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Co-Inoculation of *Rhizobium* and *Azotobacter* on Growth Indices of Faba Bean under Water Stress in the Green House Condition

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Abstract

Biofertilizers can be expected to decrease the use of chemical fertilizers. The effects of native *Rhizobium legominosarum* bv *viciae* strain F46 and *Azotobacter chroococcom* strain AGO11 inoculation and nitrogen fertilizer was studied on growth and growth indices of faba bean *(vicia faba* L.*)* in water stress condition. The trial was conducted as a split plot factorial experiment in agricultural research station of Boroujerd. The results indicated that inoculation alone and co-inoculation of *Rhizobium* and *Azotobacter* increased most of growth indices such as: number of nodule, nodulation, total nitrogen content, relative water content, root dry weight, mean day germination and day germination speed. Co-inoculation of Rhizobium and Azotobacter enhanced water and nutrient uptake under the water stress, consequently it alleviated effect of shortage of water, hence it can be suggested that co-inoculation of *Rhizobium Legominosarum* and *Azotobacter chroococcum* can improved some of the growth indices faba bean under the water stress conditions.

Keywords: Inoculation, *Rhizobium, Azotobacter,* Water stress, Faba bean

Introduction

Legumes are well known for its important roles in maintaining productivity in agricultural systems (O'Hara 1998; Graham and Vance, 2000). Grain Legumes have a nitrogen fixing symbiosis with soil root nodule bacteria (Vance, 1997). Faba bean (*Vicia faba*, L.) is an annual legume that is consumed as plant foods for human and animal nutrition, because it is rich in protein. Faba bean cultivation in Iran is about 100,000 ha and its dry seed yield is about 2100 kg/ha. Faba bean like other legumes has a nitrogen fiaxation symbiosis relation with *Rhizobium Leguminosarum* bv. *viciae*. Zapata and et all, was reported that faba bean was obtained nitrogen needs itself , 79% by means of nitrogen fixation, 20% from soil and only 1% by means of fertilizer (Zapat et al.,1987).

Nitrogen is one of the most important elements for growth of plants. Nevertheless, there is 78000 tons of N_2 above on one hectare of the earth; nitrogen deficiency is one of the most important limited factors in agricultural production in Iran. Nitrogen is one of the most consumed chemical fertilizers in the world and Iran; in other hand these fertilizers are considered as a major environmental pollutant (Mc Isaac, 2003). Drought is one of the main limiting factors in agricultural development in arid and semi-arid regions such as Iran. Inoculation with *Rhizobium* increased the seed yield of faba bean in six areas in Australian (Carter et al., 1994) and the seed yield and weight of hundred seeds in Sudan (Elsheikh et al., 1997). Seed inoculation of some native *Rhizobium Leguminosarum* bv. *viciae* on faba bean in the south of Iran indicated that seed yield increased from 35% to 69% due to the inoculation (Khosravi et al., 2001). *Azotobacter chroococcum* is a free-living nitrogen fixing rhizobacteria that can promote the growth of various crops by some mechanisms such as production of gibberellic acid, indole-3 acetic acid (IAA), and cytokinin (Paul et al., 1999; Gonzalez-lopez et al., 1986) A. chroococcum strain DR_{26} increased ARA (acetylene reduction activity) of nodulated roots in faba bean over 100% when compared with control plants inoculated with Rhizobium alone ((Rodelas et al., 1999). The bacteria, as a controlling factor for pests, plant disease and having a stimulating effect on plant growth, help to seeds germination through producing hormones which encourage plant growth (Zahir et al., 2004).

The impact of plant growth promoting rhizobacteria on their ability to germinate and plantlet strength was analyzed and verified (Biswas et al., 2000; Apte and Shend, 1981).

Reported hat, maze seeds germination capacity is increased by means of inculation with different strains of *Azotobacter chroococcum*. Too claimed that synchronous inoculation of *Brady rhizobium japonicum and Azotobacter chroococcum* was augment the number and weight of root nodules and the weight of the shoot in soy (Vesey, 2003). It was demonstrated that *Bradyrhizobium. Japonicum* increases the germination among economically important plants of different families like maze, rice, sugar beet, soy, bean and cotton in laboratory, greenhouse and field conditions. Also, as one of he mast important effects and mechanism of the bacteria, the increase in root area and length following the application of plant growth promoting rhizobacteria was reported as these features can influence the penetration and searching abilities of roots in a larger mass of soil (Zahir et al., 2004). Inoculation with the strains of Brady rhizobium *improves nodulation, vegetative growth and nitrogen up take in soy, it was observed that the nitrogen containing the shoot increased move significantly through using a coat of similar bacterium than the control seeds (Meghvansi et al., 2005).The increase in the root expansion was perceived as the increasing ability to uptake food substances by application some of plant growth promoting rhizobacteria (Barbieri et al, 1986).*

Inoculation of Rhizobium, pseudomonas putida, P.fluorescens and Bacillus cereus increased significantly the plant growth and nodulation of faba bean (Tilak et al., 2004). Seed inoculation of faba bean with Rhizobium Leguminosarum bv.viceae and five different Azotobacter chroococcum under gnotobiotic conditions of culture resulted in significant effects on nodulation and plant growth at the flowering stage (Rodelas, 1999).

Mixed inoculation of vicia faba L. with four different Rhizobium / Azospirillum and Rizobium / Azotobacter combinations led to changes in total content , concentration and distribution of the mineral macro-and micronutrients, K, P , Ca, Mg, Fe, B, Mn, Zn and Cu, when with respect to plants inoculated with Rizobium lonely (Rodelas et al.,1999).

*Three – week – old nodulated faba bean plants were subjected to different levels of drought stress (one – half, one quarter, or one eighth field c*apacity) for five weeks. Half the stressed plants were treated with kcl at 10 mg kg^{-1} soil or 150 mg kg^{-1} soil at the beginning of the drought stress, nodulation and nitrogenase activity were significantly decreased by increasing drought stress. (Wahab & et al, 1995).

The main purpose of this study was evaluation of the effects of biofertilizers on growth and growth indices of faba bean for decreasing the effects of water stress in Boroujerd. This region is located in the west of Iran which is one of the main areas under faba bean cultivation.

Materials and Methods

Bacterial strains and cultural conditions

Azotobacter chroococcum strain AGO11 and *Rhizobium Leguminosarum* strain F46 prepared from gene bank of soil and water research institute of Iran. For preparing of Inoculants, *Azotobacter chroococcum* was growth on winogradsky medium (Garrity et al., 2005) and the *Rhizobium Leguminosarum* bv. *viciae* was grown on yeast manitol Agar (Vincen, 1970) for 48 hours in $28C^0$ in rotary shaker. The suspension of *Rhizobium* and *Azotobacter* throughly mixed with perlite as carrier separately and incubated in $28C^0$ for 72h. The weight of package *Rhizobium* and *Azotobacter* was considered 500gr and contained at least 10^8 viable cells of *Rhizobium* and *Azotobacter* per gram of inoculant. Before inoculation the seeds surface was mixed with 15% sugar completely for more adhesion of inoculums. Finally the seeds were inoculated and mixed thoroughly with *Azotobacter* and *Rhizobium* inoculums. Formerly some of plant growth promoting characteristics of *Rhizobium* and *Azotobacter* which used in this study was evaluated (Khosravi et al., 2010; Khosravi, 2009)

Irrigation

All of the pots were irrigated immediately after sowing. The other irrigation was applied after 3, 5 and 7 day for each block with the same amount water for each plots.

Experimental Design and Statistical Methods

The experiment was carried out as a split plot factorial experiment in a completely randomized block design with 3 replicates. The treatments included three irrigation levels after 3, 5 and 7 day with the same amount water as main plots and *Rhizobium, Azotobacter* and nitrogen fertilizer each contain two levels (used and not used) as sub plot (2×2×2×3×3 = 72 pots). The weight of each pot was 5 kilogram. The data were analysed by SAS/STAT software and the means were compared by Duncan’s multiple range test.

Plant Sampling

The crops were harvested in middle of flowering stage. In this stage, growth indices such as: mean dry germination, day germination speed, coefficient velocity germination, the nodulation, number of nodule/pots, total N content, relative water content, root dry weight/pots and shoot dry weight/pots were determined. Nodulation was measured in based of table 1.

Measurement of nodulation

To measure nodulation, the following table is used:

Measurement of relative water content

To measure the relative water content, the following formula is used:

$$RWC = \frac{fresh\ leaf\ \ weight - \ dried\ leaf\ \ weight}{swollen\ leaf\ \ weight - \ dried\ leaf\ \ weight} \times 100$$

Measurement of mean time for germination

To measure the mean time for germination which is a factor or index of velocity and germination acceleration, the following equation is used (Ellis and Robert, 1981).

$$MTG = \frac{\sum (nd)}{\sum N}$$

Where n is the number of seeds germinated in d days, d is the number of days since the early germination,ΣN is the total number of germinated seeds.

Measurement of Mean daily germination

The following equation is used to measure the mean daily germination which is an indicator of germination velocity Scott, 1984).

$$MDG = \frac{FGP}{d}$$

Where FGP is the final germination percentage and d is the total number of days (duration of experiment period).

Measurement of daily germination speed

This index is the opposite of mean daily germination and is measured by the following equation. (Hunter, 1984).

$$DGS = \frac{1}{MDG}$$

Measurement of coefficient germination velocity (CvG)

This index which is the indicator of velocity and acceleration of germination is measured by the following equation (Maguire, 1962).

$$CVG = \frac{G_1 + G_2 + G_3 + \cdots + G_n}{(1 \times G_1) + (2 + G_2) + (3 + G_3) + \cdots + (n \times G_n)}$$

Where G_1- G_2 is the number of seeds germinated from the first day until the last day.

Results: The impact of different irrigation treatments on the mean time germination and mean daily germination suggests that as the irrigation period increases, the mean time germination augments so that in irrigation after 3 day treatment has the lowest time (days 7.45) and after 3 day treatment has the highest. (14.92 days). The mean daily germination increases as irrigation period

decreases, with the highest for treatment after 3 day treatment (9.92) and the lowest for treatment after 5 day treatment (6.9 days) (Table 2). As irrigation period increases (higher water stress), some was added to the percentage of nitrogen in the shoot so that the maximum and minimum percentages of nitrogen observed in the shoot were in treatments after 7 day treatment (3.9) and after 3 day treatment (3.38) respectively.using Rhizobium has increased the nitrogen content in the shoot significantly, this increase was 4.8 percent higher than the control treatment.(Table, 2). Further more, Rhizobium use increases such indices as the number of nodule, nodulation and relative water content of leaf. (Table, 2). The results were verified by Dakora (Dakora, 2003). Shorter mean time germination and more mean daily germination were brought about by the using of Rhizobium (Table, 2). Inoculated with Azotobacter induced A 10 – percent increase in root dry weight and 1% probability for the significant increase in nitrogen content of the shoot (Table, 2). In a study carried the same results were acquired (Subba Rao and et al, 1993). Azotobacter using has increased the number and the degree of the nodules, in which the highest values with using Azotobacter treatment were 2.91 and 1.45 respectively, and the lowest values with no using Azotobacter treatment were 2.65 and 0.95 respectively (Table, 2). *Azotobacter chroococcum* strain AGO11 had significant effects on growth and yield of wheat in field study in some parts of Iran (Khosravi, 2009). The interaction of irrigation and Rhizobium on root dry weight was significant, in which the highest values in treatment irrigation with a 3- day period and Rhizobium use were 8.12 g, while the lowest values in treatment 7-day irrigation period and no Rhizobium use
Were 568g, meanly synchronous inoculation Azotobacter and Rhizobium raised root dry weight amount of 26.4% Compared to control treatment (figure 1, d)
(Figure 1, d). Also, the longer and shortest for mean time germination in treatment 7-day irrigation period and no Rhizobium use and 3- day period and Rhizobium use treatments were 16 and 7.5 days respectively (Figure 1,c). The synchronous impact of Azotobacter and irrigation on root dry weight was significant at 5% level, in which the highest weight in 3- day irrigation period and Azotobacter use treatment was 8.01 g and the lowest in 5 – day irrigation period and with no Azotobacter use was 5.28 g. (figure 1, e) The result was confirmedly (Zahir et al., 2004).

Too, argued that the application of PGPR increased root area and length. Moreover, using both Azotobalter and rhizobium simultaneously has increased the relative water content in the leaf. A 3% - increase in co-inoculated Rhizobium and Azotobacter treatment
(0.77) was found compared to control treatment (0.74). It suggests that the plant is tolerant of water deficiency. (Figure 1, g). The mean germination time and the mean daily germination were affected by the interaction of irrigation, nitrogen and Rhizobium, and the difference was significant at 1 % level. Here, the highest and lowest number of days for synchronous nitrogen, Rhizobiom and 3- day irrigation period were 10.76 and 6.95 days respectively (Table 3). It was proved that germination ability and plantlet and strength were raised by PGPR (Biswas, 2000). The results obtained after the triple action of irrigation, Rhizobium and

Azotobacter indicate that the highest nodule dry weight, 0.67g , was obtained under stress-free condition and the synchronous uptake of Azotobalter and Rhizobium, namely via using co-inoculation Rhizobiom and Azotobacter and 3-day irrigation period treatment. In an experiment, it was pointed out that an increase in soy number and nodule weight was made via the synchronous inoculation of *Bradi rhizobium japonicum* and *Azotobacter chroococcum* (vessey, 2003).The results show that water stress conditions have reduced nodule number and dry weight. However, the impact of stress was diminish by means of the synchronous use of Azotobacter and rhizobium so that in no use Rhizobium ,Azotobacter and 7-day irrigation period treatment, the nodule's dry weight was 0.37g but rose to 0.52 g through the synchronous use of Azotobncter , Rihizobium and 7-day irrigation period treatment. Wahab et al. (1995) posited that the growth in land stress reduces nodulation.

Furthermore, relative water content in leaf was affected by the stress and the difference between the treatments was significant at 1%. In this case, the highest level in co-inoculation Rhizobiom and Azotobacter and 3- day irrigation period treatment and the lowest in with out Rhizobiom ,Azotobacter use and 7- day irrigation period treatment were 0.91 and 0.6 respectively (Table, 3) there fore, this synchronous use of Azotobacter and rhizobium has partially reduced the negative effects of land stress due to such factors as the increase in water and nutrient absorption as well as root expansion; fore example, in with out Rhizobiom and Azotobacter and 7- day irrigation period treatment, relative water content in leaf is 0.61 where as that of co-inoculation Rhizobiom and Azotobacter and 7- day irrigation period treatment is 0.74. (Marcia et al. 2008) postulated that compound inoculation can partially reduce the effects of land stress on faba bean.

Discussion: Root dry weight was augment by Rhizobium, the result is logical because the application of plant growth promoting bacteria can result in larger root area and longer root(Zahir et al; 2004) has studied and confirmed the conclusion. Too the mean time germination and the mean daily germination were affected by Rhizobium. It was proved that germination ability were raised by plant growth promoting bacteria such as Rhizobium (Biswas, 2000). Prior to this research (Khosravi et al., 2001 and 2004). Reported that inoculation of faba bean with indigenous *Rhizobium Leguminosarum* bv. *viciae* in the south and north of Iran increased significantly the seed yield. Previously some of the plant growth promoting characteristics of *Rhizobium* strain was evaluated which used in this study such as production of auxin and ACC deaminase enzyme (Khosravi et al., 2010).

Co-inoculation of *Rhizobium* and *Azotobacter* significantly increased some of the growth indices such as total N content, root dry weight, nodule dry weight, and relative water content and reduce mean time germination. Mixed inoculation of faba bean with *Rhizobium* / *Azospirillum* and *Rizobium* / *Azotobacter* combinations led to changes in total content of K, P, Ca, Mg , Fe, B, Mn, Zn and Cu, (Rodelas & et al., 1999). Seed inoculation of faba bean with *Rhizobium Leguminosarum* bv.*viceae* and five different *Azotobacter chroococcum* under gnotobiotic conditions of culture resulted in significant effects on nodulation and plant growth at the flowering stage (Rodelas, 1999). In an experiment, it was pointed out that an increase in soy number and nodule weight was made via the synchronous inoculation of *Brady rhizobium japonicum* and *Azotobacter chroococcum* (vessey, 2003).

Conclusion

The results show that water stress conditions have reduced nodule number and nodule dry weight. However, the impact of water stress was diminish by means of the co-inuculation *Azotobacter* and *Rhizobium.* Free stress irrigation increased most of the growth parameters, as well as co-inoculation with *Rhizobium* and *Azotobacter* increased water and nutrient uptake under the deficit-irrigation because it alleviated effect of shortage of water. In general, application of *Rhizobium legominosarum* bv. *viciae* strain F46 and *Azotobacter chroococcum* strain AGO11 on faba bean under deficit-irrigation in Boroujerd region is recommended.

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Table 1: Measurement of nodulation (Beck, etal.1993)

	Distribution and number of effective nodules	
Nodulation score	Crown of root*	Elsewhere of root
0	0	0
0.5	0	1-4
1	0	5-9
1.5	0	>10
2	Few	0
2.5	Few	Few
3	Many	0
4	Many	Many
5	Many	Many

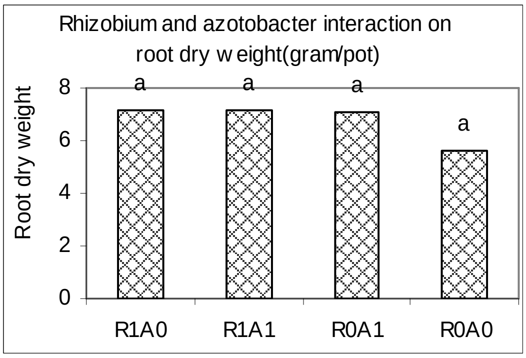
* Consist of 7cm top of the root system

Table 2: Mean comparisons of main effect of Irrigation, Nitrogen, *Rhizobium* and *Azotobacter*, Means with the same letter are not significantly different

Treatments	N (%)	RWC	MTG	MDG	CVG	DGS	NN/ pot	N	RDW (g / pot)	SDW (g / pot)
Irrigation										
I3	3.9a	0.62c	14.92a	6.9c	0.21b	0.14a	2.58a	1.2a	6.59a	8.52a
I2	3.59ab	0.74b	12.15b	8.15b	0.23b	0.12b	2.71a	1.14a	6.15a	7.97a
I1	3.38b	0.86a	7.45c	9.92a	0.30a	0.10c	3.06a	1.27a	7.52a	9.75a
Nitrogen										
-N	3.69a	0.73b	11.65a	8.2a	0.25a	0.12a	2.74a	1.23a	6.96a	8.89a
+N	3.56a	0.75a	11.37b	8.44a	0.25a	0.12a	2.83a	1.18a	6.54a	8.61a
Rhizobium										
-R	3.54B	0.73b	11.09b	8.05B	0.24A	0.12a	2.54B	1.16A	6.36A	9.41a
+R	3.71A	0.75a	11.92a	8.59A	0.25A	0.118b	3.03A	1.25A	7.14A	8.08b
Azotobacter										
-A	3.35b	0.73a	11.4a	8.4a	0.25a	0.121a	2.65A	0.95B	6.4A	9.02a
+A	3.9a	0.75a	11.62a	8.24a	0.25a	0.123a	2.91A	1.45A	7.11A	8.47a

(N %): Total nitrogen content (%); RWC: Relative water content; MTG: Mean time germination; MDG: Mean day germination; CVG: Coefficient velocity germination; DGS; Day germination speed; NN/Pot: number of nodule per pot; N: Nodulation; RDW: Root dry weight; SDW: Shoot dry weight.

b


Rhizobium and azotobacter interaction on
root dry w eight(gram/pot)
Root dry weight
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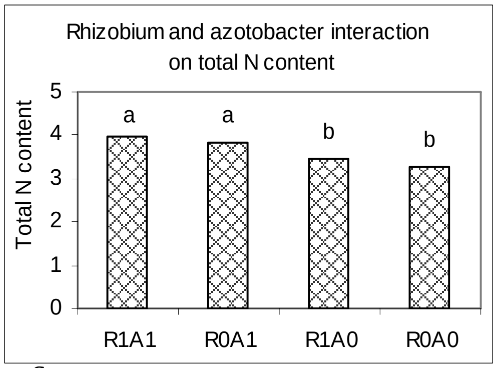
Rhizobium and azotobacter interaction
on total N content
Total N content
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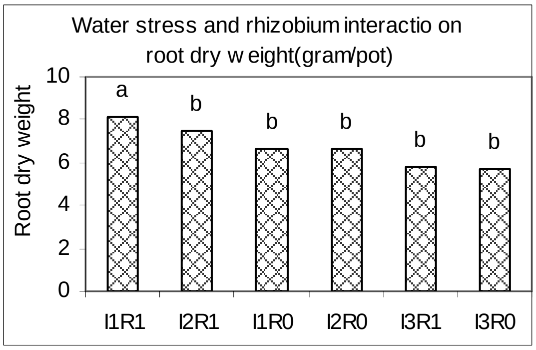
d


Water stress and rhizobium interactio on
root dry w eight(gram/pot)
Root dry weight
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6
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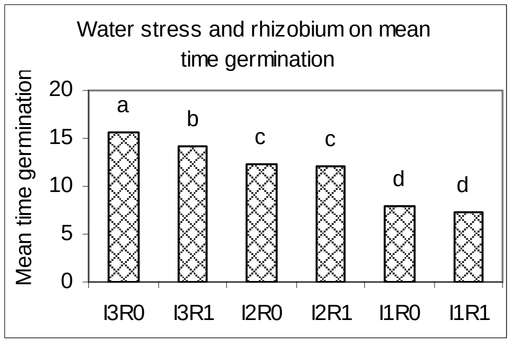
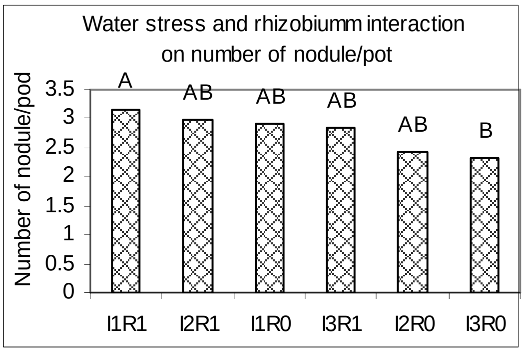
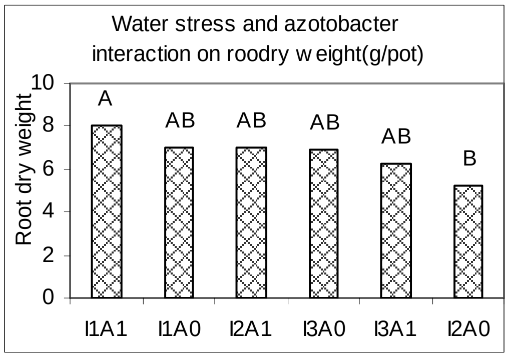
c


Water stress and rhizobium on mean
time germination
Mean time germination
20
15
10
5
0
a
b
c
c
d
d
I3R0
I3R1
I2R0
I2R1
I1R0
I1R1

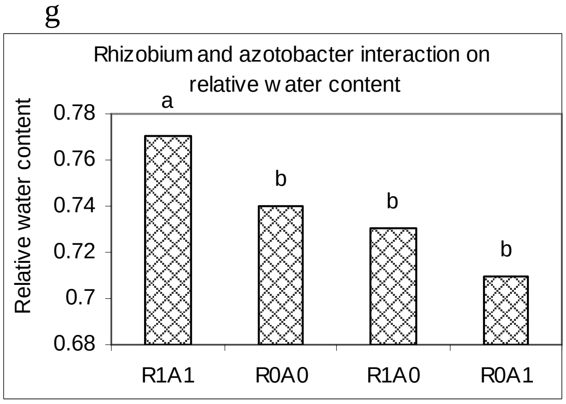
f


Water stress and rhizobiumm interaction
on number of nodule/pot
Number of nodule/pod
3.5
3
2.5
2
1.5
1
0.5
0
A
AB
AB
AB
AB
B
I1R1
I2R1
I1R0
I3R1
I2R0
I3R0

e


Water stress and azotobacter
interaction on roodry w eight(g/pot)
Root dry weight
10
8
6
4
2
0
A
AB
AB
AB
AB
B
I1A1
I1A0
I2A1
I3A0
I3A1
I2A0

g


Rhizobium and azotobacter interaction on
relative w ater content
Relative water content
0.78
0.76
0.74
0.72
0.7
0.68
a
b
b
b
R1A1
R0A0
R1A0
R0A1

Treatments	N	RWC	MTG	MDG	Treatments	RWC	NDW (g/pot)	MTG
I1N0R0	0.83 AB	0.84 b	8.24 d	9.18 ab	I1R0A0	0.85 b	0.56 ab	7.18 d
I1N0R1	1.41AB	0.86 b	7.25 d	9.93 a	I1R1A0	0.85 b	0.65 a	6.97 d
I1N1R0	0.75 B	0.84 b	7.17 d	9.81 a	I1R0A1	0.82 b	0.56 ab	8.41 d
I1N1R1	2.08 A	0.91 a	6.95 d	10.76 a	I1R1A1	0.91a	0.67 a	7.22 d
I2N0R0	1.50 AB	0.73 c	12.18 c	8.11 bcd	I2R0A0	0.74 c	0.47 ab	12.55 bc
I2N0R1	0.83 AB	0.73 c	12.03 c	8.21 bc	I2R1A0	0.73 c	0.61 ab	12.17 c
I2N1R0	1.08 AB	0.74 c	12.36 c	8.03 bcd	I2R0A1	0.73 c	0.55 ab	12 c
I2N1R1	1.16 AB	0.76 c	12.05 c	8.23 bc	I2R1A1	0.76 c	0.65 a	11.91c
I3N0R0	1.16 AB	0.60 d	15.48 b	6.69 ed	I3R0A0	0.61 d	0.37 b	15.45 a
I3N0R1	1.60 AB	0.63 d	14.55 b	7.09 cde	I3R1A0	0.62 d	0.48 ab	14.08 ab
I3N1R0	1.16 AB	0.61d	15.93 a	6.50 e	I3R0A1	0.60 d	0.47ab	15.96 a
I3N1R1	0.83 AB	0.63 d	13.75 a	7.34 cde	I3R1A1	0.74 c	0.52ab	14.21 ab

Fig.1:Mean comparisons of double interactions for total N content (a), root dry weight (b, d and e), mean time germination (c), number of nodule/pot (f) and relative water content (g) by Duncan' s multiple range test. Means with the same letter are not significantly different

Table 2: Mean comparisons for triple interaction for: Relative water content (RWC); Mean time germination (MTG); Mean day germination (MDG); Nodulation (N) and Nodule dry weight (NDW).
By Duncan' s multiple range test. Means with the same letter are not significantly different.

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