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RESPONSE OF FABA BEAN (*VICIA FABA* L.) TO COMBINED INOCULATION WITH RHIZOBIA, VA-MYCORRHIZAE AND PHOSPHATE SOLUBILIZING BACTERIA UNDER SANDY SOIL CONDITIONS

[24]

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ABSTRACT

A pot experiment was conducted at controlled greenhouse of Bio-fertilizers production Unit; Soils, Water and Environment Research Institute, ARC, Giza through the winter growing season of 2006/2007 to evaluate the effects of symbiotic nitrogen fixing bacteria (*Rhizobium leguminosarum* bv. *viceae*), phosphate solubilizing bacteria (*Bacillus megaterium* var. *phosphaticum*) and VAM fungus (*Glomus* sp.) on nodulation, N_2-fixation, growth, N and P uptake of faba bean plants grown in phosphorus-deficient sandy soils. The obtained data resulted in significant increases in VA- Mycorrhizal colonization ratios when soil inoculated with rhizobia and VA-Mycorrhizae as compared with the other treatments. In addition, rock-P fertilized plants exhibited significant increases in VAM colonization ratios when compared with those supplemented with super-P. Concerning of dual inoculation with *Rhizobium* and *Bacillus*, the results indicated higher increases in VA- mycorrhizae colonization ratios as compared with the uninoculated or inoculated treatments with rhizobia alone, being most pronounced in the rock-P amended soil. The obtained results showed clearly that combined inoculation of faba bean with rhizobia and VA-Mycorrhizae in a soil amended with rock-P or super-P, gave the highest significant increases in nodulation, nitrogen fixation, plant biomass, N and P uptake when compared with the plants inoculated with *Rhizobium* alone. On the other hand, combined inoculation with *Rhizobium* and *Bacillus megaterium* did not record significant influence on the abovementioned parameters as compared to inoculation with *Rhizobium* alone.

INTRODUCTION

Significant areas of cultivated sandy soils in Egypt are deficient of available N, P and K. In modern agriculture, the replenishment of soil nitrogen involves most commonly extensive application of chemical fertilizers **(Peoples *et al* 1995)**, an approach which has several serious drawbacks, high cost and severe negative environmental impacts. These considerations stimulated research on other alternatives, such as biological nitrogen fixation which contributes more than 170 million tons of fixed nitrogen to the biosphere. Eighty percent of the stable biologically fixed nitrogen is a direct result of the rhizobiaceae and some actinomycetes symbioses with leguminous and certain non-leguminous plants, respectively **(Earl and Ausubel, 1993)**.

The dual inoculation with both rhizobia and VA mycorrhizae induced more significant increases in plant dry weight, N and P content of lentil and faba bean as well as seed yield of soybean than inoculation with either VA mycorrhizae or *Rhizobium* alone **(Badr El-Din and Moawad, 1988)**.

The use of plant growth promoting rhizobacteria (PGPR), including phosphate solubilizing bacteria as biofertilizers was suggested as a sustainable solution to improve plant nutrient and production **(Vessey, 2003)**. Direct application of rock phosphate may be agronomically more useful and environmentally more feasible than soluble P **(Rajan *et al* 1996)**.

Phosphorus is one of the major nutrients limiting plant growth. Most soils throughout the world

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are P deficient **(Batjes, 1997)** and therefore require P to replenish the P demand by crop plants. To circumvent the P deficiency in soils, P fertilizers could be applied. However, after application, a considerable amount of P is rapidly transformed into less available forms by forming a complex with Al or Fe in acid soils **(Norrish and Rosser, 1983)** or Ca in calcareous soils **(Lindsay *et al* 1989)** before plant roots have a chance to absorb it. Further, the use of rock phosphate as a phosphate fertilizer and its solubilization by microbes **(Kang *et al* 2002)**, through the production of organic acids **(Maliha *et al* 2004)**, have become a valid alternative to chemical fertilizers. Rock phosphate is widely distributed throughout the world, both geographically and geologically **(Zapata and Roy, 2004)**. In conjugation with phosphate solubilizing microorganisms (PSM), rock phosphate provides a cheaper source of P fertilizer for crop production, however most of them are not readily available to a plant. In this regard, several studies showed that PSM solubilizes the fixed soil P and applied phosphates, resulting in higher crop yields **(Zaidi, 1999 and Gull *et al* 2004)**. The alternative approach is to use these PSM along with other beneficial rhizospheric microflora to enhance crop productivity. In this context, the simultaneous application of *Rhizobium* and PS microorganisms **(Perveen *et al* 2002)** and arbuscular mycorrhizal VAM fungi **(Zaidi *et al* 2003)** has been shown to stimulate plant growth more than inoculation of each microorganism alone in certain situations when the soil is P deficient. On the other hand, VAM fungi encourage the plant roots to absorb rapidly the solubilized P. Accordingly, the increase in plant growth may be due to the release of certain plant growth promoting substances **(Kucey *et al* 1989)** by the PS organisms or VAM development and mycorrhizal formation **(Azcon-Aguilar and Barea, 1985)**. However, the inoculation effect of the tripartite interaction between N_2- fixing bacteria, PSM and VAM fungus on legume crops are relatively scarce **(Zaidi *et al* 2004)**.

Because the minerals are released slowly and their use as fertilizers often causes insignificant yield increases of current crop **(Zapata and Roy, 2004)**. Phosphate solubilizing bacteria (PSB) have been used to improve rock P value because they convert insoluble rock P into soluble forms available for plant growth **(Bojinova *et al* 1997)**. This conversion is through acidification, chelation and exchange reactions **(Gerke, 1992)** and produces strong organic acids, which have become indicators for routine isolation and selection procedures of PSB **(Illmer *et al* 1995)**. *Bacillus megaterium* var. *phosphaticum* is known for its ability to solubilize rock P material **(Schilling *et al* 1998)**.

The aim of the present study was to evaluate the response of faba bean to *Rhizobium* inoculation combined with VA-mycorrhizae and phosphate solublizing bacteria under sandy soil conditions.

MATERIALS AND METHODS

Microbial cultures

Rhizobium leguminosarum bv. *viceae*, strain ICARDA 441; *Bacillus megaterium* var. *phosphaticum* (local isolate) and VA- mycorrhizal fungus (local isolate of *Glomus* spp.) were supplied by Biofertilizer Production Unit; Soils, Water and Environment Research Institute; ARC; Giza; Egypt.

Preparation of microbial inoculants

Rhizobium strain was grown on yeast extract mannitol broth medium (Vincent, 1970) at 28 ºC under shaking at 250 rpm for 3 days until early log phase ($5X10^9$ cfu/ml culture). Vermiculite mixed with 10% peat was packed in polyethylene bags (180 g carrier per bag), then sealed and sterilized by gamma irradiation ($5.0 X 10^6$ rads). *Rhizobium* culture was injected into the carrier to satisfy 60% of the maximal water holding capacity.

Bacillus megaterium was grown on nutrient broth medium under shaking at 250 rpm for 48 hrs at 28 ºC. Viable bacterial cells ($4X10^8$ cfu/ml culture) was injected in the abovementioned carrier materials used in *Rhizobium* inoculants.

Viscular Arbuscular mycorrhizal (VAM) fungi spores were propagated for 16 weeks on *Sorghum bicolor* in sterilized pot cultures prior to the start of the experiment. Soil from 16 week-old pot culture was used as a crude inoculum consisted of soil, mycorrhizal spores and infected roots.

Pot experiment

Pot experiment was conducted at the controlled greenhouse of Biological Nitrogen Fixation Unit; Soils, Water and Environment Research Institute, Agricultural Research Center, Giza through the winter growing season of 2006/2007 to study the Effect of *Rhizobium* inoculation combined with VM-mycorrhizae and *phosphate* dissolving bacteria on growth, nodulation, nitrogen fixation and phosphorus uptake in faba bean plants under sandy soils.

The soil used in pot experiment was collected from Ismailia Research Station. The main physico-chemical characteristics of the soil were determined according to **Jackson (1973)** and **Page *et al* (1982)**, **(Table 1).**

Table 1. Some physical and chemical properties of the used soil

Physical properties		Chemical properties	
Sand	90.20%	pH	7.30
Silt	5.68%	E.C. (dS m^{-1})	0.35
Clay	4.12%	Soluble cations (meq l^{-1})	
Textural class	Sandy	Ca^{++}	0.57
$CaCO_3$	0.57%	Mg^{++}	0.48
SP	19.65%	Na^+	1.27
		K^+	1.10
		Soluble anions (meq l^{-1})	
		CO_3^{--}	0.00
		HCO_3^-	1.09
		Cl^-	0.76
		SO_4^{--}	1.58
		Total Solble N ppm	25.3
		Available P ppm	8.2

Plastic pots (30 cm diameter) were filled with 10 kg of the soil. All pots received the recommended dose of potassium sulphate (48.5% K_2O) at the rate of 100 kg fed^{-1} applied at two equal doses (10 and 20 days of planting). The pots were divided into two main plots. The first main group was fertilized with super-phosphate (15.5% P_2O_5) at a rate of 200 kg fertilizer/fed. The second group was fertilized with rock phosphate (27% P_2O_5) at a rate of 115 kg fertilizer/fed. All treatments received ammonium sulphate (20.5%N) at a rate of 20 kg N fed^{-1} as a starter dose of nitrogen. The nitrogen control treatment fertilized with 40 kg N fed^{-1}. The N-fertilizer applied at two equal doses (10 and 20 days of planting). Pots were arranged in a complete randomized design with four replicates.

Faba bean seeds variety Giza 40 were supplied from Food Legume Research Department, Field Crops Research Institute, ARC and inoculated with vermiculite-based inoculants containing *Rhizobium* or *Bacillus megaterium*. Each inoculant was applied at the rate of two bags per seeds of feddan using 16% Arabic gum solution as a sticking agent.

Mycorrhizal inoculation of experimental pots were achieved by placing 75g of the crude inoculum, 2-4 cm below the soil surface before planting. The used inoculum contained approximately 25 viable propagules per gram of mycorrhizal inoculum, which were estimated using the Most Probable Number (MPN) technique **(Daniels and Skipper, 1982)**.

The following treatments were applied:

1- Uninoculated + superphosphate
2- Uninoculated (N-control) + superphosphate
3- *Rhizobium* + superphosphate
4- *Rhizobium* + Mycorrhizae + superphosphate
5- *Rhizobium* + *Bacillus megaterium* + superphosphate
6- Uninoculated + rock phosphate
7- Uninoculated (N-control) + rock phosphate
8- *Rhizobium* + rock phosphate
9- *Rhizobium* + Mycorrhizae + rock phosphate
10- *Rhizobium* + *Bacillus megaterium* + rock phosphate

After 60 days of planting, plants were uprooted and assayed for number and dry weight of nodules, nitrogenase activity, shoots and roots dry weight. Nitrogen and phosphorus uptake by faba bean plants were determined according to **Page *et al* (1982)**.

Mycorrhizal colonization ratios

Representative samples of fresh roots were stained after washing several times according to the procedures of **(Phillips and Hayman, 1970)**. Mycorrhizal colonization ratios of plant roots were determined using the gridline intersect method after staining the root samples **(Giovannetti and Mosse, 1980)**. Data were subjected to analysis of variance according to **Snedecor and Cochran (1980)**.

RESULTS AND DISCUSSION

Data in **Table (2)** clearly showed that the used soil in this study contained already indigenous Viscular Arbuscular mycorrhizal (VAM) fungi populations. However, inoculation of soil with rhizobia and mycorrhizae (*Glomus* sp.) supported higher levels of VAM colonization ratios as compared with the other treatments. Both super-P and rock-P amended soil were more pronounced with super-P amended soil. In this respect, **Aryal *et al* (2003)** and **Jalaluddin (2005)** reported that, dual inoculation of legumes with both rhizobia and VAM fungi maintained high levels of mycorrhizal colonization over than those achieved with rhizobia alone.

In the uninoculated treatments, rock-P fertilized plants exhibited significant increases in VAM colonization ratios when compared with those supplemented with Super-P, irrespective of the nitrogen

Table 2. Arbuscular-mycorrhizal colonization ratios in faba bean plant roots after 60 days of planting

Treatments	Phosphate-Source		Mean
	Super-P	Rock-P	
Uninoculated	16.24	30.30	23.27
Uninoculated (N-control)	20.50	30.45	25.48
Rhizobium	26.41	32.61	29.51
Rhizobium + AMF*	60.63	46.00	53.32
Rhizobium + BM**	37.52	48.33	42.92
Mean	32.26	37.54	
LSD 0.05 Inoc.			5.89
P-source	3.73		
Inoc. X P-source		8.34	

* Arbuscular Mycorrhizal Fungus

** *Bacillus megaterium* var. *phosphaticum*

dose added. These results are in harmony with those obtained by **Nikolaou *et al* (2002)** and **Covacevich *et al* (2006)**, who concluded that, the percent of mycorrhizal root colonization was higher in insoluble P-form treatments compared to control or to soluble P-form ones.

Irrespective of superiority of rock-P to support VAM colonization ratios in all treatments except in the case of (rhizobia + VAM) treatment which exhibited opposite trend, being 60.63 and 46.00 % for super-P and rock-P, respectively. This could be due to the high stimulation effect of rhizobia and mycorrhizal inoculation which may lead to more activation for N_2-fixation that requires more phosphorus to support plant growth. An enhancement of the N_2 fixation rates in *Rhizobium*-inoculated VAM-plants, over that achieved by *Rhizobium* in nonmycorrhizal plants was previously reported.

Concerning of dual inoculation with *Rhizobium* and *Bacillus*, the obtained data showed higher increases in mycorrhizal colonization ratios as compared with noninoculated or only rhizobial inoculated treatments, being most pronounced in the rock-P amended soil.

Data presented in **Table (3)** presented that N_2 –ase activity of root nodules of faba bean inoculated with *Rhizobium* alone or combined with A-mycorrhizae or phosphate solubilizing bacteria gave the highest significant increases when compared to the uninoculated plants. Under rock phosphate treatment, inoculation with *Rhizobium*, *Rhizobium* plus VA-mycorrhizae and *Rhizobium* plus *Bacillus megaterium* var. *phosphaticum* stimulated N_2-ase activity of root nodules to be 32.00, 33.55 and 10.93 µ mole C_2H_4 /plant/hr compared to 11.91, 22.45 and 6.91 µmole C_2H_4/plant/hr under superphosphate fertilizer, respectively. On the contrary, the uninoculated plants gave the lowest nitrogenase activity of 0.26 and 1.06 µ mole C_2H_4/plant/hr under both super phosphate and rock phosphate, respectively. On the other hand, combined inoculation of faba bean with Rhizobium and phosphate solubilizing bacteria did not gave any significant effect on N_2-activity of faba bean plants compared to those obtained either by *Rhizobium* or inoculation alone or in combination with VA-mycorrhizae. The increased nodulation and N_2-fixation of legume crops following inoculation with N_2-fixing bacteria and phosphate solubilizing microorganizms have been reported by many workers **(Algawadi and Guar, 1988; Gupta, 2004)**.

The obtained results in **Table (4)** showed clearly that combined inoculation of faba bean with *Rhizobium* and VA-mycorrhizae gave the highest significant increases in number and dry weight of nodules when compared with the plants inoculated with *Rhizobium* alone. These increases in number and dry weight of nodules reached to 32.6 and 37.1%, respectively under superphosphate fertilizer, compared to 30% and 27% under rock phosphate treatment. These results are in harmony with those obtained by **Aryal *et al* (2003)** they found that *Rhizobium* inoculation influenced positively nodulation of bean plants. On the other hand, combined inoculation of faba bean with *Rhizobium* and phosphate solubilizing bacteria did not record any significant effect on nodulation of faba bean plants compared to those obtained by *Rhizobium* inoculation alone.

Table 3. Effect of inoculation with rhizobia, VA-mycorrhizae and phosphate solubilizing bacteria on N_2-ase activity of faba bean plants (μ mole C_2H_4 /plant/hr)

Treatments	Phosphate-Source		Mean
	Super-P	Rock-P	
Uninoculated	1.06 ±0.15	0.26 ±0.13	0.66
Uninoculated (N-control)	0.88 ±0.10	1.91 ±0.87	1.40
Rhizobium	11.91 ±0.74	32.00 ±4.30	21.96
Rhizobium + AMF	22.45 ±3.07	33.55 ±2.90	28.00
Rhizobium + BM	6.91 ± 1.38	10.93 ±1.73	8.92
Mean	8.64	15.77	

Table 4. Effect of inoculation with rhizobia, VA-mycorrhizae and phosphate solubilizing bacteria on nodulation of faba bean plants

Treatments	Nodules per plant					
	Number			Dry weight (mg)		
	Super-P	Rock-P	Mean	Super-P	Rock-P	Mean
Uninoculated	5.0	2.7	3.8	14.67	4.67	9.67
Uninoculated (N-control)	3.7	3.0	3.3	13.33	6.00	9.67
Rhizobium	38.7	33.3	36.0	35.00	31.67	33.33
Rhizobium + AMF	51.3	43.3	47.3	48.33	38.67	43.50
Rhizobium + BM	29.3	35.0	32.2	34.67	30.00	32.33
Mean	25.6	23.5		29.20	22.20	
LSD 0.05 Inoc.			4.56			9.05
P-source	2.89			5.72		
Inoc. X P-source		6.45			12.79	

Data in **Table (5)** reveceled that faba bean plants inoculated with *Rhizobium* alone or combined with VA-mycorrhizae recorded the highest shoot dry weight as compared to the uninoculated plants. Combined inoculation of faba bean with *Rhizobiun* and VA-mycorrhizae gave increases of 24.2% and 15% in shoots dry weight over the uninoculated ones under the application of superphosphate and rock phosphate fertilizers, respectively. With respect to dry weight of roots, *Rhizobium* inoculation alone or combined with VA-mycorrhizae increased significantly dry weight of roots when compared to the uninoculated plants.

Combined inoculation with *Rhizobium* and A-mycorrhizae under rock phosphate treatment increased significantly upto 14.9% over those obtained in plants fertilized with superphosphate. These results are in agreement with those obtained by **Badr El-Din and Moawad (1988)** in which the dual inoculation with both rhizobia and Mycorrhizae induced more significant increases in plant dry weight of lentil and faba bean. On the other hand, there were no significant differences in shoot or root dry weight between combined inoculation with *Rhizobium* and *Bacillus megaterium* var. *phosphaticum* compared to *Rhizobium* inoculation alone.

Combined inoculation **(Table 6)** with rhizobia and mycorrhizae significantly increased N-uptake in shoots up to 9.0 and 17.2% over rhizobial inoculation alone under super-P and rock-P, respectively. In addition, rhizobial inoculation increased significantly N-uptake in roots up to 62.7 and 77.5% over N-control treatments under super-P and rock-P, respectively. On the other hand, dual inoculation with rhizobia and phosphate solibilizing

Table 5. Effect of inoculation with rhizobia, VA-mycorrhizae and phosphate solubilizing bacteria on biomass of faba bean plants

Treatments	Dry wt. (g/plant)					
	Shoot			Root		
	Super-P	Rock-P	Mean	Super-P	Rock-P	Mean
Uninoculated	6.28de	5.93e	6.10	2.85	3.29	3.07
Uninoculated (N-control)	7.32ab	6.83bc	7.07	4.02	4.40	4.21
Rhizobium	7.80a	6.77cd	7.28	4.41	4.89	4.65
Rhizobium + AMF	7.62a	6.82bc	7.22	4.62	5.31	4.97
Rhizobium + BM	6.89bc	6.22e	6.55	3.39	4.19	3.79
Mean	7.18	6.52		3.86	4.42	
LSD 0.05 Inoc.			0.46			0.39
P-source	0.29			0.25		
Inoc. X P-source	0.64			0.55		

Table 6. Effect of inoculation with rhizobia, VA-mycorrhizae and phosphate solubilizing bacteria on nitrogen content of faba bean plants

Treatments	Shoot			Root		
	Super-P	Rock-P	Mean	Super-P	Rock-P	Mean
	Nitrogen content (mg/plant)					
Uninoculated	187.4	162.3	174.8	29.4	30.9	30.2
Uninoculated (N-control)	240.7	236.1	238.4	50.9	55.9	53.4
Rhizobium	268.2	245.8	257.0	82.8	99.2	91.0
Rhizobium + AMF	292.4	288.0	275.2	71.8	91.5	81.6
Rhizobium + BM	208.2	199.5	203.9	45.9	88.8	67.3
Mean	239.4	220.3		56.15	73.26	
LSD 0.05 Inoc.			22.9			9.93
P-source	14.50			6.27		
Inoc. X P-source	32.44			14.03		

bacteria had significant effect on N-uptake in both shoots and roots when compared to uninoculated treatments, received the same dose of N-fertilizer. With respect to P-uptake in faba bean plants **(Table 7)**, combined inoculation with rhizobia and mycorrhizae increased significantly P-uptake in both shoots and roots of faba bean plants under the two sources of phosphorus, when compared to rhizobial inoculation alone. These increases reached to 37.5 and 31.3% in shoots and roots, under super-P, respectively. Under rock-P these increases reached to 38.0 and 47.8% in the same order. These results are in agreement with those obtained by **Aryal *et al* (2003)** who indicated that VAM infection showed a significant positive correlation to the shoot phosphate or nitrogen in bean plants.

Also, **Badr El-Din and Moawad (1988)** found that the dual inoculation with both rhizobia and mycorrhizae induced more significant increases in N and P contents of lentil and faba bean. On contrary, combined inoculation with rhizobia and phosphate solubilizing bacteria did not record any significant effect on P-uptake in faba bean plants when compared to the other inoculated treatments.

Table 7. Effect of inoculation with rhizobia, VA-mycorrhizae and phosphate solubilizing bacteria on phosphorus content of faba bean plants

Treatments	Shoot			Root		
	Super-P	Rock-P	Mean	Super-P	Rock-P	Mean
	Phosphorus content (mg/plant)					
Uninoculated	9.64	9.05	9.3	1.30	1.21	1.3
Uninoculated (N-control)	37.96	33.40	35.7	2.75	2.75	2.8
Rhizobium	48.80	50.20	49.5	3.20	2.91	3.1
Rhizobium + AMF	67.10	69.3	68.3	4.20	4.30	4.2
Rhizobium + BM	35.51	30.14	32.8	2.11	2.00	2.0
Mean	39.8	38.4		2.7	2.6	
LSD 0.05 Inoc.			4.34			0.45
P-source	2.74			0.29		
Inoc. X P-source	6.14			0.64		

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