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Microorganisms (AMF and PSB) interaction on linseed productivity under water-deficit condition

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Abstract

The relationship between arbuscular mycorrhizal fungi (AMF) and their associated bacteria can be has great importance for sustainable agriculture especially in the case of highly mycorrhizal plants such as linseed. To evaluate the possible effect of AMF in association with phosphate solubilizing bacteria (PSB) on linseed plants, a 2-yr factorial experiment was conducted based on a randomized complete block design with three replications at Urmia University, Urmia, Iran (37° 39′ 24.82″ N 44° 58′ 12.42″ E). The treatments included two AMF species (*Glomus mosseae*, *G. intraradices* and non-mycorrhizal control), PSB (*Pseudomonas putida* P13 and non-inoculated control) and various irrigation regimes (irrigation after 60, 120 and 180 mm of evaporation from Class A pan). A significant increase in mycorrhizal linseed plants yield indicated the effectiveness of the two AMF species more than bacterial inoculation. The cumulative (second year) soil spores were maximally observed in mycorrhizal (single AMF and dually inoculation) treatments. The reduction in bacterial population was found with an increase in water deficit. Dual infections caused an increase in leaf P content more than the one in PSB and AMF inoculations. Drought stress-induced yield reduction in seed and in oil was significantly compensated by mycorrhizal symbiosis for all irrigation levels. We found over 25% increase for seed yield and 30% for oil yield in mycorrhizal plants as well as co-inoculated plants. The yields improvements in mycorrhizal treatments (single and dually inoculated) leading to the highest water use efficiency.

Keywords: Irrigation; Linseed; Mycorrhizae; Spore density; Water use efficiency.

Introduction

Linseed (*Linum usitatissimum* L., *Linaceae* family), is one of the ancient cultural plants in the world and basically is used in two forms: oil and fibber (Muir and Westcott, 2003). From 1993 to 2014, although the world-wide linseed harvested area and its production went down, the oil yield increased at a rate of 1.72%. Hence, it was cultivated on 2.3 million hectares of land world-wide with 560000 tons of oil production (FAOSTAT, 2014). Drought imposes one of the commonest and most significant constraints to agricultural production, seriously limiting crop growth, yield and quality. As water supplies decline and/or the cost of water increases, it is clear that producers are being driven toward deficit irrigation management and some level

of plant water stress is inevitable. The challenge is to define management systems that minimize the negative impact of the expected stress (Fereres and Soriano, 2007; Geerts and Raes, 2009).

Plant rhizosphere is known to be preferred ecological niche for soil microorganisms due to rich nutrient availability (Joseph et al., 2007). Rhizosphere microorganisms (particularly beneficial bacteria and fungi) can improve plant performance under stress environments and, consequently, enhance yield (Dimkpa et al., 2009). There is considerable evidence to suggest that arbuscular mycorrhizal fungi have the potential to increase the tolerance of their host plants to water-deficit stress (Murphy et al., 2015; Auge, 2004). Earlier, Al-Karaki et al. (2004) showed that plant recovery after water-deficit stress occurred faster in mycorrhizal plants than it did in non-mycorrhizal ones. Depending upon soil moisture, inoculation of plants with suitable AMF consortium can be beneficial (Shukla et al., 2013). The alleviating effect of the AMF symbiosis in response to drought generally relies on the uptake and transport of water and on an improved uptake of nutrients, especially of available soil phosphorus (P) and other immobile mineral nutrients, resulting in the hydration of plant tissues. Although AMF can affect the water balance of both amply watered and droughted host plants (Auge, 2001).

The findings of Timmusk et al. (2014) demonstrate that plant inoculation with bacteria from harsh environments resulted in significantly higher survival of drought-stressed plants and in greater photosynthesis and biomass production. While total phosphorus contents of soil is typically high, the available phosphate ions (Pi), the prevalent forms of phosphorus that plant roots absorb, is usually suboptimal (Rodriguez and Fraga, 1999). A close association was evident between phosphate solubilizing ability by *Pseudomonas putida* strain P13 and growth rate which is an indicator of active metabolism (Malboobi et al., 2009). AM inoculation improves the establishment of both inoculated and indigenous phosphate-solubilizing rhizobacteria acting as helping bacteria (Barea et al., 2002). The cooperation of bacteria and AM fungi in nutrient uptake by plants (Barea et al., 2005) is probably due to specific attributes of microorganisms and there is a growing interest in improving our understanding of their involvement in nutrient cycling and non-nutritional physiological values that make the plant more tolerant to drought stress.

Linseed is less efficient in absorbing P from soil compared with other crops because of its shallow root system (Casa et al., 1999), that it may limited by water deficit (Shukla et al., 2013). Thus, the present study aimed to evaluate the drought tolerance and effectiveness of two AM fungi species alone or in association with *Pseudomonas putida*. This may provide a basis for developing strategies (AM root colonization, spore density and bacterial population) in order to reduce the drought-induced risks and maintain a sustainable plant production.

Materials and Methods

Site Description, Experimental Design and Crop Management

A 2-year field experiment was conducted at the Agricultural Faculty of Urmia University, Urmia, located at North-West of Iran (37° 39′ 24.82″ N 44° 58′ 12.42″ E)

during the years 2014 and 2015. The experimental design was factorial (three factors) based on a randomized complete block with three replications. Monthly climatic parameters recorded at a weather station located adjacent to the experimental site for two years are given in Table 1. This region enjoys a semi-arid climate. The main soil physicochemical properties are presented in Table 2. The number of AMF spores (30) and *P. putida* strain P13 population (2×10^3) were observed per 10 g of initial soil of the experimental site.

Table 1. Climatic parameters at the experimental site during the 2-yr of the study.

Parameter	2014						2015					
	April	May	June	July	August	September	April	May	June	July	August	September
Highest temperature, °C	16.7	24.0	27.9	32.5	33.2	31.4	17.1	22.4	29.5	33.5	34.4	28.5
Lowest temperature, °C	2.9	9.2	11.0	15.8	15.7	12.6	3.0	7.9	12.3	17.0	15.5	12.7
Sum of sunny hours, no.	242	304	378	374	357	326	270	283	378	374	341	259
Precipitation (mm)	1.8	0.9	0.8	0.0	0.0	0.0	0.4	1.3	0.2	0.0	0.0	0.3

Table 2. Chemical status and physical characteristics of the initial soil of the experimental site.

Soil Texture	Silt Sand Clay (%)	T.N.V (%)	Organic mater (%)	Organic carbon (%)	N (%)	Available P (mg kg^{-1})	Available K (mg kg^{-1})	EC (dS m^{-1})	pH	FC (%)	BD (g cm^{-3})	WP (%)
Loam	32 44 24	10.3	1.34	0.78	0.09	25	166	0.9	8.1	24	1.2	15

T.N.V: Total Neutralizing Value, EC: Electrical Conductivity, FC: Field Capacity, BD: Bulk Density, WP: Wilting Point.

The treatments included irrigation regimes (irrigations after 60, 120 and 180 mm of evaporation from Class A pan as well-watering, mild and severe stress, respectively), arbuscular mycorrhizal fungi inoculum (non-inoculated, *G. mosseae* and *G. intraradices*) and bacteria strain (non-inoculated and *P. putida* strain P13). Seeds were sown into a loamy soil (a fine-loamy, active, calcareous, mixed, mesic, Typic Haploxerepts) at a depth of 2 cm in plots of 150- by-200 cm size, with plant spacing of 20 by-2 cm on 19 April 2014 and 14 April 2015.

The mycorrhizal inoculum (initially isolated from endemic AMF communities on maize farm) was a mixture of sterile sand, mycorrhizal hyphae and spores (20 spores g^{-1} inoculum) and colonized root fragments, which were produced on maize (*Zea mays* L.) host plants by Dr. Y. Rezaee Danesh at Urmia University, Urmia, Iran. Inoculum was placed in the planting rows below the seeds and lightly covered with soil. For non-mycorrhizal control treatment plants, seeds were sown with no inoculation. For phosphate solubilizing bacteria (PSB) treatments, the seeds were inoculated with bacterial suspension of *Pseudomonas putida* strain P13 (10^8 cfu ml^{-1}) provided by Dr. Malboobi, Green Biotech, Iran, before being immediately planted. Wet seeds were rolled into the bacterial suspension until they uniformly coated.

The irrigation water supply at each plot was measured through a water counter. Irrigation water needed prior to irrigation (VN) is the amount of water needed during irrigation to replenish the soil moisture deficit, thereby restoring the soil back to the field capacity. VN was calculated according to Benami and Ofen (1984):

$$VN = \frac{[(FC - WP) \times BD \times D \times (1 - ASM) \times A]}{100}$$

where, VN is the irrigation water needed before irrigation (m^3), FC is field capacity (%), WP is the wilting point (%), BD is bulk density (g cm^{-3}), D is the root zone depth (m), ASM is the available soil moisture before irrigation (a fraction) and A is the area of the field (m^2).

Seed and biological yield

At the end of the growing season, when the plants had produced mature seeds (on 6, 20 and 27 August 2014 and 2015 for irrigation after 180, 120 and 60 mm of evaporation from Class A pan respectively; different times of harvesting is because of different maturity date for three irrigation regimes), samples were taken from 1 m^2 area with ignoring border effects in every plot to determine seed and biological yield (seeds separated and weight from individual plots). Ten plants of every plot were selected to counter the number of branches and capsules per plant and measure plant height.

Seed Oil Extraction

To obtain oil samples by solvent extraction, chopped linseed were extracted with petroleum ether (Merck, 40-60 °C) in a Soxhlet apparatus and the remaining solvent was removed by distillation. After extraction, the oil samples were filtered.

Oil content of the samples is expressed in percentages based on the whole seed. Then, oil yield was determined through the following formula:

Oil yield= % oil × seed yield

Water use efficiency (WUE) was calculated as follows:

Seed WUE (kg ha^{-1} m^{-3}) = Total seed yield/Total water used

Oil WUE (kg ha^{-1} m^{-3}) = Total oil yield/ Total water used

Root Colonization

The percentage of linseed roots colonization was determined on 15 plants for each experimental plot on 1 August 2014 and 26 July 2015 (Figure 1). Root colonization was measured in fresh roots (washed with distilled water over sieve to remove soil) cleared by 10% KOH for 10 min at 90 °C and stained in 0.05% lactic acid-glycerol-Trypan Blue (Phillips and Hayman, 1970). The percentage of root colonization by AMF was microscopically determined using the gridline intersection method (Giovannetti and Mosse, 1980).

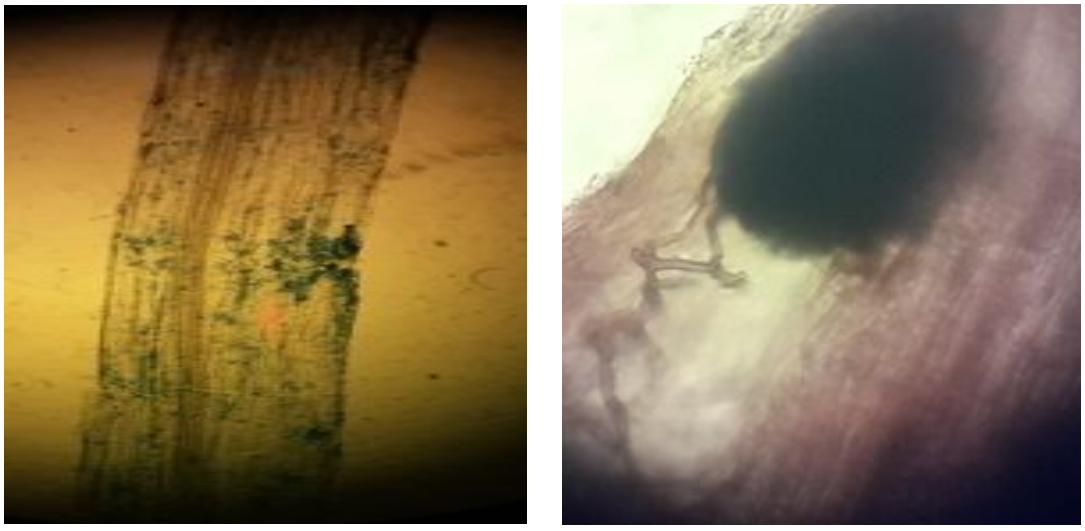

Figure 1. Colonization of linseed root by arbuscular mycorrhizal fungi.

Counting AMF Spores

Spore extraction from the soil samples (three mixed samples per treatment) were carried out using the wet sieving and decanting technique by Gerdemann and Nicolson (1963). The spores were collected on a grid pattern filter paper and washed with distilled water to spread spores evenly over the entire grid and then observed under compound microscope (Figure 2).

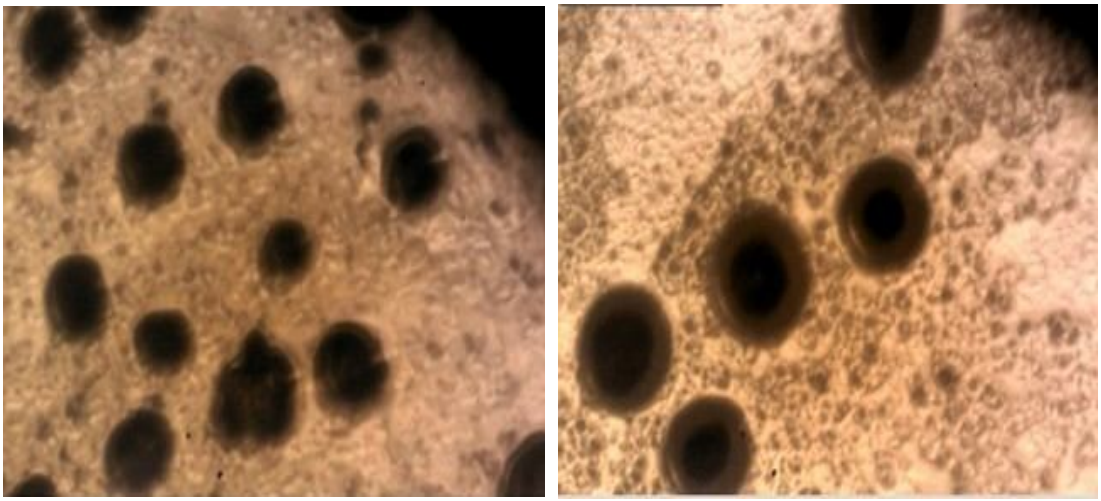

Figure 2. Spores of mycorrhizal fungi, which extracted from soil samples in linseed rhizosphere.

Counting bacterial population

The enumeration of PSB was determined from the rhizosphere soil by the dilution plate technique on Pikovskaya agar medium. Each plate was replicated three times and incubated for five days at 28 ± 2 °C. Colonies showing a clear halo around the growth indicating P solubilization were counted (Minaxi et al., 2013).

Leaf phosphorus content

To measure leaf P, dried leaves were milled, digested and analyzed as described by Watanabe and Olsen (1965) and Ohnishi et al. (1975). The method described for P involves drying, homogenization and combustion (4 h at 500 °C) of the leaf sample. The plant ashes (5 mg) are digested in 1 ml of concentrated HCl. The samples are then filtered and total P is quantified as PO_4^- using the ascorbic acid method (Watanabe and Olsen, 1965). The amount of PO_4^- in solution was determined colorimetrically at 882 nm (Graca et al., 2005).

Statistical analysis

The statistical analyses to determine the individual and interactive effects were conducted using MSTAT-C and SAS (Version 9.1.3, SAS Institute Inc., Cary, NC, USA) softwares. When the analysis of variance (ANOVA) showed significant treatments effects, Student Newman Keuls (SNK) test was applied to compare the means (at 0.05 probability level).

Results

Combined ANOVA of 2-yr data showed a significant interaction effect of irrigation regimes×bacteria×mycorrhizae on the number of capsule per plant, seed yield, oil yield, soil bacterial population ($P \leq 0.01$), plant height, the number of branch per plant,

biological yield, water use efficiency (seed and oil), root colonization, leaf phosphorus and oil percentage ($P \leq 0.05$). In addition, there were a significant interaction of irrigation×bacteria on 1000-seed weight ($P \leq 0.05$) and significant interaction of year×irrigation regimes×bacteria×mycorrhizae on the number of spore per 10 g soil ($P \leq 0.01$) (Table 3).

Inoculated plants significantly demonstrated higher levels of root colonization by *G. intraradices* and *G. mosseae*. The highest root colonization belonged to dually infected (AM plus *Pseudomonas*) plants 83% and 77% for *G. intraradices* and *G. mosseae*, respectively at an irrigation carried out after 120 mm of pan evaporation. The synergistic effect of *P. putida* on root colonization was observed only in a mild-stress condition (irrigation after 120 mm of evaporation), but there was no superiority of dual infection on root colonization in well-irrigated and sever-stressed plants. Non-inoculated plants (control) had the lowest colonization for all irrigation regimes like bacterial plants (Table 4).

Irrigation did not have any significant effect on the number of capsule and branches per plant as two yield components, so the minimum numbers (capsule and branches per plant) belonged to control treatments similar to bacterial (*P. putida*) infected in all irrigation regimes. However, it was found to be greater by the application of mycorrhizal fungi (both single and dually infected), with lesser efficiency in stressed plants (Table 4).

Plant height was decreased significantly by water deficit. However, this shortage could be compensated by some biological treatments, namely mycorrhizal and bacterial inoculation. On the other hand, these treatments lengthened plants more than they did in the case of non-mycorrhizal plants of each irrigation level. The advantage of biological treatments was more remarkable in well-watered plants. Hence, the highest plant height (39.05 cm) belonged to mycorrhizal plants (*G. intraradices*) irrigated after 60 and the shortest plants belonged to control treatment of 180 mm of evaporation (Table 4).

The increasing water deficit stress (from irrigation after 60 to 180 mm of evaporation) led to a decline in seed and biological yields. Nonetheless, mycorrhizal treatments (single and dual inoculation) could partially compensate for this reduction in all irrigation regimes. As a result, the highest seed yield (4400 kg ha^{-1}) and biological yield (12370 kg ha^{-1}) were obtained from mycorrhizal plants (*G. intraradices*) irrigated after 60 mm of evaporation. This was the same in other mycorrhizal plants (single and dual inoculation), while co-inoculation of bacteria with *G. intraradices* showed significant benefit to reach a higher yield in mild water deficit stress. The lowest seed and biological yields were obtained from non-mycorrhizal plants in all irrigation regimes similar to the bacterial infected ones (Table 4).

Another key aspect of the present study was the analysis of the seed oil content. Mild-stressed plants (irrigated after 120 mm of evaporation) contained the highest level of seed oil percentage which was reduced under severe stress (irrigation after 180 mm of evaporation) and well-watered plants (optimum for seed and biomass production). Despite non-significant effect on the yield (seed and biological) and yield components, infection with bacteria together with AM inoculation was found to be

effective in raising the oil percentage. On the other hand, the highest percent of seed oil was obtained from dually inoculated plants for each of the irrigation regimes (Table 4).

The reduction of seed yield along with an increase in irrigation intervals led to a decline in seed oil yield. Specifically, the highest (881.66 kg ha^{-1}) and lowest (544.33 kg ha^{-1}) yields were obtained from plants irrigated after 60 and 180 mm of evaporation, respectively. In all irrigation regimes, the single and dually inoculated plants indicated a high level of seed oil yield in which the highest level (1377 kg ha^{-1}) was observed following inoculation with *G. intraradices* in well irrigated (irrigation after 60 mm of evaporation) plants (Table 4).

Moreover, we found a descending trend for WUE (for both seed and oil production) with an rise in water availability from irrigation after 180 to 60 mm of evaporation. In well-watered plants (irrigation after 60 mm of evaporation), biological inoculation (single and dual infections) made a little enhancement on seed and oil WUE, but its effect was greater in mild and especially severe water deficit. Hence, the highest increase in WUE (seed and oil) occurred in co-inoculation of *G. intraradices* irrigated after 120 mm and single inoculation with *G. mosseae* in irrigation after 180 mm of evaporation. Fallen WUE was estimated under well-watered condition (0.58 and 0.16 kg m^{-3}) compared to mild (0.71 and 0.23 kg m^{-3}) and severe (0.95 and 0.25 Kg m^{-3}) water deficit stress for seed and oil yields, respectively (Table 4).

Leaf phosphorus was diluted by limited irrigation, so the minimum leaf P was observed in severe stressed plants (irrigated after 180 mm of evaporation). Ion phosphorus accumulation in leaves of inoculated plants at both fungal species was higher than those of the leaves of control and bacterial inoculation plants. A positive synergistic effect of test organisms led to the highest leaf P in dually infected plants in all irrigation regimes (Table 4).

Comparison of the means indicated a minimum number of fungi spores (spore density) in bacterially infected and non-inoculated control plants in both years. The increasing trends of fungal spore density on mycorrhizal plants were observed in the second year. The application of mycorrhizae (single and dually co-inoculated) raised fungal spore density for all three irrigation regimes equally in 2015 and in 2014 (Table 5).

Seed bacterial inoculation with *P. putida* increased the population of bacteria by more than 1000 folds in soil. The highest PSB population (85830 cfu g^{-1}), which was reduced by water stress, was recorded at single inoculated well-watered plants. Despite the efficiency of dually inoculated to control plants, PSB population in co-inoculation with *G. intraradices* was more than the one co-inoculated with *G. mosseae.* This association was the most successful in severe stress (irrigation after 180 mm of evaporation), so the highest abundance (32500 cfu g^{-1}) was obtained from co-inoculation of bacteria with *G. intraradices* (Table 4).

Table 3. Combined (2-year data) analysis of variance of linseed responses to irrigation under phosphate solubilizer and mycorrhizal infection.

Source of variation	df	Mean Square												
		Plant Height	Number of branch plant^{-1}	Number of capsule plant^{-1}	Biological yield	Seed yield	Seed oil %	Seed oil yield	Seed water use efficiency	Oil water use efficiency	colonization	Leaf phosphorus	number of spores	Population of *P. putida*
Year (Y)	1	2.58^{ns}	213.925**	3866.43**	71129598.2**	20893842.68**	1.130^{ns}	2133071.14**	1.45522**	0.1454**	104.03^{ns}	0.00049^{ns}	11438**	44583075^{ns}
Replicate/Y	4	3.70	4.240	16.57	5722780.1	872118.01	4.248	103697.06	0.08009	0.0091	31.85	0.00245	263.9	46223478
Irrigation (A)	2	579.34**	20.111**	396.55**	123672835.3**	21928164.48**	309.459**	2637628.12**	1.33129**	0.0682**	186.19**	0.37112**	982.9*	3545279269**
Y × A	2	24.37**	23.259**	93.56**	23834074.1**	1712105.04**	0.092^{ns}	199796.50**	0.00967^{ns}	0.0022^{ns}	3.009^{ns}	0.00028^{ns}	794.4^{ns}	4284658^{ns}
Pseudomonas (B)	1	10.893^{ns}	0.037^{ns}	58.37*	4508819.3^{ns}	3212.23^{ns}	88.111**	79598.37^{ns}	0.00009^{ns}	0.0076*	21.33^{ns}	0.12335**	453.2^{ns}	51479070075**
Y × B	1	0.52^{ns}	1.814^{ns}	1.97^{ns}	1833790.1^{ns}	44286.75^{ns}	0.484^{ns}	844.48^{ns}	0.00163^{ns}	0.0001^{ns}	104.03^{ns}	0.00004^{ns}	752.0^{ns}	46164556^{ns}
Mycorrhiza (C)	2	342.68**	133.861**	1768.81**	13091385.5**	4722163.81**	36.168**	754581.78**	0.34557**	0.0549**	32110.19**	0.07127**	118837**	1653301686**
Y × C	2	18.17*	0.898^{ns}	254.67**	11890933.2**	1413307.26**	0.022^{ns}	168009.78**	0.02085^{ns}	0.0029^{ns}	915.89**	0.00047^{ns}	2467.9**	980686^{ns}
A × B	2	18.30*	3.370^{ns}	17.96^{ns}	1550424.2^{ns}	732478.70*	1.423^{ns}	59475.95^{ns}	0.06868^{ns}	0.0058*	487.86**	0.00031^{ns}	253.8^{ns}	3534004269**
Y × A × B	2	7.65^{ns}	1.925^{ns}	3.56^{ns}	1761630.4^{ns}	217414.11^{ns}	1.132^{ns}	20519.89^{ns}	0.01432^{ns}	0.0019^{ns}	64.12^{ns}	0.00031^{ns}	649.8^{ns}	4513362^{ns}
A × C	4	34.76**	3.722^{ns}	42.54*	9371095.8**	1495601.04**	0.713^{ns}	191496.26**	0.13315**	0.0143**	98.26*	0.00700**	765.1*	242252547**
Y × A × C	4	23.22**	6.148*	17.34^{ns}	10085359.1**	2836213.70**	1.498^{ns}	301211.14**	0.15970**	0.0160**	35.24^{ns}	0.00009^{ns}	608.0^{ns}	1629769^{ns}
B × C	2	13.25*	5.842*	48.85*	3575761.0^{ns}	1071776.70**	3.493^{ns}	130357.51**	0.14408**	0.0154**	50.69^{ns}	0.00527**	72.09^{ns}	1685718353**
Y × B × C	2	9.43^{ns}	1.398^{ns}	35.07^{ns}	1653562.9^{ns}	155800.78^{ns}	0.804^{ns}	25118.51^{ns}	0.01735^{ns}	0.0028^{ns}	19.62^{ns}	0.00028^{ns}	763.3^{ns}	873279^{ns}
A×B×C	4	12.52*	5.259*	44.06**	5380605.8**	701741.01**	3.168*	78642.09**	0.07934**	0.0081**	91.76*	0.00160*	331.2^{ns}	242485881**
Y × A × B × C	4	3.62^{ns}	3.259^{ns}	7.86^{ns}	1646253.1^{ns}	117450.81^{ns}	1.118^{ns}	11050.76^{ns}	0.01047^{ns}	0.0010^{ns}	65.74^{ns}	0.00011^{ns}	1036.0**	1611251^{ns}
Error	68	4.22	1.711	11.94	1347149.6	194315.1	1.238	21469.91	0.01452	0.0016	32.59	0.00064	256.5	26449443
Coefficient of variation (%)		7.06	9.12	12.23	13.00	15.07	3.49	15.64	13.55	14.23	10.93	4.84	17.63	23.31

* Significant at the 5% probability level; ns, not significant, ** Significant at the 1% probability level.

Table 4. Comparison of two-year means of linseed traits by irrigation regimes, bacteria and mycorrhizae species.

Irrigation regime†	Bacteria	Mycorrhizal Fungi Species	Plant height (cm)	Number of branch plant^{-1}	Number of capsule plant^{-1}	Biological yield (kg ha^{-1})	Seed yield (kg ha^{-1})	Seed oil (%)	Seed oil yield (kg ha^{-1})	Seed water use efficiency (kg ha^{-1} m^{-3})	Oil water use efficiency (kg ha^{-1} m^{-3})	Colonization (%)	Leaf phosphorus (%)	Population of *P. putida* (cfu g^{-1})
60	Non-bacterial	non-mycorrhizal	29.28^{cde‡}	13^{bc}	22^{ef}	9310^{bc}	3019^{bc}	29.20^{gh}	881.66^{bcd}	0.58^{f}	0.16^{f}	19^{d}	0.54^{c}	125^{g}
		G. mosseae	33.58^{b}	15^{ab}	32^{bc}	11430^{a}	4319^{a}	31.50^{ef}	1362.16^{a}	0.83^{cde}	0.26^{cde}	61^{c}	0.59^{b}	191^{g}
		G. intraradices	39.05^{a}	16^{a}	36^{ab}	12370^{a}	4400^{a}	31.31^{ef}	1377.00^{a}	0.84^{cd}	0.26^{cde}	68^{bc}	0.62^{b}	308^{g}
	Pseudononas putida P13	non-mycorrhizal	27.95^{cde}	13^{bc}	22^{ef}	10520^{ab}	3170^{b}	31.52^{ef}	998.66^{b}	0.61^{ef}	0.19^{ef}	17^{d}	0.58^{b}	85830^{a}
		G. mosseae	34.06^{b}	14^{abc}	38^{a}	11233^{a}	3938^{a}	33.31^{cd}	1314.66^{a}	0.76^{cdef}	0.25^{cde}	62^{c}	0.69^{a}	47500^{c}
		G. intraradices	36.88^{a}	16^{a}	39^{a}	11250^{a}	3981^{a}	33.61^{bcd}	1341.50^{a}	0.76^{cdef}	0.25^{cde}	71^{bc}	0.70^{a}	62500^{b}
120	Non-bacterial	non-mycorrhizal	24.11^{f}	12^{cd}	18^{f}	7250^{cd}	2298^{cd}	32.24^{de}	737.83^{bcde}	0.71^{def}	0.23^{de}	17^{d}	0.43^{efg}	183^{g}
		G. mosseae	28.16^{cde}	16^{a}	30^{bc}	7900^{cd}	2626^{bcd}	35.22^{ab}	923.50^{bc}	0.82^{cde}	0.28^{bcd}	64^{c}	0.52^{c}	408^{g}
		G. intraradices	30.60^{c}	16^{a}	32^{bc}	8436^{cd}	2555^{bcd}	34.94^{abc}	893.66^{bcd}	0.79^{cdef}	0.27^{bcd}	70^{bc}	0.51^{c}	340^{g}
	Pseudononas putida P13	non-mycorrhizal	25.58^{ef}	12^{cd}	18^{f}	7255^{cd}	2298^{cd}	35.14^{ab}	808.66^{bcd}	0.71^{def}	0.25^{cde}	16^{d}	0.46^{de}	59170^{b}
		G. mosseae	30.26^{cd}	15^{ab}	32^{bc}	7906^{cd}	2434^{bcd}	34.93^{abc}	847.50^{bcd}	0.76^{cdef}	0.26^{cde}	76^{b}	0.60^{b}	26670^{e}
		G. intraradices	31.03^{c}	16^{a}	32^{bc}	10790^{ab}	3692^{a}	36.43^{a}	1343.00^{a}	1.15^{b}	0.41^{a}	83^{a}	0.59^{b}	35000^{d}
180	Non-bacterial	non-mycorrhizal	21.00^{g}	10^{d}	17^{f}	6730^{d}	2009^{d}	26.88^{i}	544.33^{e}	0.95^{bc}	0.25^{cde}	19^{d}	0.37^{h}	73^{g}
		G. mosseae	26.95^{def}	13^{bc}	24de	8070^{cd}	2969^{bc}	28.07^{hi}	833.66^{bcd}	1.41^{a}	0.39^{s}	70^{bc}	0.41^{g}	166^{g}
		G. intraradices	26.40^{ef}	16^{a}	32^{bc}	6980^{d}	2172^{cd}	29.06^{gh}	631.50^{de}	1.03^{b}	0.30^{bcd}	75^{b}	0.40^{g}	216^{g}
	Pseudononas putida P13	non-mycorrhizal	26.26^{ef}	12^{cd}	22^{ef}	7414^{cd}	2250^{cd}	29.19^{gh}	655.83^{cde}	1.07^{b}	0.31^{bc}	17^{d}	0.42^{fg}	28330^{e}
		G. mosseae	26.55^{ef}	15^{ab}	28^{cd}	7573^{cd}	2328^{cd}	30.50^{efg}	710.66^{cde}	1.11^{b}	0.33^{b}	60^{c}	0.45^{def}	17500^{f}
		G. intraradices	26.26^{ef}	14^{abc}	26^{cde}	7016^{d}	2177^{cd}	30.04^{fg}	653.50^{cde}	1.03^{b}	0.31^{bc}	68^{bc}	0.48^{d}	32500de

† Irrigation after evaporation from Class A pan.

‡ Means followed by the same letter in each column are not significantly different.

Table 5. comparison of two-year means of Spore numbers of AMF colonization affected by year × irrigation regime × bacteria×mycorrhizae.

	Irrigation regime†	Bacteria	Mycorrhizal Fungi Species	Number of spores (spore $10g^{-1}$)
2014			non-mycorrhizal	19 ^{f}
		Non-bacterial	*G. mosseae*	127 ^{abcd}
			G. intraradices	113 ^{bcd}
	60		non-mycorrhizal	12 ^{f}
		Pseudononas putida P13	*G. mosseae*	117 ^{abcd}
			G. intraradices	105 ^{cde}
			non-mycorrhizal	29 ^{f}
		Non-bacterial	*G. mosseae*	120 ^{abcd}
			G. intraradices	108 ^{cde}
	120		non-mycorrhizal	33 ^{f}
		Pseudononas putida P13	*G. mosseae*	95 ^{de}
			G. intraradices	114 ^{bcd}
			non-mycorrhizal	28 ^{f}
		Non-bacterial	*G. mosseae*	77 ^{e}
			G. intraradices	98 ^{de}
	180		non-mycorrhizal	20 ^{f}
		Pseudononas putida P13	*G. mosseae*	108 ^{cde}
			G. intraradices	125 ^{abcd}
2015			non-mycorrhizal	23 ^{f}
		Non-bacterial	*G. mosseae*	140 ^{abc}
			G. intraradices	125 ^{abcd}
	60		non-mycorrhizal	25 ^{f}
		Pseudononas putida P13	*G. mosseae*	118 ^{abcd}
			G. intraradices	119 ^{abcd}
			non-mycorrhizal	25 ^{f}
		Non-bacterial	*G. mosseae*	130 ^{abcd}
			G. intraradices	151 ^{ab}
	120		non-mycorrhizal	28 ^{f}
		Pseudononas putida P13	*G. mosseae*	155 ^{a}
			G. intraradices	138 ^{abc}
			non-mycorrhizal	27 ^{f}
		Non-bacterial	*G. mosseae*	140 ^{abc}
			G. intraradices	156 ^{a}
	180		non-mycorrhizal	25 ^{f}
		Pseudononas putida P13	*G. mosseae*	117 ^{abcd}
			G. intraradices	142 ^{abc}

† Irrigation after evaporation from a Class A pan.
Means followed by the same letter in each column are not significantly different.

Discussion

Yield components of linseed plants (plant height, the number of branches and capsules per plant) were affected by AMF. Furthermore, a mixture of AMF and bacterium improved the results more than they were for the non-inoculated control plants. However, the single presence of *P. putida* had no significant effect on the above-cited parameters. Improved plant growth parameters in AMF linseed plants and its combination with beneficial bacteria (Table 4) were reported in maize (Wu et al., 2005) and linseed (Neetu et al., 2012; Rydlova et al., 2011). Our findings suggest that dual inoculations (beneficial bacteria and AMF) seem to be more effective than single bacterial inoculations.

Mycorrhizal symbiosis clearly increased the yield of biomass, seed and seed oil compared with the non-mycorrhizal and bacterial plants under all three irrigation regimes. The results showed that AM colonization was the most important factor for linseed development, especially under water deficit condition due to a vital role in nutrient and water deficiency management of plants (Thingstrup et al., 1998; Ansari et al., 2016). The single inoculation with *P. putida* did not improve plant development. However, when this bacterium was co-inoculated with the AM fungi, we observe a positive advancement in yield and yield components as well as mycorrhizal symbiosis. Thingstrup et al. (1998) have shown that AMF is essential to flax growth at soil P levels below ca. 40 mg P kg^{-1}. In our study, the relative effects of both species of fungi (*G. intraradices* and *G. mosseae*) were almost identical. It is well documented that the application of mycorrhizal fungi led to an increase in water and nutrient absorption and transmission, special phosphorus to host plants cells and improved growth as well as photosynthesis which produce more assimilation (Mohamed et al., 2014).

Seed oil as the most important product of linseed was observed to be in its highest amount in mild-stressed plants which experienced a downward trend with severe stress and well-watered supplies. The compliance of water use efficiency with seed and/or oil yields could also be increased in mycorrhizal plants. Water use efficiency contributed to maximum yields in seed and oil of the linseed after infection and severs stress indicating water use efficiency. The consumptive use of water by the crop is dependent on availability of water and overall crop growth. The reduction in WUE with a decline in irrigation interval suggests poor efficiency of crop to utilize favorable environmental conditions towards economical yield formation (Sharma et al., 2012). It seems that inoculated plants made an optimal use of the consumed water (Habibzadeh et al., 2013).

Our microscopic study showed a high percentage of mycorrhizal colonization in roots of all mycorrhizal plants as well as the ones with dual bacterial infections. These results are in line with those reported by Rydlova et al. (2011) and Neetu et al. (2012). Both mycorrhizal species colonized linseed roots equally in all irrigation regimes. However, mild water-deficit stress increased root colonization to the highest value in dually infected plants. Despite non-significant effect of water deficit levels on spore density, it was greatly increased by AM inoculation treatments. Thus, maximum records of AMF infection percentage (83%) and numbers of AMF spores (156 spore 10 g^{-1} soil) were observed for linseed plants. Olsson et al. (1999) found that AMF made up as much as 50% of the total soil microbial biomass on a sandy soil under linseed (*Linum usitatissimum*). Previous studies showed a higher AM root infection rates in the presence of bacterial inoculation (Wu et al., 2005).

Even though the presence of bacterium had little impact on mycorrhizal colonization of linseed roots, co-inoculation with AMF significantly decreased *Pseudomonas* population in the soil. The bacterial community can change as a result of mycorrhizal organization (Marschner et al., 2001). Regardless of increasing effect of mycorrhizal inoculation on P-solubilizing bacteria density (Toro et al., 1998; Minaxi et al., 2013), further studies focusing on *Pseudomonads* showed that mycorrhizal fungi exhibited a neutral effect on bacteria depending on the AMF isolates. In the mycorrhizosphere (extra radical mycelium, leading to the formation of a specific zone of soil), the AMF might affect negatively (Cavagnaro et al., 2006; Rydlova et al., 2011), positively (Minaxi et al., 2013), or may have no effect on microbial biomass and the growth of specific microbial taxa. The combination of inoculants will not necessarily produce an additive or synergic effect, but rather a competitive process (Cavagnaro et al., 2006; Rydlova et al., 2011). These two groups of microorganisms should either interact positively with each other, or at least not be antagonistic (Ordonez et al., 2016). Negative effects of AMF on rhizosphere bacteria can be probably due to decreased carbon availability in the rhizosphere as a result of the carbon sink induced by mycorrhizal symbiosis (Rydlova et al., 2011).

Because of higher solubility of phosphorus in well irrigated soil (60 mm of evaporation), the leaf phosphorus content was reduced by greater irrigation intervals due to lower solubility, mobility (mass flow or diffusivity), transport between roots and shoots and thus P uptake in a drought condition (Sawers et al., 2008; He and Dijkstra, 2014; Wood and Silver, 2012). The greater P uptake in plants co-inoculated with PSB and AM fungus (Sabannavar and Lakshman, 2008) can be attributed to the transport of P by the AM fungus solubilized by PSB (Minaxi et al., 2013) due to a rise in root volume and more access to wider rhizosphere in order to facilitate absorption process and solution of insoluble P (Hassani et al., 2015).

Conclusion

The beneficial effects of the two mycorrhizae species in raising linseed growth and yield (biological, seed and seed oil) proved this crop to be a highly mycorrhizal plant as well as in dual inoculation (AMF plus PSB). The development of multi-functional microbial inoculants seems to be a promising method to increase the positive effects of microorganisms. In this study, the participation of microorganisms contributed to a higher yield of linseed (biological, seed and seed oil), P content, bacterial population, root colonization and fungal spore density compared to the control plants. Interestingly, the beneficial effects of bacteria were found to be significantly lower than those induced by AMF. WUE for seed and oil production, linked to the yield (seed and oil), was improved by dual inoculation treatments more than the single AMF and PSB inoculated linseed plants.

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