisms through determining their performances under biotic; disease stress.

Bio-fertilizers are known as microbial inoculants that consist of artificially multiplied cultures of certain soil organisms, which can strengthen seed germination and improve soil fertility and crop productivity. Bio-fertilizers are proved to add nutrients through the natural processes of nitrogen fixation, solubilizing phosphorus, and induce plant growth through synthesizing many growth-promoting substances (Taha *et al.,* 2016). Phosphorein and microbein are used as bio-fertilizers, especially for legumes because they are more effective in supplying legume plants with nitrogen and phosphorus compared to the conventional chemical fertilization. Microbein has greater amounts of symbiotic and non-symbiotic bacteria which are responsible for N fixation. Inoculation of faba bean seeds with such bacteria led to an increase in the availability of various nutrients that positively reflected in growth, yield and its quality (Abo El-Soud *et al.,* 2003). On the other hand, soil inoculation with phosphorein, known as phosphate dissolving bacteria, has been reported to improve soil fertility and plant productivity. Application of phosphorein with or without minerals markedly increased the available P in soil and its uptake by plants and subsequently increased plant growth and its yield on sandy loam (Mohammed 2004) or calcareous soil (Saber *et al.,* 1983). It has also been reported that inoculation of faba bean plants with phosphorein significantly increased plant weight compared to the untreated plants (Eman *et al.,* 1993).

Soil-borne diseases as one of the biotic stresses can be bio-controlled. *Pseudomonas aeruginosa* has been reported to counter biotic stresses (Pandey *et al.,* 2012). *Bacillus subtilis* N11 in addition to mature composts has been reported to control *Fusarium* infestation on banana roots (Zhang *et al.,* 2011) and *B. subtilis* (UFLA285) has been reported to provide resistance against *Rhizoctonia solani* (Medeiros *et al.,* 2011). In addition, *Paenibacillus polymyxa* (SQR-21) has been identified as a potential agent for bio-control of *Fusarium* wilt in watermelon (Ling *et al.,* 2011). It has been shown, in some cases, that mycorrhizae can confer along with bacteria resistance against fungal pathogens and inhibit the growth of many root pathogens such as *R. solani*, *Pythium spp.*, *F. Oxysporum*, *A. obscura* and *H. annosum* (Khalil and Labuschagne 2002; Riedlinger *et al.,* 2006) by improving plant nutrients profile and thereby productivity (Ansari *et al.,* 2013). For example, *Glomus mosseae* has been effective against *Fusarium oxysporum f. sp. Basilica* that causes root-rot disease in basil plants (Toussaint *et al.,* 2008). Further, *Medicago tranculata* has been conferred an induction of various defense-related genes with mycorrhizal colonization (Liu *et al.,* 2007). It has been reported that addition of *Pseudomonas fluorescens* in addition to arbuscular mycorrhizal fungi to the soil can decrease the pathogenic development of root-rot and enhance *Phaseolus vulgaris* (L.) yield (Neeraj 2011).

Based on the abovementioned, the aim of this study was to evaluate the soil-borne diseases resistance of two faba bean varieties (Giza 429 and Giza 40) by seed inoculation with some bio-fertilizers such as phosphorein and microbein (especially the combination of them at a ratio of 1:1) on borne diseases-infected soil. To support this evaluated parameter (disease tolerance), faba bean growth and productivity, as well as physio-biochemical attributes and nutrients contents were also assessed.

Materials and Methods

Plant material, growing conditions, experimental design and treatments

Two field experiments were conducted at the Experimental Farm of the Faculty of Agriculture, Fayoum University, Egypt during the two successive winter seasons of 2017/2018 and 2018/2019 to investigate the effect of two bio-fertilizers; phosphorein and microbein applied for inoculation of seeds individually or in combination (at a ratio of 1:1) on soil borne diseases suppression and enhancement of growth and productivity of two faba bean varieties (i.e., Giza-429 and Giza-40) grown under the conditions of borne disease-infected soil. Phosphorein was contained live cells of efficient bacteria strains as phosphate dissolving bacteria (*Bacillus megatherium*). Microbein was contained live cells of efficient bacterial strains of N-fixing bacteria (*Azotobacter chroccocum*, *Azospirillum braselence*, *Pseudomonas* sp., *Rhisobium* sp., and *Bacillus megaterium*). Both bio-fertilizers were prepared in the laboratory of Microbiology and Biotechnology Department, Faculty of Agriculture, Fayoum University, Fayoum, Egypt. They were used at the rate of 700 g phosphorein or microbein per 100 kg seeds for the individual inoculations and 350 g phosphorein + 350 g microbin per 100 kg seeds for combination inoculation. Arabic gum (16%) was used as a sticking agent.

To test soil infection, diseases survey was conducted on 2016/2017 and 2017/2018 seasons for percentages of infected 2 and 4 months old faba bean plants. Disease syndrome, i.e. withering, discoloration or yellowing, stunting, wilting, rotted roots occurred on plants growing under field conditions were recorded in (Table 2). Damping-off and root-rot diseases were always found in all plantations examined in the surveyed soil. Samples from infected plants were collected for isolation trials in laboratory.

The experimental design was split plot arrangement in randomized complete blocks design with three replications, where varieties were allotted to the main plots, while bio-fertilizers were arranged in the sub plots. Healthy seeds of two varieties (i.e., Giza 429 and Giza 40) of faba bean (*V. faba* L.) were sown on 17 and 21 October 2017 and 2018, respectively. Seeds were obtained from Legumes Crop Research Department, Field Crop Research Institute, Agricultural Research Centre, Giza, Egypt and were selected for uniformity by choosing those of equal size and of the same colour. The selected seeds were washed with distilled water, sterilized in 1% (v/v) sodium hypochlorite for approx. 2 min, washed thoroughly again with distilled water, and left to dry at room temperature. Seeds were subjected to inoculation treatments with phosphorein (Phrn), microbein (Mibn) or distilled water (as a control) for 4 h, and then soaked seeds were air-dried again at room temperature overnight. Uniform, air-dried faba bean seeds were sown in hills spaced 20-25 cm apart, in rows spaced 70 cm apart in 3.0 m × 3.5 m plots, using an equivalent of 120 kg seed ha^{-1} to generate the recommended planting density. Thinning was done before the first irrigation to remain two plants per hill.

During soil preparation and plant growth, the soil was supplemented with the full dose of NPK fertilizer according to the recommendations of the Ministry of Agriculture and Land Reclamation. These recommendations were for 360 kg ha^{-1} calcium superphosphate (15.5% P_2O_5), 240 kg ha^{-1} ammonium sulfate (20.5% N) and 120 kg ha^{-1} potassium sulfate (48% K_2O). Irrigation water was added to 100% of the reference crop evapotranspiration (ETo), values from the Fayoum Meteo Station. Seven irrigations were applied in each season, with total water rates of about 2800 m^3 ha^{-1} in each growing season. All other recommended agricultural practices were followed as recommended by the Ministry of Agriculture and Land Reclamation. Soil samples were taken at the two depths of 30 and 60 cm for mechanical and chemical analyses as described by Chapman and Pratt (1978), and data are presented in (Table 1).

Disease assessment

To isolate and identify damping-off and root-rot causal organisms, faba bean plants with symptoms of root-rot infection were collected in plastic bags from the field a year before the experiment and brought to the laboratory. The infected samples were rinsed in tap water and the necrotic portions were excised and cut into at least 2 mm, then surface sterilized with 5% sodium hypochlorite (NaClO) for 30 s and rinsed in 4 successive changes of sterile distilled water. These were then plated on potato dextrose agar (PDA) and incubated at 25 ± 2 °C for up to 5 days under 12 hr photoperiod. Hyphal type transfer and the single spore technique were adopted whenever possible. Pure cultures and morphological features were done referring to Gilman (1957), Burnett and Hunter (1972) and Nelson *et al.*, (1983). The disease assessment was performed periodically by examining and recording damping off after 30 and 45 days from sowing date. Percentage of root-rot disease incidence was assessed 30 days after sowing.

Disease severity (DS) was estimated visually by assessing the necrotic regions on the roots and hypocotyls using rating scale of 0-5 as described by Filion *et al.*, (2003).

$$DS = [\Sigma (ab) / AK] \times 100$$

Where "a" is the number of diseased plants having the same degree of infection, "b" is the degree of infection, "A" is the total number of examined plants, and "K" is the highest degree of infection.

Plant growth and yield measurements

From each experimental plot, Fifty-day-old plants (n = 10) were carefully removed and dipped in a bucket of water. Plants were shaken gently to remove all adhering soil particles and the lengths of their shoots were measured using a meter scale. Numbers of branches $plants^{-1}$ were counted. Using a graph sheet, leaf area per plant was measured manually where the squares covered by the leaf were counted. Plant shoots were weighed for fresh weights and were then placed in an oven at 70 °C until constant weight and the dry weights were recorded.

At the end of each experiment (3 and 5 April 2018 and 2019, respectively) for dry yield, all pods on each plant of each experimental plot were collected to air-dry and they were then counted. The seeds in all pods were extracted and weighed to calculate average 100-dry seed weight, and dry seed yield $plant^{-1}$ and ha^{-1}.

Determination of relative water content and membrane stability index, and the contents of total soluble sugars, free proline, and protein

RWC [according to the method of Osman and Rady (2014)] and MSI [as described in the method of Rady (2011)] were assessed using fresh fully-expanded leaves excluding the midribs. The following equations were used for calculating both RWC and MSI:

RWC (%) = [(FM − DM) ÷ (TM − DM)] × 100

MSI (%) = [1 − (C_1 ÷ C_2)] × 100

Free proline content (mg g^{-1} DW) in dried faba bean leaves was measured using the Bates *et al.*, (1973) method using 3% (v/v) sulphosalicylic acid for plant material extraction and freshly prepared acid–ninhydrin solution and toluene for separating the upper toluene phase to read on 520 nm using a UV-160A UV-visible spectrophotometer (Shimadzu, Kyoto, Japan), and the content of proline in each sample was determined using a standard curve based on analytical-grade proline. Total soluble sugars content (mg g^{-1} DW) was determined according to Irigoyen *et al.*, (1992) method using 96% (v/v) ethanol for extraction and freshly-prepared anthrone reagent [150 mg anthrone plus 100 ml of 72% (v/v) sulphuric acid], and reading was performed on 625 nm using a UV-160A UV-visible spectrophotometer (Shimadzu, Kyoto, Japan). Determination of total nitrogen (N) in seeds was carried out with Micro-Kjeldahl method (A.O.A.C. 1995). Using total N content, protein was calculated by multiplying total N by a factor of 6.25.

Determination of leaf photosynthetic pigment contents

The photosynthetic pigments (i.e., chlorophyll "a", chlorophyll "b" and total carotenoids in mg g^{-1} FW) were estimated by the spectrophotometric method recommended by Lichtenthaler (1987). Leaf samples (0.2 g from each replicate of each treatment (n = 10) were homogenized in 50 ml 80% (v/v) acetone and centrifuged at 10,000 ×g for 10 min. The absorbance of each acetone-extracted sample was measured at 665, 649, and 440 nm using a UV-160A UV-visible spectrophotometer (Shimadzu, Kyoto, Japan).

Determination of leaf nitrogen (N), phosphorus (P), potassium (K), calcium (Ca) and sodium (Na) contents

Leaf N and P (mg g^{-1} DW) were determined according to A.O.A.C. (1995) and Jackson (1967), respectively. After digesting the leaf samples using perchloric and sulfuric acids at a ratio of 1: 3, K^+ and Na^+ ion contents (in mg g^{-1} DW) were assessed using a PerkinElmer Model 52-A Flame Photometer (Glenbrook, Stamford, CT, USA; Page *et al.*, 1982). Leaf Ca^{2+} content was determined using a Perkin-Elmer Model 3300 Atomic Absorption Spectrophotometer (Chapman and Pratt 1978).

Statistical analysis

All data of the present study were subjected to analysis of variance (ANOVA) for a split-plot arrangement in randomized complete blocks design, after testing for homogeneity of error variances as described in the methods of Gomez and Gomez (1984). Combined analysis of data for the two seasons was performed and significant differences between each two treatments were compared at $P \leq 0.05$ by the Duncan's Multiple Range Test.

Results and Discussion

Pathogenicity tests of faba bean varieties

Samples of both faba bean varieties (i.e., Giza 429 and Giza 40) used for fungi isolation and identification showed clear symptoms of root-rot, and its percentage was recorded. Data in Figure 1 show that *Rhizoctonia solani* was the dominant pathogen occurred by 25.5%, followed by *Fusarium solani* by 19.0%, and then *Macrophomina phaseolina* and *Alternaria alternata* by with 15.4 and 13.1%, respectively, and the lowest pathogen occurred was *Fusarium moniliforme* by 11.8%.

Growth characteristics of faba bean varieties

Seed inoculation of both faba bean varieties (i.e., Giza 429 and Giza 40) with a mixture of phosphorein + microbein at a ratio of 1:1 significantly increased all tested growth traits (i.e., shoot length, number of branches plant^{-1}, leaf area plant^{-1}, shoot fresh weight and shoot dry weight plant^{-1}) compared to the individual inoculations (phosphorein or microbein), which in turn significantly increased these growth characteristics compared to the control; seeds without bio-fertilizers (Table 3).

The combined (phosphorein+microbein) was the best treatment, increasing the above growth characteristics by 74.1, 37.6, 53.2, 92.7 and 89.7%, respectively for Giza 429, and by 82.7, 54.25, 60.0, 97.0 and 90.6%, respectively for Giza 40 compared to the control.

For varieties, there was significant growth characteristics increases in Giza 429 compared to those in Giza 40.

Physio-biochemical attributes of faba bean varieties

Plants generated from phosphorein+microbein bio-fertilized seeds of both faba bean varieties showed significant increases in all physio-biochemical attributes (i.e., relative water content; RWC, membrane stability index; MSI, the contents of total soluble sugars; TSS, free proline, proteins, chlorophyll "a", chlorophyll "b" and total carotenoids) compared to those obtained from plants generated from phosphorein or microbein bio-fertilized seeds, which in turn significantly exceeded those obtained from the control plants that generated from untreated seeds (Table 4 and 5). The best treatment was phosphorein+microbein combined application that increased RWC% by 47.4%, MSI% by 32.9%, TSS content by 60.4%, free proline content by 79.6%, protein content by 90.3%, chlorophyll "a" content by 72.6%, chlorophyll "b" content by 67.9%, and total carotenoids content by 78.8% for Giza 429, and increased these parameters by 50.9, 34.2, 66.5, 81.0, 47.3, 77.2, 72.3 and 77.8%, respectively for Giza 40 compared to those of the control. Giza 429 showed significant higher values of physio-biochemical attributes than those of Giza 40.

Nutrient status of faba bean varieties

Phosphorein+microbein combined treatment for seeds of both faba bean varieties showed significant increases in the contents of N, P, K^+, and Ca^{2+}, while revealed significant reductions in the content of Na^+ compared to the individual treatment of phosphorein or microbein that in turn significantly exceeded the control (Table 6).

The combined phosphorein+microbein seed inoculation as the best treatment increased the N, P, K^+, and Ca^{2+} contents by 56.8, 81.1, 69.1 and 55.1%, respectively for Giza 429, and by 60.4, 51.7, 65.7 and 55.3%, respectively for Giza 40 while.

In contrast, the content of Na^+ was reduced 46.8 and 40.8% in Giza 429 and Giza 40, respectively by the best combined treatment compared to those of the control. The faba bean variety of Giza 429 collected higher macro-nutrient contents and lower Na^+ content than those collected by Giza 40 variety.

Yield and its components of faba bean varieties

The combined phosphorein+microbein inoculation for faba bean seeds of both varieties significantly increased faba bean yield and its components (i.e., number of pods plant^{-1}, number of seeds pod^{-1}, average 100-dry seed weight, dry seed yield plant^{-1} and per ha^{-1}) compared to the individual phosphorein or microbein treatment, which in turn significantly surpassed the control (Table 7). The increases in the yield and its components by the combined phosphorein+microbein application as the best treatment were 78.6, 88.2, 52.6, 60.0 and 54.5%, respectively for Giza 429, and were 87.2, 93.6, 50.8, 60.6 and 53.8%, respectively for Giza 40 compared to those of the control. Giza 429 variety conferred higher green yield and its components than those conferred by Giza 40 variety.

Damping-off and root-rot diseases in faba bean varieties

Plants developed from both Giza 429 and Giza 40 seeds inoculated by the combined phosphorein+microbein showed significant decreases in damping-off, root-rot diseases and disease severity, while showed significant increases in survival ratio compared to the individual phosphorein or microbein inoculation, which in turn exceeded the control plants developed from non-inoculated seeds (Table 8). The combined phosphorein+microbein was the best treatment, decreasing pre- and post-emergence damping-off, root-rot incidence and disease severity by 31.7, 45.2, 30.6 and 53.3%, respectively for Giza 429, and by 40.1, 41.7, 29.9 and 49.8%, respectively for Giza 40, and increasing survival ratio by 27.3 and 31.0% for Giza 429 and Giza 40, respectively compared to those of the control. The variety of Giza 429 showed higher survival ratio and lower pre- and post-emergence damping-off, root-rot incidence and disease severity than those shown by Giza 40 variety.

Decreasing the infection of faba bean by soil-borne diseases and increasing its yield are important indicators to find out the effect of bio-fertilizers on improving soil and faba bean health. Effective approaches for improving plant performances using bio-fertilizers supplementations are highly requested because they are an acceptable approach for higher yield with good quality in addition to that they are eco-friend and safe substances. Our results show that either individual or combined inoculation of phosphorein and microbein for seeds conferred higher positive responses of all investigated parameters. Sustainable farming systems that imbedded certain practices are reported to reduce the dependency on the conventional farming systems such as chemical fertilization and pesticides, which became nowadays unpleasant from the point of view of preserving the environment from pollution, as well as maintaining the soil fertility status (Rekha *et al.,* 2018; Saikia *et al.,* 2018). Therefore, the essential need for alternative practices that ensure the environmental safety and soil sustainability has become a must. Bio-fertilization farming is one of the environmental technologies, including phosphorein and microbein bio-fertilizers to provide soil fertility and more resistance for plants growing under biotic stress such as soil-borne diseases (Ellafi and Gadalla 2010; Simarmata *et al.,* 2016).

The soil used for the current study has shown to be infested with many of the soil-borne diseases (Fig. 1; Table 2). Damping off and root rot fungal diseases have shown high percentages (29.2% as a mean of the two studied seasons) in the investigated soil. However, application of phosphorein and/or microbein bio-fertilizers, as seed inoculations, markedly resulted in clear improvements of the growth characteristics and yield and its components of faba bean plants compared to the control; seed without any bio-fertilizer (Table 3). Moreover, combined phosphorein+microbein seed inoculation significantly exceeded the individual inoculations for these parameters.

These positive results could be explained based on the positive integrative roles of the two bio-fertilizers (e.g., the integrative action of various beneficial micro-organisms found in both bio-fertilizers) in providing growing plant by increased available nitrogen (N), phosphorus (P), potassium (K^+), and calcium (Ca^{2+}) (Table 6).

This may be attributed to the role of phosphorein in solubilizing the phosphates in the soil and the role of microbein in fixing N_2 because it contains N_2-fixing bacteria such as *Azotobacter spp.*, *Azospirillum spp.*, and *Pseudomonas spp.*, and phosphate-dissolving bacteria such as *Bacillus megaterium*, as well as it contains photosynthetic bacteria (El-Wakeil and El-Sebai 2007; Yao *et al.*, 2010; Farahat *et al.*, 2014). Phosphorein and microbein bio-fertilizers have gained, together, the best nutritional values due to that these bio-fertilizers contain a great number of microorganisms that provides N_2 fixation either symbiotic or non-symbiotic, in addition to release of some macro-nutrients to be available to plant roots such as P and K^+ (Abd El-Ati and El-Hadidy 2013). The important characteristic of bio-fertilizer is that they excrete ammonia into the rhizosphere in the presence of root exudates.

The increase in plant P content might be due to the P-solubilizing potential of the isolates used in bio-fertilizer. This might be attributed to the production of organic acids, chelating Oxo-acids and solubilization of inorganic insoluble phosphates by microorganisms (Rekha *et al.*, 2018). Bio-fertilizers also improve the availability of K^+ nutrient in soil. This may be due to the presence of potash releasing bacteria in the bio-fertilizer that release the soluble K^+ from K-bearing minerals. The mechanism of K releasing from potash-bearing minerals is by organic acids production, rapidly dissolving rocks and chelate silicon ions and leads to releasing K^+ ions into the soil (Bennett *et al.*, 1998).

In addition, microbes in bio-fertilizers release organic acids into the soil to decrease soil pH that is suitable to the availability of nutrients to plant roots.

Using bio-fertilizers, especially the integrative phosphorein+microbein for faba bean seeds conferred higher protein and soluble sugars contents (Table 3).

This result may be due to the increased content of N (Table 6) as essential nutrient for formation of amino acids (Agamy *et al.*, 2012), as well as due to the increased soluble sugars, which use with N to synthesize amino acids for protein formation.

In addition, this integrative bio-fertilizers application induced increases in relative water content (RWC, membrane stability index (MSI), and free proline content (Table 3).

These realities may be due to certain features in bio-fertilizers, playing a very important role in its mode of action (Abd El-Ati and El-Hadidy 2013). One of these is the stimulated increase in the K^+ content, playing a crucial role as an osmolyte in increasing water absorption that increase the cell water content, maintaining its membranes from toxic elements by dilution and consequently increase of MSI. Moreover, bio-fertilizers improve plant physiological properties that enhance water and plant relationships, thus plant growth and metabolism (Rakha and El-Said 2013).

Table.1 Mechanical and chemical analyses of the experimental soils of two seasons*

Properties		Season of 2017/2018			Season of 2018/2019	
		30 cm depth	60 cm depth		30 cm depth	60 cm depth
Mechanical						
Sand (%)		25.7	27.7		27.0	29.9
Silt (%)		19.1	18.0		17.9	19.2
Clay (%)		55.2	54.3		55.1	50.9
Soil texture		Clay				
Chemical						
ECe (dS m^{-1})		1.79	1.79		1.63	1.67
Organic matter (%)		1.35	1.23		1.41	1.33
N	**mg kg^{-1}**	71.1	73.1		75.1	77.1
P		21.0	22.0		23.1	25.0
K		695	697		731	722

*All analyses were done in the Central Lab of Soil, Water and Plant Analyses (Iso-17025), Faculty of Agriculture, Fayoum University, Fayoum 63514, Egypt.

Table.2 Natural infections (%) by damping off and root rot fungal diseases shown on faba bean plants grown on borne diseases-infected soil in two seasons

Season & plant age	Season of 2016/2017			Season of 2017/2018			Grand mean
	2 months old	4 months old	Mean	2 months old	4 months old	Mean	
Infections (%)	20.5	36.7	28.6	22.4	37.2	29.8	29.2

Table.3 Response of growth characteristics of two *Vicia faba* varieties to combined application of phosphorein (Phrn) and microbein (Mibn) under borne diseases-infected soil conditions

Treatments		Shoot length (cm)	No. of branches plant^{-1}	Leaves area plant^{-1} (dm^2)	Shoot fresh weight (g)	Shoot dry weight (g)
Variety	**Bio-fertilizer**					
Giza-429	**Control**	34.0c	13.3c	11.3c	43.2c	5.13 c
	Phrn	57.0b	16.1b	15.1b	80.1b	9.03 b
	Mibn	55.2b	15.9b	15.9b	79.2b	8.27 b
	Phrn+Mibn	59.1a	18.2a	17.2a	83.2a	9.73 a
	Mean	**51.3A**	**15.9A**	**14.9A**	**71.4A**	**8.04 A**
Giza-40	**Control**	31.2c	11.0c	10.0c	41.1c	3.73 c
	Phrn	54.0b	14.7b	14.2b	77.1b	7.89 b
	Mibn	51.1b	14.0b	14.1b	76.9 b	7.03 b
	Phrn+Mibn	57.1a	17.0a	16.1a	81.0 a	7.11 a
	Mean	**48.3B**	**14.2B**	**13.6B**	**69.0 B**	**6.44 B**

Mean values in the same column for each trait with the same lower small or upper bold-case letters are not significantly different by Duncan's Multiple Range Test at $P \leq 0.05$.

Table.4 Response of physio-biochemical attributes of two *Vicia faba* varieties to combined application of phosphorein (Phrn) and microbein (Mibn) under borne diseases-infected soil conditions

Treatments		Relative water content (%)	Membrane stability index (%)	Protein (mg^{-1} DW)	Total soluble sugars (mg^{-1} DW)	Free proline (µg g^{-1} DW)
Variety	**Bio-fertilizer**					
Giza-429	**Control**	45.5 c	64.1 c	2.17 c	5.13 c	105.3 c
	Phrn	64.2b	81.0 b	3.25 b	7.01 b	171.2 b
	Mibn	63.9 b	80.2b	3.23 b	6.93 b	170.2b
	Phrn+Mibn	67.1 a	85.2a	4.13 a	8.23 a	189.1 a
	Mean	**60.2A**	**77.6 A**	**3.20 A**	**6.83 A**	**159.0A**
Giza-40	**Control**	43.2c	62.0c	2.07 c	4.03 c	102.3c
	Phrn	63.0b	77.9 b	3.01 b	5.93 b	167.3b
	Mibn	62.1b	76.9 b	2.97 b	5.05 b	166.2b
	Phrn+Mibn	65.2a	83.1 a	3.05 a	6.71 a	185.1 a
	Mean	**58.4B**	**75.0B**	**2.78 B**	**5.43 B**	**155.2 B**

Mean values in the same column for each trait with the same lower small or upper bold-case letters are not significantly different by Duncan's Multiple Range Test at $P \leq 0.05$.

Table.5 Response of leaf photosynthetic pigments contents of two *Vicia faba* varieties to combined application of phosphorein (Phrn) and microbein (Mibn) under borne diseases-infected soil conditions

Treatments		Chlorophyll "a" (mg g^{-1} FW)	Chlorophyll "b" (mg g^{-1} FW)	Carotenoids (mg g^{-1} FW)
Variety	**Bio-fertilizer**			
Giza-429	**Control**	1.13 c	0.53 c	0.33 c
	Phrn	1.43 b	0.74 b	0.47 b
	Mibn	1.41 b	0.73 b	0.46 b
	Phrn+Mibn	1.95 a	0.89 a	0.59 a
	Mean	**1.48 A**	**0.72 A**	**0.46 A**
Giza-40	**Control**	1.01 c	0.47 c	0.27 c
	Phrn	1.29 b	0.61 b	0.39 b
	Mibn	1.27 b	0.60 b	0.39 b
	Phrn+Mibn	1.79 a	0. 81 a	0.48 a
	Mean	**1.34 B**	**0.56 B**	**0.38 B**

Mean values in the same column for each trait with the same lower small or upper bold-case letters are not significantly different by Duncan's Multiple Range Test at $P \leq 0.05$.

Table.6 Response of macro-nutrients and sodium contents of two *Vicia faba* varieties to combined application of phosphorein (Phrn) and microbein (Mibn) under borne diseases-infected soil conditions

Treatments		N (mg g^{-1} DW)	P (mg g^{-1} DW)	K^{+}(mg g^{-1} DW)	Ca^{2+}(mg g^{-1} DW)	Na^{+}(mg g^{-1} DW)
Variety	**Bio-fertilizer**					
Giza-429	**Control**	23.7 c	2.17 c	21.2b	7.11 c	7.95 a
	Phrn	33.3b	3.05 b	31.9 b	9.79 b	5.09 b
	Mibn	32.7b	3.03 b	31.1b	9.21 b	5.17 b
	Phrn+Mibn	37.1 a	3.93 a	35.8a	11.03 a	4.23 c
	Mean	**31.7 A**	**3.05 A**	**30.0A**	**9.29 A**	**5.36 B**
Giza-40	**Control**	21.9 c	2.01 c	20.0 c	5.97 c	8.93 a
	Phrn	31.0b	2.83 b	27.9 b	7.89 b	6.61 b
	Mibn	30.1b	2.75 b	27.0b	7.25 b	6.91 b
	Phrn+Mibn	35.2a	3.05 a	33.2a	9.27 a	5.29 c
	Mean	**29.5 B**	**2.66 B**	**27.0B**	**7.60 B**	**6.69 A**

Mean values in the same column for each trait with the same lower small or upper bold-case letters are not significantly different by Duncan's Multiple Range Test at $P \leq 0.05$.

Table.7 Response of yield and its components of two *Vicia faba* varieties to combined application of phosphorein (Phrn) and microbein (Mibn) under borne diseases-infected soil conditions

Treatments		No. of pods $plant^{-1}$	No. of seeds pod^{-1}	Average 100-seed weight (g)	Dry seed yield $plant^{-1}$(g)	Dry seed yield ha^{-1} (ton)
Variety	**Bio-fertilizer**					
Giza-429	**Control**	15.3c	2.29 c	53.3c	33.3c	1.91 c
	Phrn	25.0b	3.73 b	76.2b	50.2b	2.27 b
	Mibn	24.7 b	3.65 b	75.3b	49.1b	2.25 b
	Phrn+Mibn	27.3 a	4.31 a	81.3a	53.3a	2.95 a
	Mean	**23.1A**	**3.50 A**	**71.5 A**	**46.5 A**	**2.35 A**
Giza-40	**Control**	13.8c	2.03 c	51.2 c	31.3c	1.71 c
	Phrn	21.9b	3.07 b	73.9 b	48.9 b	2.11 b
	Mibn	21.0 b	3.05 b	73.9 b	48.1b	2.08 b
	Phrn+Mibn	25.8 a	3.93 a	77.3a	50.2a	2.63 a
	Mean	**20.6 B**	**3.02 B**	**69.1B**	**44.6 B**	**2.13 B**

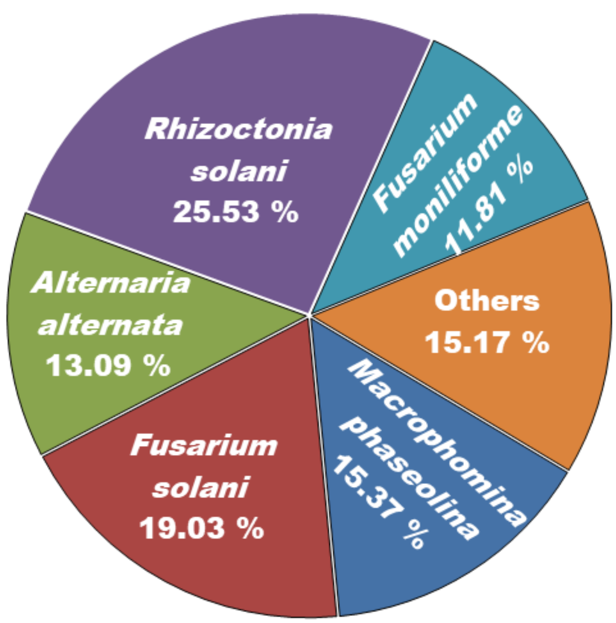
Mean values in the same column for each trait with the same lower small or upper bold-case letters are not significantly different by Duncan's Multiple Range Test at $P \leq 0.05$.

Table.8 Combined application of phosphorein (Phrn) and microbein (Mibn) influences on damping-off and root-rot diseases of two *Vicia faba* varieties grown under borne diseases-infected soil conditions

Treatments		Damping off (%)		Root-Rot Incidence (%)	Survival Ratio	Disease severity
Variety	**Bio-fertilizer**	**Pre**	**Post**			
Giza-429	**Control**	5.49 a	11.25 a	27.5 a	55.76 c	1.95 a
	Phrn	4.41 b	7.67 b	23.3b	64.62 b	1.19 b
	Mibn	4.43 b	7.73 b	23.1 b	64.74 b	1.23 b
	Phrn+Mibn	3.75 c	6.17 c	19.1 c	70.98 a	0.91 c
	Mean	**4.52 B**	**8.21 B**	**23.3B**	**63.97 A**	**1.32 B**
Giza-40	**Control**	6.53 a	12.43 a	28.4 a	52.64 c	2.65 a
	Phrn	5.39 b	8.65 b	24.3 b	61.66 b	2.17 b
	Mibn	5.41 b	8.71 b	24.2b	61.68b	2.19 b
	Phrn+Mibn	3.91 c	7.25 c	19.9 c	68.94 a	1.33 c
	Mean	**5.31 A**	**9.26 A**	**24.2 A**	**61.23B**	**2.09 A**

Mean values in the same column for each trait with the same lower small or upper bold-case letters are not significantly different by Duncan's Multiple Range Test at $P \leq 0.05$.

Fig.1 Percentage (as a mean of two seasons; 2016/2017 and 2017/2018) of fungi isolated from faba bean roots grown in the Experimental Farm of the Faculty of Agriculture, Fayoum University, Fayoum, Egypt



The significant increases occurred in the contents of chlorophyll "a", chlorophyll "b", and carotenoids in faba bean leaves by seed inoculation of both bio-fertilizers, especially the combined application compared with the untreated control (Table 5) may be attributed to the role of bio-fertilization in enhancement of N content (Table 6) as an important component for chlorophyll molecule formation (Agamy *et al.,* 2012) and acceleration of chloroplasts differentiation. This explanation reflects in increasing photosynthetic process, conferring more photosynthates to support strong plant growth, especially root system to fight well the soil-borne pathogens. In addition, bio-fertilizers have some hormonal effects for accelerating plant growth, escaping from the soil-borne pathogens.

In legumes, bio-fertilizers not only enhance the soil fertility but also improve the nodulation that play a role in a high suppression of soil-borne pathogens (Table 8). Besides, it releases some bio-fertilizers to encourage the uptake of nutrients by plant roots (Javaid and Mahmood, 2010). Moreover, it considers as organic manure containing N, P, K^+, and many other essential nutrients, so it enhances retention of nutrients, consequently promotes growth of beneficial organisms that help plants to resist soil-borne diseases, producing more yields (Ross 2008). Bio-fertilizers have the ability to suppress the soil-borne diseases such as *Fusarium* propagation which is a harmful microorganism that causes high disease problem in continuous cropping. Also, *Fusarium* pathogens encourage the promotion of harmful nematode increases. Thus, bio-fertilizers enhance quality and sustain soil environment by increasing antimicrobial activity of the soil. Application of bio-fertilizer, especially in integration of phosphorein and microbein can effectively increase the induced resistance of faba bean plants to soil borne pathogens and improve its productivity. It seems that the increase in the induced resistance plays a crucial role in improving plant health by suppressing the major soil-borne diseases. Where, bio-fertilizers suppress the pathogens; *Rhizoctonia solani*, *Fusarium solani*, *Macrophomina phaseolina*, *Alternaria alternate,* and others to protect faba bean plants and increase its productivity.

In contrast, without using bio-fertilization under the conditions of the biotic stress of the current study (the control) the virgin environment and human health are negatively affected and the probability of infection by and development of root-rot pathogens are occurred, leading to plant cell restriction, rapid cell mitosis, thinness of cell walls. These conditions could be perfect for the soil-borne diseases to generate its mass of injury (Xu *et al.,* 1996).

Our results show also that, the faba bean variety of Giza 429 showed more resistance to the mentioned soil-borne pathogens, conferring more growth and yield compared to the Giza 40 variety. Therefore, using the seed of Giza 429 variety and inoculating them with both bio-fertilizers (phosphorein and microbein) in integrative application considers as an effective strategy to increase faba bean yield significantly under the biotic stress of soil-borne diseases.

More specifically, after inoculation with bio-fertilizers and seedling growth, a symbiotic living occurs between plants and microorganisms found in bio-fertilizers. Fifteen genes have been up-regulated during symbiosis that identified as putative hexose transporters in *L. bicolor* (Bonfante and Genre 2010). Transporter gene up-regulation during symbiosis has indicated the action of useful compounds transportation like polyamines, amino acids, and oligopeptides through the symbiotic interface from one organism to other. Cysteine-rich proteins (MISSP7) of fungus play a crucial role as effectors and facilitators in the formation of symbiotic interfaces (Plett *et al.,* 2011). Many auxin biosynthesis- and root morphogenesis-related genes have showed up-regulation during mycorrhizal colonization (Splivallo *et al.,* 2009; Abdel-Raouf *et al.,* 2012). Further, *G. versiforme* possesses inorganic phosphate (Pi) transporters on its hyphae which help in the direct absorption of phosphate from the soil and a glutamine synthase gene was found in *G. intraradice*, which strengthens the possibility of nitrogen metabolism in fungal hyphe that can be transported later to the plant (Salvioli *et al.,* 2012). Bioactive compounds so-called "Myc factors" in addition to "Nod factors" of *Rhizobium* have been suggested to be secreted by mycorrhiza and *Rhizobium* and perceived by host roots for the activation of signal transduction pathway or common symbiosis (SYM) pathway (Kosuta 2003; Roberts *et al.,* 2013). This common symbiosis (SYM) pathway prepares the host plant to bring about changes at the molecular and anatomical level with the first contact of fungal hyphae. Heretofore, Ca^{2+} is supposed to be the hub of secondary messengers via Ca^{2+} spiking in the nuclear region of root hairs (Sieberer *et al.,* 2009). In addition, it has been reported that *Rhizobium leguminosarum biovar viciae* can induce various genes in the plants like pea, alfalfa and sugar beet as evident from microarray studies (Ramachandran *et al.,* 2011). Plant growth-promoting rhizobacteria (PGPR) produce IAA that, in turn, induces production of nitric oxide (NO). NO acts as a second messenger to trigger a complex signaling network leading to improved root growth and developmental process (Molina-Favero *et al.,* 2007) to cope with the biotic stress like soil-borne pathogens. Expression of ENOD11 and many defense-related genes and root remodelling genes get up-regulated during entry. Consequently, this allows formation of a pre-penetration apparatus or PPA (Bucher *et al.,* 2009). Many disease resistance genes that work via jasmonate/ethylene signaling as well as osmotic regulation via proline synthesis genes have been differentially expressed with *Bacillus subtilis* (UFLA285) induction (Baharlouei *et al.,* 2011).

Various differentially expressed genes have been identified including metallothionein-like protein type 1, a NOD26-like membrane integral protein, ZmNIP2-1, a thionin family protein, an oryzain gamma chain precursor, stress-associated protein 1 (OsISAP1), probenazole-inducible protein PBZ1 and auxin and ethylene-responsive genes (Brusamarello-Santos *et al.,* 2012). Expression of the defense-related proteins PBZ1 and thionins have been reported to get repressed in the rice–H seropedicae association, suggesting the modulation of plant defense responses during colonisation (Brusamarello-Santos *et al.,* 2012). Among PGPR species, *Azospirillum* has been suggested to secrete gibberellins, ethylene, and auxins (Perrig *et al.,* 2007). Some plant associated bacteria can also induce phytohormone synthesis in roots (Bent *et al.,* 2001) supporting plants against biotic stress conditions. Rhizobium and Bacillus have been reported to synthesize IAA at different cultural conditions such as pH, temperature and in the presence of agro waste as substrate (Sudha *et al.,* 2012). Interestingly, the potential of PGPRs has been further improved by introducing genes involved in the direct oxidation (DO) pathway and mineral phosphate solubilization (MPS) into some useful strains of PGPRs. Gene encoding glucose dehydrogenase (gcd) involved in the DO pathway, as well as soluble form of gcd gene has been cloned and characterized from some PGPRs (Tripura *et al.,* 2007; Sashidhar and Podile 2010). All of these mechanisms encourage plant growth strength and consequently cope effectively with biotic stress conditions such as soil-borne disease under study.

This work can provide an acceptable explanation for the significant increase in faba bean growth, productivity and resistance to soil-borne diseases by application of bio-fertilizers, especially the combined application of phosphorein and microbein at a ratio of 1:1 to be the most appropriate bio-fertilization application under the biotic stress conditions (soil-borne diseases). To improve faba bean production under abovementioned stress conditions, the inoculant types should depend on the selection of effective bio-fertilizer. The best performing type was the combined phosphorein+microbein than their individuals. Therefore, we recommend using this combined application as commercial inocula for improving the production of faba bean and soil-borne diseases resistance.

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