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Effectiveness of inoculation with arbuscular mycorrhizai fungi and phosphate-solubilizing bacteria on growth and nutrition of soybean in calcareous soil amended with rock phosphate

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Abstract

This research aimed at evaluation of the effects of inoculation with arbuscular mycorrhizal fungi (*Glomus intraradiaces* and *G. fasciculatum*), rhizobacteria (P-solubilizing *Bacillus megaterium* and N_2-fixing *Bradyrhizobium japonicum*)-and their interactions on the growth and nutrient content of soybean plants in calcareous soil. The investigation was conducted in potted –soil under greenhouse conditions. Soybean growth was significantly improved by mycorrhizal and/or rhizobacterial inoculation but dual inoculation with both was far more effective. *Bradyrhizobium* inoculation significantly improeved number and dry weight of nodules, especially in presence of mycorrhizal fungi. The concentrations of macronutrients (N, P and K) and micronutrients (Fe, Cu, Mn and Zn)-in soybean shoots increased due to inoculation with any of the inoculants used but N content was lower in treatments inoculated with mycorrhizae in presence of rhizobacteria in comparison to those receiving mycorrhizae alone. In general, dual inoculation with *G. intraradiaces* and P-solubilizing - *Bacillus megaterium* resulted in the best stimulatory effect on soybean growth and nutrient content in calcareous soil.

Key-Words: Soybean, AM fungi, Rhizobacteria, *Bradyrhizobium japonicum*

Introduction

Microorganisms are involved in a range of processes that affect the transformation of soil P and are thus an integral part of the soil P cycle. In particular, soil microorganisms are effective in releasing P from inorganic and organic pools of total soil P through solubilization and mineralization (Hilda and Fraga, 1999). Recently, phosphate solubilizing microorganisms have attracted the attention of agriculturists as soil inoculums to improve the plant growth and yield (Goldstein et al., 1999; Fasim et al., 2002). Plant growth promoting bacteria (PGPB) are soil and rhizosphere bacteria that can benefit plant growth by different mechanisms (Glick, 1995), increasing costs, the use of PGPB is advantageous in the sustainable agricultural practices.

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and P-solubilization ability of the microorganisms is considered to be one of the most important traits associated with plant P nutrition. Given the negative environmental impacts of chemical fertilizers and their It is generally accepted that the mechanism of mineral phosphate solubilization by PSB strains is associated with the release of low molecular weight organic acids such as oxalic, citric, butyric, malonic, lactic, succinic, malic, acetic,.. etc (Goldstein, 1995; Kim et al., 1997a), which through their hydroxyl and carboxyl groups chelate the cations bound to phosphate, thereby converting it into soluble forms (Kpomblekou and Tabatabai, 1994). However, P-solubilization is a complex phenomenon, which depends on many factors such as nutritional, physiological and growth conditions of the culture (Reyes et al., 1999).

Mycorrhizal symbioses are known to play a critical role in plant nutrition, based on the ability of the external mycorrhizal mycelium developing around the host plant roots to efficiently explore a larger volume of soil, thereby enhancing mineral acquisition by the plant (Smith and Read, 1997).

Dual inoculation of AMF and PSB stimulates plant growth better than inoculation with either microorganism alone (Azcon et al. 1976). Increased uptakes of P and plant growth due to these organisms have been demonstrated by several researchers with several different plant species (Piccini and Azcon 1987).

One of the most important leguminous crops is soybean. its production is rapidly expanded as a result of the high demand for the seeds, that serve as a major and excellent source of oil and protein for human and livestock consumption .it is well known that legumes have higher requirements of phosphorus nutrition for growth and effective nodulation and consequently N_2-fixation.

Phosphorus, a major plant nutrient is required for various metabolic processes such as cell division and development, energy transport, signal transduction, macro-molecular biosynthesis, photosynthesis, and respiration (Khan et al. 2009b; Ahemad et al. 2009; Shenoy and Kalagudi 2005). However, a large portion of soluble inorganic phosphate applied to the soil as chemical fertilizer is immobilized rapidly and becomes unavailable to plants (Goldstein, 1986).

The aim of this research was to evaluate the interactive effects of arbuscular mycorrhizai fungi (AMF) and phosphate solubilizing microorganisms on the growth and nutrient absorption of soybean in calcareous soil**.**

Material and Methods

Soil

Surface calcareous soil sample (0-15 cm) was collected from EI-Banger, Village 27 Alexandria Governorate., the soil was sterilized through fumigation using methyl bromide. The soil sample was air- dried, ground to pass into 2mm sieve and thoroughly mixed. Some characteristics of the experimental soil are presented in Table (1).Particle size distribution was estimated according to Jackson (1973).while, chemical analysis was determined according to Black et al (1982).

Table 1: Some characteristics of the experimental soil

Parameters	Values	Parameters	Values
Mechanical analysis (%)		**Soluble ions meq L^{-1}**	
Clay	26.38	Calcium	**12.4**
Silt	24.40	Magnesium	**5.70**
Sand	49.22	Sodium	**2.29**
Soil texture		Potassium	**0.60**
Organic matter (%)	1.72	Carbonate	**0.95**
P^H (1:2.5 suspension)		Bicarbonate	**5.7**
Total-N (%)	0.21	Chloride	**3.91**
Total-P (%)	0.17	Sulphate	**8.67**
Available phosphours	3.5 mg kg^{-1}		
Total-K (%)	0.38	**Microelements mg kg^{-1}**	
$CaCO_3$ (%)	17	Available Fe	**14.39**
EC (dSm^{-1})	1.90	Available Zn	**2.64**
		Available Mn	**3.21**
		Available Cu	**2.95**

Microorganisms

Mycorrhizal inocula (AMF) species belonging to the genus *Glomus: Glomus intraradiaces* (AMF1) and *Glomus fasciculatum*(AMF2) (Soil Goettingen strain) were provided from Tropical Institute, Goettingen University, Federal R.Germany*;* phosphate-solubilizing bacteria, (PSB) *Bacillus megatherium* **;**and N2-fixing bacteria, (NFB) *Bradyrhizobium japonicum*-Soybean were used. The second and third microbial cultures were obtained from the Unit of Biofertilization, Fac. Agric., Ain Shams Univ., Cairo.

For preparation of *Glomus intraradiaces* and *Glomus fasciculatum* inocula, pots of 30cm diameter were filled with autoclaved sandy soil.

The soil of each pot was inoculated with every AMF1 and AMF2 alone.

Five onion seedlings were transplanted in each pot as a host plant. After 12 weeks, spores of each AMF were collected from the rhizosphere and roots of onion were extracted by wet sieving and decanting technique (Gerdmann and Nicolson,1963).AMF spores were counted by the method desribed by Daniels and Skipper (1982).For preparation of *Bradyrhizobium* inoculum,yeast mannitol broth medium (Vincent,1970) was inoculated with the effective strain (*Bradyrhizobium japonicum*),then incubated at 32C° for 7 days.

Pot experiment

Pot experiment using Completely Randomized Design with four replications and 9 treatments as follows: (1) control, without inoculation, (2) inoculated with *Glomus intraradiaces,* (3) *Glomus fasciculatum* (4) *Bradyrhizobium japonicum*, (5) *Bacillus megatherium*, (6) *G. intraradiaces* +(NFB),(7) *G. intraradiaces* +(PSB), (8) *Glomus fasciculatum* +(PSB) (9) *Glomus fasciculatum* +(NFB).

Plastic pots were filled with 10 kg soil with holes in their bottom.

Seeds of Soybean (*Glycine max* .L) were kindly provided by the Dept. of Legume Research, ARC, Egypt. They was successively washed and then surface sterilized by $HgCl_2$

(0.1%) for 30 min .Surface sterilized seeds were washed 10 times with sterile water. They were then germinated on moist filter paper at 28 C° until the radicles appeared for 2 days, and then removed two plants into each pot experiment. The extracted mycorrhizal spore suspension containing about 120-150 spores/ml was used as a standard inoculum (20ml/pot) for mycorrhizal treatments. Five ml of phosphate-solubilizing bacterial inoculum suspension was added per pot after three days of plant growth. Soybean seeds were soaked in cell suspension of *Bradyrhizobium japonicum* (1ml contains about 6.4×10^7 viable cells) for 30 min.Gum Arabic (16%) was added as an adhesive agent perior to inoculation.

All pots were irrigated with tap water every three days to keep the soil at 70% of its field capacity by regular weighing of pots.

The experiment was conducted in a greenhouse maintained at 20-22C° at daytime and 12-17C° at nighttime and 16/8 h light/dark photoperiod. The

relative humidity was 70-80%. There were four replicates per treatment.

The soil of each pot was fertilized with (30 mg N kg^{-1} soil in the form of NH_4NO_3), (60 mgK kg^{-1} soil in the form of K_2SO_4). Phosphate fertilizer was applied as rock phosphate at the rate of 150 mg P kg^{-1} soil in the form of Hydroxyapatite **(**Powdered rock phosphate).

Plant height was observed every 10 days. The plant was harvested at 60 days after planting for determining of the dry weight of shoots and roots. For nutrient analysis, plant material was ground to pass through a 0.5-mm screen and digested in a H_2SO_4–H_2O_2 mixture according to Lowther (1980).Data of nodules number, nodules dry weight/plant, N_2-ase activity of nodules were estimated at vegetative stage at 60 day after cultivation. N_2-ase activity was estimated according to Hardy et al (1973).

Total nitrogen, phosphorus and potassium content were determined in soybean shoots at 60 days after planting according to A.O.A.C (1980),(A.P.H.A,1992) and Dewis &Freitas (1970),respectively.Also ferrum, copper, zinc and manganese determined by atomic absorption spectroscopy (A Analyst 400). Roots were subsampled in three 2-cm cross-sections of the upper, middle, and lower root system. To assess colonisation, roots were cleared with 10% KOH and stained with 0.05% trypan blue (Phillips and Hayman,1970). The gridlines intersect method of Giovannetti and Mosse (1980) was used to estimate the mycorrhizae infection percentages using the following equation:

$$\text{AMF infection \%} = \frac{\text{Number of segments containing AMF}}{\text{Total number of examined segments}} \times 100$$

Statistical analysis

Data were subjected to analysis of variance using the ANOVA procedures according Snedecor and Cochran (1972) Statistical significance was determined at $P < 0.05$.

Results and Discussion

Effect of dual inoculation and their interaction on plant growth, nodulation, N_2-ase activity and mycorrhizal root infection of soybean plants

The growth of soybean was significantly improved by AMF or/and rhizobacteria (N-fixing and P-solubilizing) inoculation (Table 2).

However, the inoculation of AMF improved soybean growth better than inoculation of rhizobacteria. Dual inoculation AMF and rhizobacteria resulted in significantly higher total plant dry weight than these microorganisms were used alone (single inoculation). Dual inoculation with the AMF (*Glomus intraradiaces*) and PSB resulted in the highest plant growth response. This dual inoculation was able to increase the total dry weight 201 times compared to the uninoculated treatment. It is a surprising result that all of rhizobacteria used in this study were significantly higher when inoculated together with *G. intraradiaces* than when used alone. Dual inoculation of PSB, and *G.intraradiaces* improved the total dry weight of soybean 115%, while NFB improved 63% higher than single inoculation with *G. intraradiaces* alone. It is clear from data presented in Table (2) that the nodules number and dry weight were remarkably increased in bradyrhizobal inoculated treatments compared to uninoculated ones.

Table 2: Effect of inoculation with (AMF) and (PSB) and their interaction on nodulation, N_2-ase activity, Plant growth and AMF root infection of soybean plants after 60 day of cultivation

Treatments	AMF infection%	(g/plant)			Plant height (cm)	No. of nodules/ plant	Dry weight of nodules(mg/plant)	N_2-ase activity(n moles C_2H_4/hr/g dry nodules
		Roots Dry weigth	Shoots Dry weigth	Total dry weight				
Uninoculated	0	0.48f	1.12ef	1.60	10.30	8	96	**19.3**
***G.intraradiaces*(AMF1)**	49	0.93de	1.34 d	2.27	61.20	15	138	**32.6**
***G. fasciculatum*(AMF2)**	43	0.82cd	1.47 d	2.29	47.35	11	125	**15.0**
***B.. japonicum*(NFB)**	0	0.74 c	1.33 d	2.07	17.65	20	211	**63.0**

***B.megatherium* (PSB)**	0	0.65 c	1.27e d	1.92	25.69	7	59	**13.2**
***G.intraradiaces*+(NFB)**	71	1.19 b	2.46 b	3.65	73.16	41	475	**97.8**
***G.intraradiaces*+(PSB)**	63	1.71 a	3.10 a	4.81	77.50	18	201	**39.0**
***G. fasciculatum*+(NFB)**	67	1.21 b	2.24 c	3.45	59.21	33	312	**86**
***G. fasciculatum*+(PSB)**	57	1.10 b	2.96 a	4.06	62.30	16	164	**30**
LSD $_{0.05}$		**0.13**	**0. 19**					

Number and dry weight of nodules in bradyrhizobal inoculation treatments were greater than mycorrhizal inoculation. The highest number and dry weight of nodules were observed with dual inoculation especially (both AMF and NFB).In AMF treatment, inoculation of rhizobacteria also increased the colonization of AMF.these results showed that both AMF and rhizobacteria have significant role on plant growth promotion, and their roles were higher when applied together. seemly, all rhizobacteria used in this behaved as mycorrhizal helper bacteria that promote the colonization of AMF.These results are in agreement with reported previously(Toro et al .,1997) who found that the PSB behaved as mycorrhizal -helper bacteria,which improve the colonization of introduced AMF (Toro et al., 1997). However, the
mechanisms by which these bacteria stimulated AMF colonization are still poorly understood (Toro et al., 1997). The production of plant growth promoting substances by rhizobacteria, such as vitamins, amino acids, and hormones may be involved in this interaction (Barea et al., 1997).

It is not a surprising result that, N_2-ase activity was higher in case of bradyrhizobal inoculated treatments than mycorrhizal inoculated ones. This result is in harmony with those obtained by Mikhaeel *et al* (2000) who found increases of soybean nodulation and N_2-ase activity due to rhizobal inoculation .the highest records of N_2-ase activity was observed in case of dual inoculation compared to inoculation with either *Bradyrhizobium* or AMF.

Effect of inoculation and their interaction on concentration of macro, micro nutrients in soybean shoots. Data in Table (3) show that concentration of nitrogen, phosphorus and potassium in shoots of soybean plants were increased in the treatments inoculated with either *Bradyrhizobium* , *B. megatherium* or mycorrhizae compared to uninoculated treatments.

Table 3: Effect of inoculation with (AMF) and (PSB) and their interaction on concentration of macro-nutrients in soybean shoots

Treatments	Nitrogen (%)	Phosphorus (%)	Potassium(%)
Uninoculated	1.2 d	0.01cd	1.04d
***G.intraradiaces*(AMF1)**	3.26a	0.40a	1.80bc
***G. fasciculatum*(AMF2)**	3.12ab	0.29b	1.59c
***B.. japonicum*(NFB)**	3.51a	0.18bc	1.72c
***B.megatherium* (PSB)**	2.89b	0.21b	2.10b
***G.intraradiaces*+(NFB)**	2.11c	0.12c	2.42a
***G.intraradiaces*+(PSB)**	1.98c	0.21b	2.16b
***G. fasciculatum*+(NFB)**	2.13c	0.10c	1.98b
***G. fasciculatum*+(PSB)**	1.87c	0.19bc	2.10b
LSD $_{0.05}$	0.27	0.09	0.23

The concentration of N in soybean shoots was significantly improved by inoculation, of AMF and NFB, *B. japonicum*. However, the concentration of N in soybean shoots was lower when inoculated by AMF and rhizobacteria (dual inoculation) than when inoculated by AMF only.
These results are in harmony with those obtained by Abd El-Fattah (2001) who found that bradyrhizobal inoculation increased total nitrogen in plant shoots in comparison with uninoculated plants.
The concentration of P in soybean shoots was improved by inoculation AMF but not by inoculation of rhizobacteria.However, dual inoculation of PSB and AMF were significantly increased the concentration of P in soybean shoots.
Data presented in Table (4) show that concentration of micronutrients (iron ,copper and Manganese) of soybean

shoots were higher in mycorrhizal inoculated treatments than rhizobacteria(PSB and NFB) inoculated ones.whereas,zinc concentration of soybean shoots was higher in NFB inoculated treatments than mycorrhizal ones, the same trend was observed in the their interaction especially between (AMF and NFB)

Table 4: Effect of inoculation with (AMF) and (PSB) and their interaction on concentration of micro-nutrients in soybean shoots

Treatments	Iron (ppm)	Zinc (ppm)	Copper(ppm)	Manganese(ppm)
Uninoculated	40	18	5	22
***G.intraradiaces*(AMF1)**	67	30	20	16
***G. fasciculatum*(AMF2)**	54	23	16	13
***B.. japonicum*(NFB)**	39	54	10	15
***B.megatherium* (PSB)**	62	39	7	17
***G.intraradiaces*+(NFB)**	74	58	45	32
***G.intraradiaces*+(PSB)**	69	40	15	20
***G. fasciculatum*+(NFB)**	76	52	42	29
***G. fasciculatum*+(PSB)**	65	43	12	22

This resulted in total nutrients taken up by plant increased (data not shown). Inoculated treatments by AMF and rhizobacteria did not have such a high nutrient concentration because of a dilution effect associated with growth (Table 2). In general, the dual inoculation of *G.intraradiaces* and PSB provided the best stimulation effect on plant growth in calcareous soil used in this study. It is possible that the available and transportation of P are main key for plant growth in this soil. The PSB released the fixed phosphorus and subsequently this released P was transported by the external mycorrhizal mycelium to the plant root system, thereby enhancing mineral P acquisition by the plant (Smith and Read, 1997).

Conclusion

In conclusion, it appears that the described interaction between AMF and rhizobacteria contributed to the plant growth promotion in calcareous soils due to the plant growth promotion and improvement of nutrient uptake. The potential of dual inoculation with AMF and rhizobacteria needs to be further evaluated under different crop and agroclimatic conditions, particularly in the field.

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