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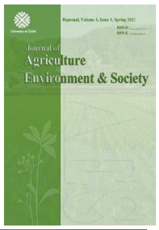

Effects of mycorrhiza symbiosis on seed yield and some physiological responses of chickpea genotypes

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ABSTRACT

An experiment was conducted in 2014 in the research field of Ferdowsi University of Mashhad, Iran, to investigate the triplet symbiosis of chickpea, mycorrhiza, and rhizobium, as well as the responses of chickpea genotypes to these inoculations. The experimental design was a split-plot with three replications based on a randomized complete block design. The main plot included three mycorrhiza levels as a biological fertilizer (Glomus mosseae, *Piriformospora indica*, and non-used), while the subplot included nine chickpea genotypes. When compared to other treatments, G. mosseae significantly increased seed yield and dry matter of chickpeas from mid-season onward. Arbuscular mycorrhiza significantly increased chlorophyll a and b levels, as well as carotenoids and SPAD levels. MCC537 outperformed the other genotypes in terms of seed yield and dry matter during the growing season and at harvest time. MCC537, MCC427, and MCC392 genotypes had the highest levels of carotenoids and SPAD readings. It appears that using G. mosseae in conjunction with rhizobium can improve the physiological traits and seed yield of chickpea.

1. Introduction

During the last few decades, the indiscriminate use of chemical fertilizers to achieve greater agricultural productivity has had adverse environmental consequences due to the increasing contamination of soil and water resources (Kranz et al., 2020). However, the idea of returning to nature and less use of chemical fertilizers and pesticides, and the incremental tendency for people to use organic products, have drawn more attention to the use of biological fertilizers. The use of biological resources instead of chemical resources has an important role in soil fertility and environmental protection (Zaidi et al., 2003). The use of bio-fertilizers is considered, including strategies to improve nutrient supply in sustainable agriculture. In other words, one of the main pillars of sustainable agriculture is the use of bio-fertilizers in agricultural ecosystems, with the approach of minimizing the use of chemical inputs (Sharma, 2002).

The importance of pulses in agricultural systems has long been known, but because of the environmental problems that are created in conventional agricultural systems, their importance has been increased. In today's agriculture, nitrogen is one of the main limiting factors for agricultural production (Lucisk et al., 2002). It has been indicated that nitrogen has the highest effect on increasing leaf area index and crop growth rate among essential elements. Nitrogen deficiency often occurs at critical growth stages of the plant, with symptoms such as yellowing of the leaf tissue (called chlorosis) (Arshadi and Asgharipour, 2011). However, pulses, because of the symbiosis with rhizobium bacteria, are self-sufficient in terms of supplying required nitrogen as carbon supply and have a significant role in maintaining nitrogen balance in the world (Voisin et al., 2013). Chickpea (Cicer arietinum L.) is the second most important food legume after dry beans, which are cultivated on more than 13.5 million ha in the producer countries and produce more than 8 million tons with a high protein content (22–24%) (FAOSTAT, 2018; Gaur et al., 2014). About 3.2% of the world's chickpeas are produced in Iran, and in this country, chickpeas among pulses have been allocated the biggest

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area under cultivation, at 641000 ha (FAOSTAT, 2018). However, both the irrigated and rain-fed yields of chickpea are low compared to other producing countries of this crop.

One of the most effective factors for increasing the seed yield of chickpea is a symbiosis with mycorrhiza. Rhizobium colony formation in roots by mycorrhizal fungi can provide a suitable condition for nodulation of rhizobium, because they enhance the availability of phosphorus for nitrogenase enzymes involved in rhizobium bacteria (Diouf et al., 2003). Mycorrhizal fungi are divided into two categories: endo-mycorrhiza (AMF: Arbuscular Mycorrhizal Fungi) and ecto-mycorrhiza (Abbott and Murphy, 2007). AMFs generally belong to the Zygomycetes classes (that are newly named Glomeromycetes) and ecto-mycorrhiza are mainly Basidiomycetes (Abbott and Murphy, 2007). Recently, a new species of ecto-mycorrhiza has been identified with the name *Piriformospora indica* that acts as an AMF and is an endophyte fungus (Verma et al., 1998). That is why it is called "pseudomycorrhiza." Some recent research has shown that symbiosis with P. indica increases the tolerance of crops to adverse environmental conditions (Baltruschant et al., 2008; Stein et al., 2008). Therefore, this study was performed to investigate the effects of different genotypes inoculated with rhizobium, AMF, and pseudo-endomycorrhiza on seed yield and some physiological characteristics.

2. Materials and methods

The experiment was carried out in 2014 at the Faculty of Agriculture, Ferdowsi University of Mashhad (Iran). For one year before conducting the experiment, the land was left fallow. The experimental design was a split-plot based on a randomized complete block design with three replications. Main plots consisted of three levels of mycorrhiza as a biological fertilizer (arbuscular mycorrhiza of Glomus mosseae, pseudo-endomycorrhiza of *Piriformospora indica*, and non-used mycorrhiza) and subplots consisted of nine genotypes of chickpea (high potential yield genotypes selected by the Institute of Plant Sciences, Ferdowsi University of Mashhad seed bank (Table 1).

Table 1. Traits of chickpea genotypes that were used in the experiment.

Identifying code in seed bank of lant Institute	Origin and name of genotypes
MCC* 80	Iran - 5311
MCC 358	Iran – Karaj cv.
MCC 361	Iran – Jam cv.
MCC 392	Iran – native lats of Kermanshah
MCC 427	Iran – native lats of Bojnurd
MCC 537	Iran – native lats of Gonabad
MCC 693	Iran
MCC 696	Iran
MCC 950	Iran – Hashem cv.

* Mashhad Chickpea Collection

Before planting, soil samples were taken to a depth of 0.3 m and characteristics including pH, organic matter, EC, soil texture, macro (N, P, K) and micro (Fe, Cu, Mn, Zn) elements were determined (Table 2). The soil texture of the experimental site was silt loam. Based on laboratory fertilizer recommendations, the phosphorus and potassium content of the soil was enough, so there was no need to apply phosphorus and potassium fertilizer. However, at the time of planting 40 kg/ha of urea as a starter and, a month before planting, 20000 kg/ha of manure were applied to improve soil organic carbon. After land preparation (including plow, disk, leveling, and furrower handling), plants were sown on March 11, 2014. The distance between and within rows was 0.1 and 0.5 m, respectively, and five rows were considered in each plot, with two rows set aside as a margin. The width of the plots was 7.5 m, and in each row, 75 seeds were planted by hand at a depth of 0.05 m. Seeds were disinfected with a 5% sodium hypochlorite solution. In order to infect the seeds with the symbiotic rhizobium bacteria of chickpea-*Mesorhizobium ciceri*, the bacterial inoculum was spread with seeds and uniformly used for all treatments.

In order to infect the soil with *G. mosseae*, in the treatment involved, 30 g of soil infected with fungal mycelium was poured onto the seed placement. To infect the soil with *P. indica*, after developing roots from 2-day-old germinating, seeds of chickpea were immersed in the source of the *P. indica* and then planted (Harrach, 2009). To apply *P. indica*, first its culture medium as a solid medium was prepared (Kumar et al., 2011). Then *P. indica* was inoculated on Petri dishes, and the plates were moved into incubators to grow and propagate.

Table 2. Characters of studied soil.

pH	EC (dS/m)	OC (%)	N (ppm)	P (ppm)	K (ppm)	Fe (ppm)	Cu (ppm)	Zn (ppm)	Mn (ppm)
7.29	4.26	0.87	861	38	413	7.62	1.52	1.41	17.15

Weeding was performed three times on May 7, 13, and 25 during the growing season. On May 14 and 20, diazinon concentrate was sprayed twice to control the Heliothis worm. For all treatments, irrigation was applied after planting, beginning of flowering, and beginning of pod forming (when the three plants of the three central rows of each plot had gone to flower, it meant flowering, and when three plants of the three central rows of each plot had gone to pod, it meant pod forming (IBPGR, 1993)).

The amounts of a, b, and total chlorophyll and carotenoids of leaves were determined by using a spectrophotometer after chlorophyll extraction with 80% acetone at 50% flowering, from a developed leaf at the top of the canopy (Wellbum, 1994; Nikolaeva et al., 2010). The results were expressed as 1 g of leaf fresh weight.

In the mid-flowering, the SPAD chlorophyll content of leaves was measured by monitoring three plants per plot. The tip of the leaflet of the first leaf, fully developed on the upper stem, was used for the chlorophyll meter. Measurements were made at a central spot on the leaflet, between the midrib and the leaf margin (Wu et al., 2006; Pepó and Vári, 2016). During the growing season at 32, 51, 65, 75, 81, and 87 days after planting, three plants were randomly selected from the upper half of each plot, and then the dry matter was assessed by placing them in an oven at 75°C for 48 h. Plants were harvested on the bottom half of each plot allocated for the yield evaluation, and grain yield was measured. It is noteworthy that the MCC80 genotype was ready to be harvested on June 21, 2014. On June 27, 2014, the MCC696, MCC693, MCC950, MCC358, MCC361, MCC392 and MCC427 genotypes were ready to be harvested, and the MCC537 genotype was ready to be harvested on June 29. Data analysis was performed by using M-STAT-C software, and mean comparisons were performed by using the Duncan test. Treatments were compared at the probable level that was significant as 1 or 5%.

3. Results and discussion

3.1. Dry Matter

At first sampling, biological fertilizer and chickpea genotypes did not significantly affect the dry matter (DM) of chickpeas. However, at the second (51 DAS–Day After Sowing), third (65 DAS), fourth (75 DAS), and fifth (81 DAS) samplings, the interaction of biological fertilizer and chickpea genotypes was significant ($P \leq 0.01$). The simple effects of biological fertilizer and chickpea genotypes were also significant at the sixth (87 DAS) sampling (Table 3). In these samplings, the DM of those exposed to rhizobium and mycorrhizal treatments was significantly higher than that of other plants. During the growing season, the most DM was considered in treatments of MCC537 and MCC427 + rhizobium with mycorrhiza (Table 5). On the other hand, since the second sampling (51 DAS) towards the end of the growing season, the DM of MCC537 and MCC427 genotypes were treated with rhizobium, and mycorrhiza was higher than the other genotypes (Table 5, Figure 1). It seems that these two genotypes have been more successful in communicating symbiosis with rhizobium and mycorrhiza than other genotypes. In the sixth sampling, there was no significant difference between treatments of rhizobium (alone) and rhizobium with pseudo-endomycorrhiza, but DM was significantly higher in the integrated application of rhizobium and mycorrhiza. Also, in the last sampling, the DM of MCC537 and MCC427 was significantly higher than the other genotypes, and MCC80 showed the lowest DM among all nine study genotypes. It seems that MCC537 and MCC427 genotypes were ranked first and second for DM production.

Table 3. ANOVA for DM.

S.O.V	df	1^{st} sampling of DM	2^{nd} sampling of DM	3^{rd} sampling of DM	4^{th} sampling of DM	5^{th} sampling of DM	6^{th} sampling of DM
Replication	2	0.120	0.436	11.43	147.4	34.2	485.7
Bio-fertilizer (A)	2	0.030 ^{ns}	37.79 **	3596 **	8233 *	15387 *	15912*
Error (a)	4	0.459	0.229	5.74	942.3	1016	1065
Genotype (B)	8	0.011 ^{ns}	38.30 **	4570 **	10420 **	16153 **	20873 **
A * B	16	0.017 ^{ns}	2.26 **	363.2 **	1082 **	2211 **	1811 ^{ns}
Error (b)	48	0.172	0.818	10.55	321.5	699.4	1200
Total	80	-	-	-	-	-	-
C.V. (%)	-	16.19	6.30	2.22	7.03	7.31	9.93

ns: non-significant *: significant in 5% level **: significant in 1% level

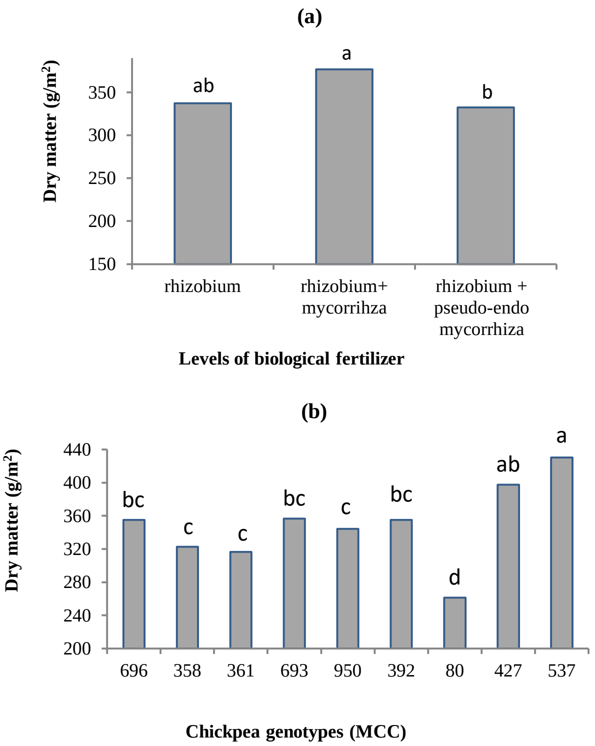
Table 4. ANOVA for traits of chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, SPAD readings and seed yield.

S.O.V	df	Chlorophyll a	Chlorophyll b	Total chlorophyll	Carotenoids	SPAD readings	Seed yield
Replication	2	0.0005	0.004	0.0002	0.001	0.197	9236
Bio-fertilizer (A)	2	0.012 **	0.007 *	0.021 ^{ns}	0.088 *	78.70 **	602275 **
Error (a)	4	0.001	0.001	0.004	0.010	1.495	11537
Genotype (B)	8	0.003 **	0.010 **	0.150 **	0.178 **	162.4 **	2313273 **
A * B	16	0.0003 ^{ns}	0.003 *	0.003 ^{ns}	0.024 ^{ns}	6.70 ^{ns}	72262 **
Error (b)	48	0.0004	0.001	0.002	0.018	12.01	15762
Total	80	-	-	-	-	-	-
C.V. (%)	-	7.04	12.66	7.56	6.46	12.72	8.29

ns: not-significant *: significant in 5% level **: significant in 1% level

3.2. Chlorophyll Content

The effect of biological fertilizer on the chlorophyll a of chickpea leaves was significant at a 1% probability level (Table 4). Chlorophyll a of rhizobium and mycorrhizal treatments was significantly higher than the other treatments, and in comparison with treatments of rhizobium (alone) and rhizobium with pseudo-endomycorrhiza, they increased chlorophyll a by about 11.53% and 12.30%, respectively. There was no significant difference between using rhizobium alone and rhizobium



Means with a common letter have not significantly different together based on Duncan's test at 5% for biological fertilizer and at 1% for chickpea genotypes.

Figure 1. Effect of biological fertilizer (a) and chickpea genotypes (b) on 6th sampling of DM.

with pseudo-endomycorrhiza (Figure 2a). The effect of biological fertilizer on chlorophyll b was significant at a 5% probability level (Table 4). The amount of chlorophyll b using rhizobium and mycorrhizal treatment was significantly higher than using rhizobium alone (Figure 2b). However, the effect of biological fertilizer on the total chlorophyll of the leaf was not significant (Table 4). The effect of chickpea genotypes on chlorophyll a of the leaf was significant (P<0.01) (Table 4). MCC537 showed the highest chlorophyll content of 0.3101 mg/g, but no significant difference was found between genotypes of MCC427, MCC392, MCC696, and MCC693 with MCC537. There were no significant differences between MCC361, MCC358, MCC80, and MCC950 (Figure 3a).

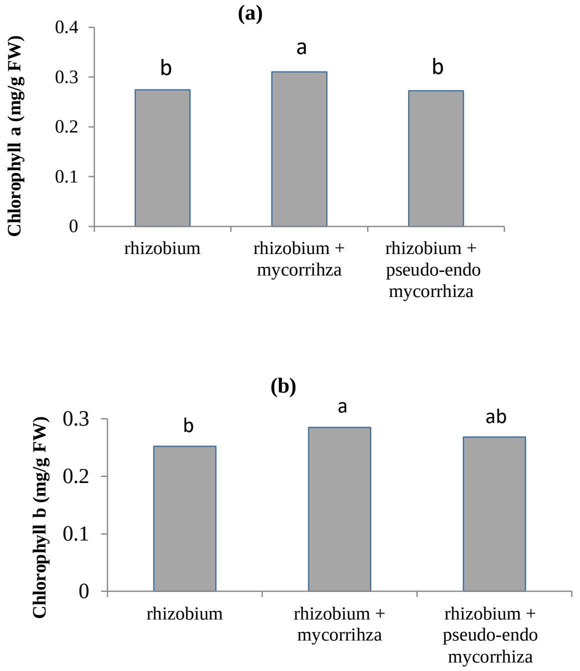
For chlorophyll b, genotypes of MCC427, MCC392, MCC80, and MCC537 were in the same significant class, and genotypes of MCC358, MCC361, MCC696, MCC693, and MCC950 were in the other significant class (Figure 3b). Moreover, the effect of chickpea genotypes on the total chlorophyll of chickpea leaves was significant at the 1% probability level (Table 4). The highest total chlorophyll was obtained for MCC537 and the lowest for MCC950. However, between genotypes of MCC537, MCC427, MCC392, MCC80, and MCC693 and also between genotypes of MCC950, MCC358, MCC361, and MCC696, there was no significant difference (Figure 3c).

Table 5. Mean comparisons for interactions of biological fertilizer and chickpea genotypes on DM, chlorophyll b and seed yield.

Biological fertilizer	(MCC) genotype	2nd sampling of DM (g/m²)	3rd sampling of DM (g/m²)	4th sampling of DM (g/m²)	5th sampling of DM (g/m²)	Chlorophyll b (mg/g FW)	Seed yield (kg/ha)
Rhizobium	696	14.80 c-g	141.9 gh	240.2 e-h	348.4 b-d	0.243 c-f	1134 i-k
	358	11.51 jk	118.1 mn	226.1 f-h	330.6 c-f	0.256 a-f	925 kl
	361	12.26 h-k	126.3 kl	230.8 f-h	325.7 d-f	0.213 fg	876 kl
	693	14.08 d-i	139.3 gh	246.1 d-g	349.3 b-d	0.150 g	1370 g-j
	950	13.36 e-j	137.0 hi	233.2 f-h	348.2 b-d	0.228 ef	790 l
	392	14.42 c-h	146.7 fg	249.2 d-g	360.0 b-d	0.283 a-f	1907 de
	80	13.33 e-j	124.4 k-m	208.0 gh	281.0 ef	0.291 a-f	1488 gh
	427	15.58 b-e	156.8 e	268.3 c-f	375.2 b-d	0.308 a-e	1957 c-e
	537	17.33 b	171.9 d	290.9 cd	396.3 bc	0.301 a-e	2085 b-e
Rhizobium+ Mycorrihza	696	15.22 b-f	152.5 ef	244.6 e-h	368.5 b-d	0.235 d-f	1187 h-k
	358	11.93 i-k	127.0 kl	237.4 f-h	342.0 b-e	0.259 a-f	1285 h-j
	361	12.94 f-k	128.3 j-l	231.3 f-h	332.9 b-f	0.246 b-f	1185 h-k
	693	15.22 b-f	151.7 ef	265.6 d-e	377.2 b-d	0.329 a	1431 g-i
	950	15.21 b-f	153.9 ef	270.6 c-f	367.9 b-d	0.228 h-j	1124 i-k
	392	16.28 b-d	166.8 d	284.3 c-f	390.5 b-d	0.325 ab	2352 b
	80	14.39 c-h	131.3 i-k	223.5 f-h	324.2 d-f	0.315 a	1615 fg
	427	20.26 a	205.7 b	338.5 b	480.1 a	0.301 e	2226 bc
	537	19.44 a	217.7 a	381.2 a	520.8 a	0.326 b-d	2730 a
Rhizobium+ Pseudo-endo mycorrihza	696	14.81 c-g	137.8 hi	247.3 d-g	355.1 b-d	00.241 h-j	1085 j-l
	358	10.69 k	140.6 gh	257.5 c-f	369.5 b-d	0.254 i-k	924 kl
	361	11.90 i-k	120.9 l-n	224.3 f-h	323.8 d-f	0.255 f-h	1094 j-l
	693	14.08 d-i	134.8 h-j	241.4 e-h	363.2 b-d	0.251 f	1375 g-j
	950	12.72 g-k	123.3 lm	239.1 e-h	353.7 b-d	0.232 f	1347 g-j
	392	12.40 h-k	138.6 hi	240.9 e-h	343.6 b-e	0.296 cd	1817 ef
	80	11.61 jk	113.7 n	198.8 h	273.1 f	0.280 de	1302 h-j
	427	15.18 b-f	158.3 e	269.0 c-f	369.4 b-d	0.303 a-c	2099 b-e
	537	16.67 bc	180.3 c	300.3 bc	400.6 b	0.302 a-c	2203 b-d

Means that have a common letter, have not significantly difference together based on Duncan's test at 1%.

The effect of chlorophyll a as a major factor in the center of photosynthetic reaction, the role of chlorophyll b as an antenna pigment, and the phenomenon of energy funnel have long been known (Taiz and Zeiger, 2006), and increasing their amounts by using a combination of rhizobium and mycorrhizal will affect the photosynthesis rate and thus crop photosynthetic capacity. As regards the three study genotypes of MCC537, MCC427, and MCC392, they accounted for the maximum amounts of three types of chlorophyll a, b, and total. It seems that these three genotypes have a high potential for the production of chlorophyll.



Means with a common letter have not significantly different together based on Duncan's test at 5% for chlorophyll b and at 1% for chlorophyll a.

Figure 2. Effect of biological fertilizer on contents of chickpea chlorophyll. (a) and (b) are effects of biological fertilizer on chlorophyll a and chlorophyll b, respectively.

3.3. SPAD Readings

The effect of biological fertilizer on the SPAD readings of chickpea leaf was significant at the 1% level (Table 4). So, SPAD readings of rhizobium and mycorrhizal treatments were significantly higher than the other treatments, and this treatment, in comparison with treatments of rhizobium (alone) and rhizobium with like-endo mycorrhiza, increased SPAD readings by an amount of 8.03% and 11.39%, respectively (Figure 4). According to the superiority of the characteristics of rhizobium and mycorrhizal treatment in tissue nitrogen of plants and chlorophyll (especially chlorophyll a), it seems reasonable to the superiority of SPAD readings of this treatment compared to other treatments. Because nitrogen is an integral part of the chlorophyll structure, and the chlorophyll meter SPAD-502 shows an estimate of the amount of nitrogen that is in leaf chlorophyll content.

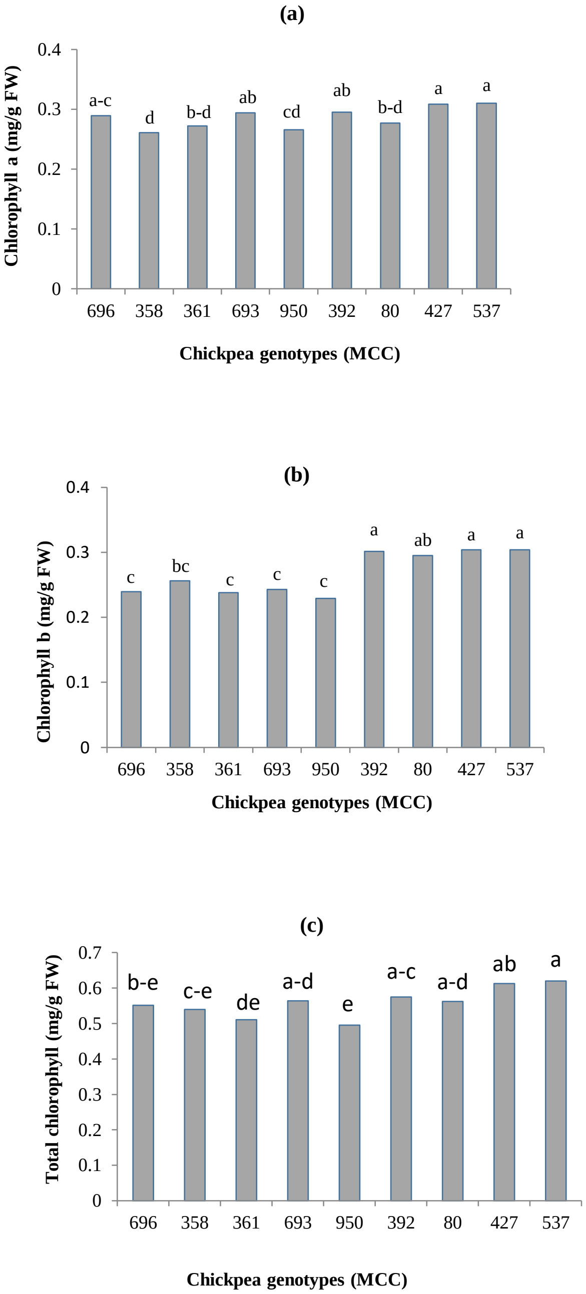


Figure 3. Effect of chickpea genotypes on contents of chickpea chlorophyll. (a), (b) and (c) are effects of chickpea genotypes on the chlorophyll a, chlorophyll b and total chlorophyll, respectively.

The effect of chickpea genotypes on the SPAD readings of chickpea leaves was significant at the 1% level (Table 4). The MCC537 genotype, with 33.84%, showed the highest SPAD readings. Of course, this genotype was in the same significant group as MCC427 and MCC392. On the other hand, the MCC358 and MCC361 genotypes compared with other genotypes in this study showed the lowest SPAD readings (Figure 5). Given that the chlorophyll meter SPAD-502 is an estimate of the amount of leaf chlorophyll (Martinez and Guiamet, 2004). It seems that some chickpea genotypes have a higher ability to synthesize chlorophyll in their leaves.

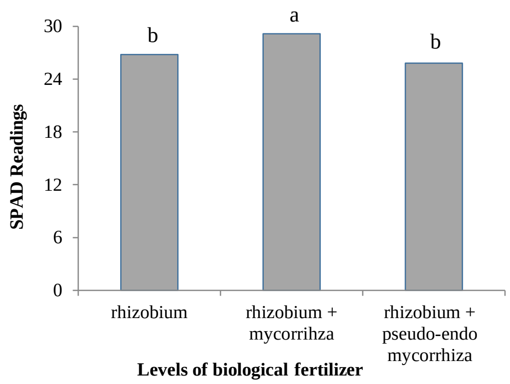


Figure 4. Effect of biological fertilizer on SPAD readings of chickpea carotenoids.

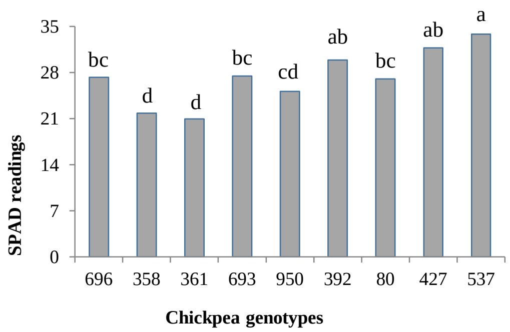


Figure 5. Effect of chickpea genotypes on SPAD readings of chickpea carotenoids.

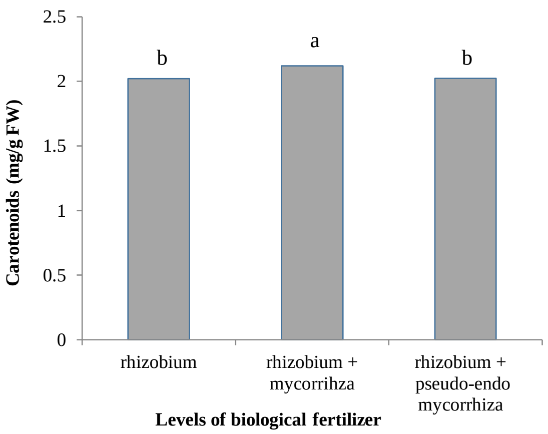


Figure 6. Effect of biological fertilizer on contents of chickpea carotenoids.

3.4. Carotenoids

The Effect of biological fertilizer on the carotenoids of leaves was significant at a 5% probability level (Table 4). Carotenoids of rhizobium and mycorrhizal treatments were significantly higher than the other treatments, and this treatment, in comparison with treatments of rhizobium (alone) and rhizobium with pseudo-endomycorrhiza, increased carotenoids by 4.71% and 4.62%, respectively. Indeed, there was no significant difference between treatments of rhizobium alone and rhizobium with pseudo-endomycorrhiza (Figure 6). At the 1% probability level, the effect of chickpea genotypes on leaf carotenoids was significant (Table 4).MCC537 had the highest carotenoid content, while MCC950 had the lowest. Indeed, between the genotypes of MCC537, MCC427, and MCC392, there was no significant difference. Also, there were no significant differences between MCC950, MCC358, MCC361, and MCC696 (Figure 7).

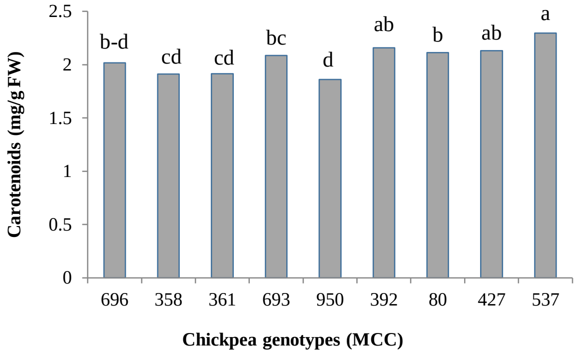


Figure 7. Effect of chickpea genotypes on contents of chickpea carotenoids.

Carotenoids are a large group of isoprene molecules that are made by all photosynthetic tissues and some non-photosynthetic organs. Carotenoids are divided into hydrocarbon carotenes, such as lycopene and beta-carotin or G-xanthophyll (Andrew et al., 2008). It seems that the combined use of rhizobium and mycorrhiza in providing substrate for the biosynthesis of carotenoids has been more successful than the other two treatments. Considering that the three genotypes of MCC537, MCC427, and MCC392 accounted for the maximum amount of carotenoids, it seems that the three genotypes have a high potential for the production of carotenoids. These results indicate that the chickpea genotypes have different capabilities in the production of photosynthetic pigments. In another study, the physiological responses of 35 chickpea genotypes were examined under drought stress and non-stress conditions. Carotenoids content of genotypes Flip03-63C, Flip03-87C, Flip05-59C, Flip05-153C, Flip05-74C, and Flip05-143C decreased less under drought stress than other genotypes in this study (Talebi et al., 2013).

3.5. Seed Yield

The interaction effect of biological fertilizer and chickpea genotypes on the seed yield of chickpea was significant at a 1% probability level (Table 4). Seed yield in the treatment of MCC537 + rhizobium with mycorrhiza was significantly higher than in other treatments (Table 5). Seed yield differences between genotypes studied in this research were ascribed to different capabilities of production potential in these genotypes. In other words, there was a considerable difference between chickpea genotypes in terms of yield potential. In general, almost all genotypes' seed yields were higher in treatments of integrated application of rhizobium and mycorrhiza than in treatments of rhizobium (alone) and rhizobium with pseudo-endomycorrhiza. These findings reflect the success of chickpea, mycorrhiza, and rhizobium triplet symbioses, as well as the better role of mycorrhiza in providing nutrients required for plant growth and the better distribution of photosynthesis products between chickpea photosynthetic organs and sinks compared to the other treatments. Zaidi et al. (2003) reported that the use of rhizobium along with the phosphate-solubilizing microorganisms and AMF causes an increase in rhizobium nodule dry weight, and that this function can enhance the seed yield of chickpea. It seems that rhizobium with mycorrhiza treatment, rather than the other two treatments, has been more successful in the absorption of water and nutrients for chickpea. This phenomenon increases the photosynthetic capacity of the plant and leads to a better allocation of photosynthetic products to the seeds of chickpea. Solaiman et al. (2005) concluded that the use of rhizobium along with arbuscular mycorrhiza for chickpea could cause an increase in seed yield because of increasing nodule dry weight in comparison to the application of rhizobium alone.

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