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Growth and yield of lentil (*Lens culinaris* L.), as affected by mycorrhizal symbiosis and *Azospirillum brasilense* under rainfed conditions

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Abstract

Microbial interactions are being used to improve the yield and drought tolerance of cultivated plants in some semi-arid regions. A field experiment was conducted aiming to determine the possibility of improving the lentil performance as co-inoculated with Vesicular Arbuscular Mycorrhiza (VAM) fungi and Azospirillum under rain-fed conditions, in Iran. A factorial experiment conducted on the basis of randomized complete block design with four replicates, in which two VAM fungi (*Glomus intraradices* and *G. mosseae*) and *Azospirillum brasilense* inoculated with two lentil cultivars; Mashad and Naaz (large seed and small seed, respectively). Results imparted the substantial impact of VAM fungi on grain protein, root colonization and shoot dry weight. Highest value for shoot dry weight recorded in plants which inoculated with *G. intraradices* and highest values for root colonization and grain protein content was observed in plants which inoculated with *G. mosseae*. Also, Azospirillum had a significant effect on shoot dry weight and root colonization. A significant differences on grain protein content observed when combination of both microorganisms have been used. The tripartite interaction between VAM fungi, Azospirillum and lentil cultivars found to be significant for grain protein content and root colonization.

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Introduction

A series of malpractices has jeopardized the natural processes of soil to recover, rebuild and restore the potential soil fertility and environmental abnormalities have occurred as an inevitable consequence. During the last few decades uses of organic and biological fertilizers had faced drastic declines but the extensive use of chemical fertilizers and the dire consequences have put the environmental friendly fertilizers back on the scientific agenda (Al-Karaki *et al.*, 2004). Sustainable agriculture on the pillar of using biofertilizers and with the aim of elimination or at least a significant decline of Chemical inputs seems an eligible answer to the problems the world faces today. A biofertilizer is a substance containing living microorganisms colonizing the rhizosphere or the interior of the plant, enriching soil fertility and fulfilling plant nutrient requirements by supplying the organic nutrients through microorganism and their byproducts. Seed inoculation with *Azospirillum brasilense* and soil inoculation with the vesicular arbuscular mycorrhizal fungi have been long recognized as biofertilizer technologies. Albeit biofertilizer is a contemporary term in scientific literature but the initial applications can be traced back to much older ages. Aside from the positive externalities on soil characteristics, many benefits from economic, environment and social aspects are associated with biofertilizer applications (Gary and Williams, 1975). The classical mutualism relationship of arbuscular mycorrhizal fungi and a majority of crop roots plays a direct role in nutrient cycling rates and patterns in agroecosystems contributing markedly to nutrient capture and supply, water retention, alleviating the environmental abiotic stresses and improving the pathogen resistance resulting in improvements of crop health and yield in sustainable agroecosystems (Azcon *et al.,* 1997; Cox and Tinker, 1976; Ojala and Jarrell, 1983; Annaduraia *et al.,* 2002; Sharma, 2002a; Daei *et al.,* 2009; Safapour *et al.,* 2011). *Azospirillum* strains fix atmospheric nitrogen efficiently in association with plants and produce several plant hormones, Majorly (IAA) and less but biologically significant levels of (IBA), several gibberellins, (ABA) and cytokinins (Bashan and Levanony, 1990; Zahir *et al.*, 2004; Ardakani *et al.,* 2011; Zakikhani *et al.,* 2012). These properties contribute greatly to the improvement of water and nutrient absorption and solubilizing of phosphates.

Lentil (*Lens culinaris* Medik.), an old world grain legume food crop that was domesticated in the Near East and is broadly cultivated in semi-arid Mediterranean, south Asia, Indian sub-continent, south America and Iran. Seeds of lentil are among the richest sources of protein (20-36%) with an important role in the human diet in developing countries while contributing to soil nitrogen balances and a suitable alternative for agroecosystems with fallow-cereal rotations (Christou, 1993). Water deficiency is a major limiting factor of crop yield in arid and semi-arid areas. In these areas because of limited underground water resources many crops are cultivated under rain-fed conditions. Management of water and nutrient cycles and maintaining the viability of soil are of considerable importance under rain-fed conditions (Webber, 1976; Moghaddam *et al.,* 2012; Raza *et al.,* 2013). With regard to the properties of biological fertilizers and their applications in enhancing the overall performance of crops, practitioners are experimenting to reach different combinations and approaches to improve the crop yield under water deficit and rain-fed conditions. Mycorrhizal symbiosis is widely reported to enhance the water movement from soil into the host plant and host plants have shown substantial mycorrhizal dependency under lowmoisture regimes (Mark *et al.,* 1996). Plants with colonized roots with mycorrhizae vis-a-vis the noncolonized have demonstrated faster absorption of soil water content, greatly because of the expected shoot development and leaf area, resulting in increased levels of plant transpirations needs while colonized roots in comparison with non-colonized roots are more extensive in structure and provide a greater volumes of soil to interact with (Gao *et al.,* 2001). Most of studies done on the applications of biofertilizer were on cereals and forages while few experiments investigated such applications on legumes in general and particularly lentil especially under field and rain-fed conditions. Neuman *et al.,* (2004) on a pot experiment with Sorghum shown the significant difference of plant phosphorous content in mycorrhizal plants and non-mycorrhizal plants. Ghazi and Kraki (1998) indicated that mycorrhizal plants produce more dry matter per unit of consumed water, in other words showing elevated levels of water use efficiency under water deficit conditions. It has been shown that the inoculation of wheat with (AMF) enhanced the growth, yield and nutrient absorption and acted to alleviate the effects of drought stress under farming conditions(Al-karaki *et al.,* 2004). Gupta *et al.,* (2002) reported that (AMF) inoculation of Mint substantially increased the biological yield and root symbiosis percentage in comparison with noninoculated plants. Another study (Subramanian *et al.,* 2006) on Tomato revealed that the plant-Mycorrhiza symbiosis increased the number of flower per bush statistically significant. (Ruiz-lozano., 2003) also reported the increased activity of (SOD) enzyme in Myco-inoculated plants which acted to provide a defense mechanism against imposed drought stress. A report (Omar *et al.,* 2009) imparted that (SOD) enzyme activity of Barely decreased when inoculated with Azospirillum. An experiment conducted on grain yield of wheat co-inoculated by (VAM) and Azospirillum revealed that the leaf relative water content, leaf area and chlorophyll content were increased when co-inoculated. Also the Biomass and grain yield were shown to be more than the non-inoculated control. (Panwar, 1993).

Iran has arid or semiarid climates mostly characterized by low rainfall and high potential evapotranspiration. The average annual precipitation over the country was estimated to be around 250 mm, occurring mostly from October to March (Nazemosadat *et al.,* 2006). So, shifting toward the efficient use of water and soil resources and implementing of the principles of sustainable agriculture are highlighted recently, present paper examines the effects of application of biofertilizers in order to enhance the quantity and quality of yield of the lentil with the aid of mycorrhizal mutualistic and Azospirillum associative relationships under rain-fed conditions.

Materials and methods

This field study was conducted in, Khalkhal, Iran, (37^{o} 37′ N latitude, 48^{o} 32′ E longitude and altitudes of 1796 m) on 2011-2012 growing season. The yearly average precipitation (30-years long term period) is 370 mm but the precipitation on this particular growing season has recorded as 230 mm by Khalkhal synoptic meteorology institute. Soil samples were taken to determine the physical and chemical properties from the depth of 0-30 cm and characteristics are presented in Table 1.

The treatments were arranged as factorial experiment based on randomized complete block design with 4 replicates. The experimental treatments consisted as follow:

- Mycorrhizal Inoculation in 3 levels (M_0 = without inoculation, M_1 = *Glomus intraradices* and M_2 = *Glomus mosseae*) Mycorrhizaes with the population of 10^5 *spor.g*$^{-1}$
- Azospirillum inoculation in 2 levels (A_0 = without inoculation and A_1 = inoculation with *A. brasilense*) with the population of 10^8 *cfu.g*$^{-1}$
- Lentil cultivar in 2 levels (L_1 = large grain Mashadi and L_2 = small grain Naaz). Seeds were provided by Agriculture department of the province.

Conventional field preparations were done in early spring time. Each plot was consisted of 6 rows, 25 cm apart. Distance between plots and replications were arranged to be 1 and 2 meters, respectively. Cultivation of seeds was performed in mid-spring and mycorrihzae applied under seed beds and seeds in Azospirillum dedicated plots were inoculated accordingly. No chemical fertilizers were applied during the course of experiments and weeds were eliminated with mechanical methods. The measured traits were Grain yield, biological yield, shoot dry weight, percentage of grain protein content and the percentage of root colonization. In order to measure dry weight, samples taken and placed in an oven in 75 degrees Celsius for 48 hours. In the beginning of seeding stage 20 plants were selected randomly from each plot for root studies. At the same stage for measuring percentage of root colonization root samples were taken and washed with FAA (Formalin Acetic Acid Alcohol) solution for further procedures. Philips and Hayman (1970) method was used for staining the roots. First to decolorize the roots a solution of 10% KOH used for 3 hours then roots were washed with distilled water. Coloring of roots was done with 0.05 % w/v trypan blue in lactoglycerol (1:1:1 lactic acid, glycerol and water) then the gridline intersect method was applied introduced by Giovannetti and Mosse (1980) and results expressed in percentage. Using MSTAC, data were subjected to analysis of variance. Mean comparison was conducted using the Duncan's Multiple Range Test (DMRT) at 5% level of probability.

Results and discussion

Shoot Dry Weight

As it is illustrated in (Table 2), direct effects of Mycorrhizae and Azospirillum inoculations and the interactive effects of both factors found to be statistically significant ($P \leq 0.01$). Mean comparison of treatments imparted significant differences between different levels of Mycorrhizal inoculations and application of *G. intraradices* resulted in the foremost value (115.78 g.m-2)while the non-inoculated treatments showed the least value (108.61 g.m-2) for shoot dry weight (Table 3). No significant effects were observed between treatments inoculated with *G. intraradices* and *G. mosseae*. In an experiment (AL-Karaki *et al.*, 1997) on Mycorrhizal inoculated wheat, it was proposed that the enhancement of shoot dry weight is due to the increased levels of phosphorous absorption because inoculated plants store higher levels of phosphorous on the shoot. It is well documented (Gupta *et al.,* 2002) that the symbiosis between Mycorrhiza and crops root elevates the photosynthesis and shoot dry root increases in result. Mean comparison of treatments (Table 3) revealed that inoculation with Azospirillum substantially (2.8% more) affected the shoot dry weight (114.97 g.m-2) in comparison with control (111.73 g.m-2). Similar results have been reported by Veresoglou and Menexes (2001) and Mahmoud *et al.*, (2010). A report (Geneva *et al.*, 2006) on co-inoculation of chick pea with Mycorrhizae and Azospirillum indicated the enhancements in Shoot dry weight, photosynthesis rate and nitrogen fixation activities. Mean comparisons of the treatments with interaction of Mycorrhizae and Azospirillum (Table4) imparted the foremost treatment to be co-inoculated while the inferiors found to be the non-inoculated treatments. Other researchers have concluded that the dual inoculation of plants increases shoot dry weight (Gupta *et al.,* 2002; Kapoor *et al.,* 2004; Ardakani *et al.,* 2009).

Table 1. Physical and Chemical properties of the soil in the experiment site.

Cu (ppm)	Fe (ppm)	Zinc (ppm)	N (ppm)	K (ppm)	P (ppm)	Sand (%)	Silt (%)	Clay (%)	Texture	pH	EC (ds/m)	Organic matter (%)
2.2	4.4	0.4	0.16	318	9.45	37	22	41	Clay	8.1	0.5	1.5

Notes: EC= Electrical conductivity.

Percentage of Root Colonization

As it is illustrated in (Table 2), direct effects of Mycorrhizae and Azospirillum inoculations and the interactive effects ($P \leq 0.01$) of both factors and the interactive effects of Mycorrhizae and Azospirillum and lentil cultivar ($P \leq 0.05$) found to be statistically significant. Mean comparison of treatments (Table 3) imparted significant differences between different levels of Mycorrhizal inoculations as in application of *G. mosseae* resulted in the foremost percentage of root colonization (34.56%) and the inoculated treatments with *G. intraradices* showed (33.1%) of myco-colonization whilst the measured colonization in non-inoculated treatments recorded as (22.24%). This can be elucidated through the effectiveness of Myco-inoculation which has been well documented (Kapoor *et al.,* 2004), (Ratti *et al.,* 2001) and (Arriagada *et al.,* 2007). As it is depicted in (Table 3)

Inoculation with Azospirillum increased the percentage of root colonization (30.30%) statistically significant over non-inoculated treatments. Azospirillum might have promoted the colonization of lentil root and acted to improve the measured percentage of Myco-colonization. Similar trends of interaction on percentage of root colonization have been reported (Geneva *et al.,* 2006) on Co-inoculated Chick pea plants with Mycorrhizae and Azospirillum. Mean comparison of the interactive effects of Mycorrhizal inoculation and Lentil cultivar was significant (Table 4) and inoculations of Naaz Cultivar seeds with *G. mosseae* resulted in highest (35.25%) percentage of root colonization. Mean comparison of the interactive effects of the all the three factors revealed significant differences on percentage of root colonization and the highest value (35.80%) was achieved in treatments with *G. mosseae* and Azospirillum inoculations in Naaz cultivar seeds (Table 4). It be can posited that the involvement of different levels of Mycorrhizae , Azospirillum and lentil cultivar results in a booster effect, improving the symbiosis of mycorrhizae and host plant. Previous studies conducted by Omar (1998), Hazarika *et al.,* (2000) and Ratti *et al.,* (2001) have repeatedly confirmed and reconfirmed the improving role of biofertilizers on percentage of root colonization.

Table 2. Analysis of variance (ANOVA) for Measured Traits.

S.O.V	df	Shoot dry weight	Biological yield	Grain yield	Root colonization Percentage	grain protein percentage
Replication	3	1.242	265.808	85.712	0.420	0.116
Mycorrhiza (M)	2	269.978**	177.983ns	0.673ns	725.352**	102.671**
Azospirillum (A)	1	125.777**	29.453ns	26.255ns	6.978**	0.241ns
Lentil cultivar (L)	1	4.750ns	42.979ns	0.005ns	0.006ns	15.870**
A×M	2	18.068**	71.667ns	3.441ns	0.008ns	0.539*
M×L	2	1.119ns	188.223ns	24.394ns	6.850**	1.546**
A×L	1	2.394ns	12.813ns	85.600*	3.983*	0.403ns
A×M×L	2	1.057ns	81.125ns	29.760ns	2.865*	0.579*
Error	33	2.385	108.450	15.236	0.567	0.137

* and ** significantly at p < 0.05 and < 0.01, respectively; ns = non-significant.

Percentage of grain protein content

As it is illustrated in (Table 2), direct effects of Mycorrhizal inoculation and lentil cultivars and the interactive effects (P ≤ 0.01) of both factors and the interactive effects of Mycorrhizae and Azospirillum and lentil cultivar (P ≤ 0.05) found to be statistically significant. The Maximum measured value (24.31%) for percentage of grain protein content was recorded in treatments with *G. mosseae* inoculations while the minimum value was (20.03%) although no statistically significant differences was observed between the two different mycorrhizae species on the percentage of grain protein content. On a study on VAM fungi (Bethlenfalvay, 1994), it is indicated that highest values for percentage of grain content can be achieved in Myco-inoculated treatments. Also significances (Table 3) observed between lentil cultivars as (23.02%) and (22.37%) in Mashhadi and Naaz cultivar, respectively. Mean comparison of the interactive effects of Mycorrhizal and Azospirillum inoculations imparted the highest value for percentage of grain proterin content in treatments with *G. mosseae* inoculations while the least value was recorded in treatments without mycorrhiza inoculation and with Azospirillum inoculation (Table 4). Mean comparison of the tripartite factors imparted that seeds of Mashhadi cultivar treated with *G, intraradices* inoculations and Azospirillum applications obtained the highest value (25.55%) for percentage of grain protein content (Table 4).

Grain yield

The results of analysis of variances represented in (Table 2) imparted the interactive effect of

Azospirillum inoculation and Lentil cultivar was the only significant ($P \leq 0.05$) effect recorded on grain yield. In our experiment, applications of Mycorrhiza and Azospirillum did not resulted in improvement of grain yield albeit the drastic decline in annual precipitation (230 mm) vis-a-vis the long term average (370 mm) might be the reasoned to be main cause. Some studies have demonstrated the decline in mycorrhizal plants in comparison with non-mycorrhizal counterparts (Smith, 1980). Also, Al-Karaki *et al.,* (1997) reported that mycorrhiza acted to increase the shoot dry weight but no significant differences recorded on Biological yield, grain yield and root dry weight.

Table 3. Mean comparison of the main effects of Mycorrhiza, Azospirillum and Lentil cultivars on measured traits.

Treatments	Shoot dry weight (g/m^2)	Biological yield (g/m^2)	Seed yield (g/m2)	Root colonization percentage	Seed protein percentage
M0	108.61b	167.47a	58.31a	22.24c	20.03b
M1	115.78a	169.29a	57.93a	33.10b	24.01a
M2	115.66a	173.94a	58.26a	34.56a	24.31a
A0	111.73b	171.01a	58.91a	29.59b	22.88a
A1	114.97a	169.45a	57.43a	30.30a	23.02a
L1	113.03a	171.18a	58.18a	29.96a	23.02a
L2	113.66a	169.28a	58.16a	29.98a	22.37b

Means with similar letter are not significantly different.

M_0 = without inoculation, M_1 = *Glomus intraradices* and M_2 = *Glomus mosseae*

A_0 = without inoculation and A_1 = inoculation with *Azospirillum brasilense*

L_1 = large grain c.v. Mashadi and L_2 = small grain c.v. Naaz.

Table 4. Mean comparison of the interaction effects of Mycorrhiza, Azospirillum and Lentil cultivars on measured trait.

Treatments	Shoot dry weight (g/m^2)	Biological yield (g/m^2)	garin yield (g/m^2)	Root colonization percentage	grain protein percentage
A_0M_0	108.20c	168.91a	59.58a	21.84e	20.08c
A_0M_1	109.1c	17.78a	57.03a	32.71c	24.5a
A_0M_2	113.60b	172.25a	58.70a	34.21ab	24.03b
A_1M_0	113.39b	166.03a	58.4a	22.63d	19.97c
A_1M_1	118.17a	166.80a	57.47a	33.50b	24.5a
A_1M_2	117.72a	175.52a	57.87a	34.92a	24.6a
A_0L_1	111.19b	171.44a	60.20a	29.86bc	23.55a
A_0L_2	112.27b	170.58a	57.56ab	29.31c	22.21c
A_1L_1	114.88a	170.91a	56.10b	30.05ab	23.50a
A_1L_2	115.06a	167.98a	58.75ab	30.65a	22.54b
M_0L_1	108.59b	165.47a	56.9a	22.86d	20.5e
M_0L_2	108.62b	169.47a	59.72a	21.62e	19.6f
M_1L_1	115.37a	174.00a	58.73a	33.14bc	25.36a
M_1L_2	116.19a	164.08a	57.13a	33.06c	23.58d
M_2L_1	115.14a	174.07a	58.91a	33.87b	24.65b
M_2L_2	116.18a	173.80a	57.62a	35.25a	23.98c
$A_0M_0L_1$	107.92c	165.9ab	57.97a	23.38fg	20.72d
$A_0M_0L_2$	108.48c	171.93ab	61.20a	21.31g	19.55e
$A_0M_1L_1$	113.03b	174.01ab	61.00a	23.50cd	25.32a
$A_0M_1L_2$	113.70b	169.00ab	60.80a	31.92e	23.72c
$A_0M_2L_1$	112.63b	174.43ab	61.82a	33.72bcd	24.90b
$A_0M_2L_2$	114.08b	170.28ab	60.70a	34.70b	23.47c
$A_1M_0L_1$	109.26c	165.04ab	60.82a	23.33f	20.27d
$A_1M_0L_2$	108.77c	167.02ab	58.25c	21.93g	19.67e
$A_1M_1L_1$	117.71a	173.99ab	60.47a	32.79de	25.55a
$A_1M_1L_2$	118.63a	159.61b	60.47a	34.21bc	23.45c
$A_1M_2L_1$	117.66a	173.71ab	60.20a	34.03bc	24.70b
$A_1M_2L_2$	117.78a	177.33a	60.00a	35.80a	24.00b

Biological yield

Results of analysis of variances (Table 2) imparted that the main and interactive effects of studied factors were not statistically significant on biological yield. It can be assumed that application of biofertilizer under rain-fed and severe drought will not result in improvement of biological yield, but rather it aids the survival of stressed plant. Although improvement of plant nutrition status and enhancement of growth are the most widely believed roles of VAM fungi in natural ecosystems but it seems that under drought stress conditions it only thrives to survival needs of plants (Varma and Hock, 1999).

Conclusion

Application of Mycorrhizae improves the shoot dry weight, grain protein percentage and percentage of root colonization. Also Inoculation of Azospirillum enhances the percentage of root colonization and shoot dry weight although co-inoculation of plants with both Mycorrhizae and Azospirillum did not significantly affected the grain yield and biological yield of lentil. The improved root growth under severe drought conditions due to the application of studied biogertilizers can be alleged to improve the grain yield of lentil under less severe and more normal climatic conditions. Duplication of similar studies for further investigations in multiple sites and under different climatic conditions is recommended.

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