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Full Length Research Paper

Interactive effects of *Arbuscular mycorrhizal* fungi and rhizobial strains on chickpea growth and nutrient content in plant

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Legumes form a tripartite symbiosis with *Arbuscular mycorrhizal* fungi (AMF) and rhizobia. Chickpea plants were inoculated with six strains of *Mesorhizobium ciceri* and three AMF species, *Glomus intraradices* (GI), *G. mosseae* (GM) and *G. etunicatum* (GE). The plants inoculated with a number of AMF species and bacterial strains increased overall plant dry mass compared to non-inoculated plants. GE was the most efficient in increasing plant dry matter. Individual AMF species were more effective than when mixed (GI+GM+GE). Bacterial treatments had increasing effect on root colonization by GI, GM and GI+GM+GE. The results revealed that dual inoculation with AMF and rhizobium enhanced nitrogen, phosphorus, zinc, iron and copper content in plants but these increasing effects was different between fungal and bacterial treatments.

Key words: *Arbuscular mycorrhizal* fungi, *Mesorhizobium ciceri,* nutrient content, root colonization, nodule, chickpea.

INTRODUCTION

Most legumes possess two main types of root symbiosis with microorganisms, namely atmospheric N_2-fixing bacteria and mycorrhizal fungi, thus establishing a triple association capable of supplying plants, especially for N and P requirements (Silveira and Cardoso, 2004). *Arbuscular mycorrhizal* fungi (AMF) usually enhance nodulation and nitrogen fixation in legumes, but the extent of these effects depends on AMF species (Valdenegro et al., 2001). The increase in total N has been explained mainly by an increase in N_2-fixation as a result of a higher P uptake through the AM hyphae rather than increased soil N uptake (Mortimer et al., 2008). Although, there are many studies on the interactions between AMF and bacteria, the underlying mechanisms behind these associations are not yet well understood, and their functional properties still require further studies. The main objectives of this study were to investigate interactions between different strains of *Mesorhizobium ciceri* and AMF species on plant growth, nutrient content and AMF root colonization.

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Abbreviations: AMF, *Arbuscular mycorrhizal* fungi; **GI,** *Glomus intraradices*; **GM,** *G. mosseae*; **GE,** *G. etunicatum*; **DM,** dry mass; **NM,** non mycorrhizal; **Gmix,** mixed culture of fungi; **RC,** root colonization; **NFW,** nodule fresh weight; **NB,** non rhizobial.

MATERIALS AND METHODS

AM fungal and bacterial inocula

The AMF species used in this study were *Glomus intraradices* (GI) Schenck and Smith, *G. mosseae* (GM) (Nicol and Gerd) Gerdemann and Trappe, and *G. etunicatum* (GE) Becker and Gerdemann. These species have been isolated from the Tabriz Plain in North West of Iran (Aliasgharzadeh et al., 2001). The species were propagated in association with sorghum plants under standard pot culture conditions (Norris et al., 1992). The most

probable number (MPN) method was used to count AMF propagules in each inoculum using Feldmann and Idczak method (Norris et al., 1992). The MPN values for GI, GM and GE were 240, 160 and 480 propagules per cm^3, respectively. Six native strains of *M. ciceri* used in this study were obtained from culture collection of the Soil and Water Research Institute (SWRI) of Iran, which have been previously isolated from different areas of Iran.

Experimental design, plant material and growth conditions

Chickpea (C*icer arietinum* L. cv. ILC482) plants were inoculated with AMF species and *M. ciceri* strains in a factorial randomized block design with two factors (mycorrhizal and bacterial treatments) in three replicates. Mycorrhizal treatments consisted of GI, GM, GE, Gmix (GI+GM+GE) and non-mycorrhizal (NM). Fungal inoculants of GI, GM and GE were used at a rate of 50, 75 and 25 g inoculum per kg sand to achieve a density of 24000 propagules per pot and Gmix treatments were used at a ratio of 16.7, 25 and 8.3 g inoculum per kg sand of GI, GM and GE respectively. Control plants were un-inoculated. Bacterial treatments were as six individual strains (S1 to S6), mixture of six strains (Smix) and non-bacterial (NB). Bacterial suspension density was 10^8 cells/ml, and was applied at a rate of 10 ml/pot. A pre-germinated seedling of chickpea was planted into pots containing 2 kg sterile sand. The experiment was conducted in a glasshouse with average maximum day and night temperatures of 28 and 18°C, and average day length of 14 h, for 8 weeks. Pots were watered once every two days, and fertilized twice a week with a nutrient solution (Weaver and Fredrick, 1982) with half level of phosphorus and without nitrogen.

Plant harvest and nutrient analysis

Plants, harvested after 8 weeks, were cut at soil surface, then oven-dried at 65°C for 72 h. Nitrogen and phosphorus concentrations were determined by wet digestion method using H_2O_2, followed by Kjeldahl method for N and colorimetric method for P. Concentrations of Fe, Zn and Cu were determined using flame atomic absorption spectrometry (Chapman and Pratt, 1982). Then, nutrient content (NC) in plant was determined by this way: NC = nutrient concentration × dry mass (DM).

Determination of nodule fresh weight and AMF colonization

Roots were carefully removed from each pot and washed under tap water. Nodule fresh weight (NFW) was determined after nodules were detached from roots that were cut into small pieces (1 to 2 cm) and homogenized thoroughly. A subsample was fixed in fixing solution (1:1 [v/v] mixture of 99% ethanol and 60% acetic acid) and stored at 4°C pending subsequent microscopic analysis. Trypan blue dye was used to visualize the intraradical fungal structures as described by Giovannetti and Mosse (1980). A subsample of stained roots was used to quantify AMF root colonization (RC) as described by McGonigle et al. (1990). The colonization was expressed as total root length colonization percentage.

Statistical analysis

All data were statistically analyzed based on a factorial randomized block design using SPSS. When the individual and interaction effects of bacteria and fungus were statistically significant therefore, data were analyzed using two-way ANOVA for bacterial treatments in each fungal species. The means comparison were analyzed by LSD range test in each level that the treatments were significant ($p < 0.01$ and $p < 0.05$).

RESULTS

Plant growth

Bacterial and AMF treatments had significant effects (Table 1) on dry mass (DM) of chickpea. The results showed that inoculation with various AMF species increase DM compared to NM plants (Table 2). The DM increases were of 38.9% for GE, 31.1% for GM, 22.7% for GI and 21.3% for mixed culture of fungi (Gmix). Inoculation with GE produced more DM than with other AMF treatment and it had significant difference with GI and Gmix. Gmix led to lesser plant DM than the individual AMF species, although, not significantly different with GI and GM. The different bacterial strains increased the plant DM in comparison to non-bacterial control (Table 3). These growth increases were 64% for S5, 61.4% for S-mix, 57% for S6, 55.8% for S1, 49.4% for S3, 48% for S2, and 47% for S4, compared to the non-bacterial treatment.

Mycorrhizal colonization and root nodulation

AMF and bacterial treatments and their interactions had significant effects on root colonization (RC). The RCs in chickpea roots were influenced by *M. ciceri* strains. RC by GI significantly increased in the presence of *M. ciceri* S2 strain compared to the other strains (Table 5). RC by GM significantly increased by S6, S2 and S1 strains compared to Smix (Table 6). RC by GE was not affected by *M. ciceri* strains (Table 7). RC by Gmix significantly increased in the presence of S3, S2 and S6 strains compared to NB (Table 8). In most cases, S2 strain markedly increased AMF colonization in chickpea roots. It was indicated (Table 1) that bacterial strains have significant effect on NFW. Also, S1, S3, S4, S5 and Smix strains had higher NFW than S2, S6 strains and NB (Table 3).

Nitrogen and phosphorus content in plant

The AMF and bacterial treatments and their interaction were significant ($p < 0.01$) on N content in chickpea. In NM plants, S1, Smix, S5, S6 and S2 strains had increasing effect on nitrogen content compared to NB (Table 4). In plants inoculated with GI, GM and GE the N content of plant was higher in co-inoculation with Smix, S5 and S5, respectively. Moreover, plant inoculated with Gmix had more N content when they co-inoculated with Smix, S1 or S3. Both bacterial and fungal treatments had significant effects on phosphorus content by plants. GE was more effective than GI, Gmix and NM in P content by plant (Table 2). Also, when plants were inoculated with S5 strain, they had most phosphorus content than NB

Table 1. Effects of AM fungi (G) and rhizobial strains (S) on dry mass (DM), root colonization (RC), nodule fresh weight (NFW) and content of N, P, Zn, Fe and Cu in chickpea.

Parameter	df	Mean of square							
		RC	NFW	DM	Nutrient content				
					N	P	Zn	Fe	Cu
Block	2	406**	0.197$^{n.s}$	4.946**	2783.3**	12.0**	29842**	631130**	4079**
G	4	12850**	0.549$^{n.s}$	2.594**	2289.5**	14.2**	15862**	356689**	1581**
S	7	246**	10.15**	2.169**	944**	10.5**	6362**	17767**	497**
S × G	28	177**	0.367$^{n.s}$	0.179$^{n.s}$	114.2**	0.778$^{n.s}$	992*	41382**	290**
S × NM	7	0	_	_	126.1*	_	1345*	59352**	168*
S × GI	7	160*	_	_	316.9**	_	1183$^{n.s}$	44630**	245**
S × GM	7	235**	_	_	307**	_	3091**	85606**	360**
S × GE	7	102$^{n.s}$	_	_	332.7**	_	2556**	88410**	425**
S × Gmix	7	455**	_	_	318.1**	_	2157**	65199**	458**
Error	78	60.64	0.282	0.147	48.58	15.2	596.2	13318	75

Non-mycorrhizal (NM), *Glomus intraradices* (GI), *G.mosseae* (GM), *G. etunicatum* (GE) and mixture of *Glomus* GI+ GM+GE (Gmix); Significance according to ANOVA,*,** and n.s mean significant at 0.05, 0.01 probability level and non significant, respectively.

Table 2. Effects of chickpea inoculation with AM fungi (G) on dry mass (DM), nodule fresh weight (NFW) and P content in chickpea.

Significant level	**	n.s	**
Fungi	**DM (g/pot)**	**NFW (g/pot)**	**P Content (mg/pot)**
NM	2.25^{c}	1.60	4.87^{c}
GI	2.77^{b}	1.77	5.74^{b}
GM	2.96^{ab}	1.35	6.40^{ab}
GE	3.12^{a}	1.53	6.93^{a}
Gmix.	2.74^{b}	1.51	5.96^{b}
LSD value	0.29		0.69

Non-mycorrhizal (NM), *Glomus intraradices* (GI), *G.mosseae* (GM), *G. etunicatum* (GE) and mixture of *Glomus* GI+ GM+GE (Gmix); Significance according to ANOVA,*,** and n.s mean significant at 0.05, 0.01 probability level and non significant, respectively; Different letters within columns represent significant differences according to LSD's test.

(Table 3).

Micronutrient content in plant

AMF and bacterial treatments and their interactions were significant ($p < 0.01$) on Zn, Fe and Cu Content in chickpea. In NM plants, S2 and S5 strains led to the highest Zn, Fe and Cu content compared to the other strains and NB (Table 4). In plants inoculated with GI, the Fe and Cu content was higher in co-inoculation with S1 and S6 strains, respectively (Table 5). In plants inoculated with GM, the Zn, Fe and Cu content of plant were higher in co-inoculation with S6, S2 and S4 strains, respectively (Table 6). In GE plants, the Zn, Fe and Cu contents were higher in co-inoculation with S6 and Smix strains, respectively (Table 7). Moreover, plants inoculated with Gmix had more Zn, Fe and Cu content when co-inoculated with S6, Smix and S1 strains, respectively (Table 8).

DISCUSSION

Symbiosis is a biological phenomenon involving dynamic changes in the genome, metabolism and signaling network, and a multidirectional comprehension of these interactions is required when studying symbiotic organisms (Kawaguchi and Minamisawa, 2010). This study demonstrated that co-inoculation of AMF and rhizobial strains had significant effects on plant growth. It is well known that the increase in plant growth resulting from AM association is usually due to increased soil mineral content by extraradical hyphae (Makoi and Ndakidemi, 2009). Individual AMF species had more DM production than mixed species and non-mycorrhizal plants as well. So, it could be concluded that individual AMF inoculants have better performance than mixed inoculants on plant growth in this study. The results showed that inoculation with mycorrhizal fungi increases nitrogen and phosphorus content by plant. The highest amounts of N and P content were with GE and GM

Table 3. Effects of chickpea inoculation with rhizobial strains on dry mass (DM), nodule fresh weight (NFW) and P content in chickpea.

Bacteria	Significant level		
	**	**	**
	DM (g/pot)	NFW (g/pot)	P Uptake (mg/pot)
NB	1.87 [b]	0 c	4.06 [c]
S1	2.92 [a]	2.14 [a]	6.17[ab]
S2	2.77 [a]	0.79 [b]	6.03[ab]
S3	2.78 [a]	2.31 [a]	6.00[ab]
S4	2.75 [a]	1.83 [a]	5.78[b]
S5	3.07 [a]	2.01 [a]	6.68 [a]
S6	2.94 [a]	1.21 [b]	6.51[ab]
Smix	3.02 [a]	2.17 [a]	6.58[ab]
LSD Value	0.37	0.51	0.88

Non-rhizobial (NB), *Mesorhizobium ciceri* strains (S1– S6) and mixture of S1– S6 strains (Smix); significance according to ANOVA,*,** and n.s mean significant at 0.05, 0.01 probability level and non significant, respectively; different letters within columns represent significant differences according to LSD's test.

Table 4. Effects of chickpea inoculation with rhizobial strains on root colonization (RC) and N, Zn, Fe and Cu content in Non-mycorrhizal (NM) plants.

Bacteria	RC (%)	Significant level			
		*	*	**	*
		N (mg/pot)	Zn (µg/pot)	Fe (µg/pot)	Cu (µg/pot)
NB	0	22.91[b]	92.25[c]	373.9[c]	22.94[c]
S1	0	42.85[a]	142.5[ab]	552.3[bc]	30.62[bc]
S2	0	34.64[a]	156.1[a]	831.6[a]	41.9[ab]
S3	0	33.61[ab]	132.7[ab]	713.6[ab]	40.35[ab]
S4	0	32.17[ab]	108.4[bc]	574.5[bc]	36.29[abc]
S5	0	39.97[a]	108.3[bc]	577.6[bc]	47.55[a]
S6	0	38.27[a]	117.9[abc]	738.1[ab]	39.81[ab]
Smix	0	41.77[a]	108.5[bc]	574.7[bc]	36.39[abc]
LSD value		11.33	39.69	248.8	14.07
Mean	0	35.77	120.8	617.04	36.98

Non-rhizobial (NB), *Mesorhizobium ciceri* strains (S1 – S6) and mixture of S1 – S6 strains (Smix). Significance according to ANOVA,*,** and n.s mean significant at 0.05, 0.01 probability level and non significant, respectively. Different letters within columns represent significant differences according to LSD's test.

species. This showed that some AMF species had better efficiency than others in nutrient content. Lisette et al. (2003) reported that co-inoculation with rhizobia and compatible AMF could dramatically enhance pea growth, and N and P content. The results suggested that inoculation with *M. ciceri* had positive effect on growth and P and N content in plant. Also, there were differences between bacterial strains in N content for each mycorrhizal treatment. In addition, a few studies have shown that some bacterial species respond to the presence of certain AM fungi (Artursson et al., 2005), suggesting a high degree of specificity between bacteria and AM fungi (Artursson et al., 2006).

The highest root colonization by GI, GM and Gmix were in co-inoculation with S2 and S6 strains, respectively. These two bacterial strains had lower nodule fresh mass. Competition for photosynthetic matter between bacteria and fungi may explain these results; also, these two strains may have stimulation effects on root colonization. Mortimer et al. (2008) showed that nodule growth was suppressed by the early development of AM colonization and this coincided with higher photosynthesis and respiratory rates in AM plants. On the other hand, studies by Zarei et al. (2006) showed synergistic relationships between AMF and rhizobial strains due to compatible pairing.

The highest root colonization level, N content, NFW and phosphorus content were in co-inoculation with Gmix and

Table 5. Effects of chickpea inoculation with rhizobial strains on root colonization (RC) and N, Zn, Fe and Cu content in *Glomus intraradices* (GI) inoculated plants.

Bacteria	Significant level				
	*	**	n.s	**	**
	RC (%)	N (mg/pot)	Zn (µg/pot)	Fe (µg/pot)	Cu (µg/pot)
NB	61.73^{ab}	23.87^{c}	112.34	443.2^{c}	33.3^{bc}
S1	52.47^{bc}	49.74^{ab}	131.87	808.7^{a}	42.8^{ab}
S2	68.63^{a}	38.35^{bc}	145.11	600.3^{abc}	43.4^{ab}
S3	52.3^{bc}	39.49^{b}	130.59	496.9^{bc}	23.9^{c}
S4	51.53^{bc}	48.45^{ab}	112.92	623.8^{abc}	32.6^{bc}
S5	48.07^{c}	50.31^{ab}	131.12	716.5^{ab}	34.3^{abc}
S6	60.2^{abc}	43.73^{ab}	168.15	635.8^{abc}	52.9^{a}
Smix	48.3^{c}	57.76^{a}	157.96	736^{ab}	44.2^{ab}
LSD Value	12.66	15.02		248.8	18.66
Mean	55.4	43.96	136.26	632.65	38.42

Non-rhizobial (NB), *Mesorhizobium ciceri* strains (S1– S6) and mixture of S1– S6 strains (Smix). Significance according to ANOVA,*,** and n.s mean significant at 0.05, 0.01 probability level and non significant, respectively. Different letters within columns represent significant differences according to LSD's test.

Table 6. Effects of chickpea inoculation with rhizobial strains on root colonization (RC) and N, Zn, Fe and Cu content in *Glomus mosseae* (GM) inoculated plants.

Bacteria	Significant level				
	**	**	**	**	**
	RC (%)	N (mg/pot)	Zn (µg/pot)	Fe (µg/pot)	Cu (µg/pot)
NB	43.1^{bc}	41.4^{c}	102.9^{c}	462.8^{d}	35.9^{c}
S1	57.6^{ab}	53.4^{bc}	136.2^{bc}	675.6^{cd}	59.1^{ab}
S2	59.1^{ab}	63.0^{ab}	184.1^{ab}	1000^{a}	55.5^{b}
S3	47.3^{abc}	58.2^{b}	143.5^{bc}	626.3^{cd}	62.1^{ab}
S4	44.6^{abc}	58.2^{b}	180.2^{ab}	720.2^{bc}	74.4^{a}
S5	55.4^{ab}	76.7^{a}	167.8^{ab}	704.1^{bcd}	56.3^{ab}
S6	60.7^{a}	58.5^{b}	204.9^{a}	928.4^{ab}	60.9^{ab}
Smix	36.4^{c}	51.4^{b}	153.0^{abc}	691.7^{bcd}	50.2^{bc}
LSD value	16.79	15.02	52.64	248.8	18.66
Mean	50.5	57.6	159.07	726.14	56.8

Non-rhizobial (NB), *Mesorhizobium ciceri* strains (S1– S6) and mixture of S1– S6 strains (Smix). Significance according to ANOVA,*,** and n.s mean significant at 0.05, 0.01 probability level and non significant, respectively. Different letters within columns represent significant differences according to LSD's test.

S3 strain. These results suggested that the effective AMF can enhance the performance of infection by rhizobia and vice versa. Johnny (1999) showed that some rhizobial strains increase the growth and yield of pea and lentil, whereas other strains had no effect. This author also suggested that specific AMF and rhizobia combination enhances plant growth and yield. More insight into these results will enable optimization of the effective use of AM fungi in combination with their bacterial partners as a tool for increasing crop yields.

In most cases, inoculation with mycorrhizal fungi, increase micronutrient content by plant. This increase in micronutrient content was affected by interaction between different strains of bacteria and fungal species. The highest Zn and Fe content was found in the co-inoculation of GE and S6 strain, and Cu occurred in GE and Smix. Clark and Zeto (2000) reported that AMF support nitrogen fixation by providing legumes with P and other immobile nutrients that are essential for N fixation, such as copper and zinc, although, these effects were also dependent on the specific symbiont combination.

In conclusion, our results have shown some synergistic effects of co-inoculation with rhizobia and AMF on chickpea growth. The efficiency of each AMF species on plant growth and nutrient content was influenced by rhizobial strains. Specific combination of AMF, rhizobial

Table 7. Effects of chickpea inoculation with rhizobial strains on root colonization (RC) and N, Zn, Fe and Cu uptake in *Glomus etunicatum* (GE) inoculated plants.

Bacteria	Significant level				
	n.s	**	**	**	**
	RC (%)	N (mg/pot)	Zn (µg/pot)	Fe (µg/pot)	Cu (µg/pot)
NB	41.8	36.4^{c}	149.6^{b}	617.9^{c}	36.8^{c}
S1	26.9	55.6^{ab}	200.7^{ab}	1018.9^{ab}	39.7^{c}
S2	38.7	59.8^{ab}	190.4^{b}	804.3^{bc}	35.4^{c}
S3	31.7	65.5^{ab}	187.8^{b}	1007.5^{ab}	50.4^{abc}
S4	30.2	53.4^{b}	177.7^{b}	780.7^{bc}	47.2^{bc}
S5	37.5	68.8^{a}	184.8^{b}	752.2^{c}	43.7^{b}
S6	27.7	67.9^{ab}	249.2^{a}	1135.1^{a}	62^{ab}
Smix	39.9	61.6^{ab}	166.7^{b}	795.9^{bc}	68.4^{a}
LSD value		15.02	52.64	248.8	18.66
Mean	34.3	58.62	188.36	864.06	47.95

Non-rhizobial (NB), *Mesorhizobium ciceri* strains (S1– S6) and mixture of S1– S6 strains (Smix). Significance according to ANOVA,*,** and n.s mean significant at 0.05, 0.01 probability level and non significant, respectively. Different letters within columns represent significant differences according to LSD's test.

Table 8. Effects of chickpea inoculation with rhizobial strains on root colonization (RC) and N, Zn, Fe and Cu content in plants inoculated with mixture of fungi GI+GM+GE (Gmix).

Bacteria	Significant level				
	**	**	**	**	**
	RC (%)	N (mg/pot)	Zn (µg/pot)	Fe (µg/pot)	Cu (µg/pot)
NB	35.5^{c}	34.5^{c}	115^{b}	608.8^{d}	36.7^{cd}
S1	46.1^{bc}	62.7^{a}	143.8^{ab}	799.2^{bcd}	65.4^{a}
S2	66.1^{a}	42.7^{bc}	142.1^{ab}	1035.1^{ab}	56.2^{ab}
S3	67^{a}	61.7^{a}	120.9^{b}	927.3^{abc}	59.1^{ab}
S4	53.5^{ab}	52.3^{ab}	114.7^{b}	886.2^{abc}	47.6^{abc}
S5	39.5^{bc}	52.2^{ab}	152.3^{ab}	889.1^{abc}	40.5^{bcd}
S6	65.4^{a}	54.9^{ab}	193.2^{a}	764^{cd}	52.5^{abc}
Smix	51.1^{abc}	64^{a}	161.7^{ab}	1061.1^{a}	28.66^{d}
LSD value	16.79	15.02	52.64	248.8	18.66
Mean	53	53.12	142.96	871.35	48.33

Non-rhizobial (NB), *Mesorhizobium ciceri* strains (S1– S6) and mixture of S1– S6 strains (Smix). Significance according to ANOVA,*,** and n.s mean significant at 0.05, 0.01 probability level and non significant, respectively. Different letters within columns represent significant differences according to LSD's test.

strains and legume plants would lead to efficient tripartite symbioses.

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