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CO-INOCULATION OF ARBUSCULR MYCORRHIZAE AND NITROGEN FIXING BACTERIA ENHANCE ALFALFA YIELD UNDER SALINE CONDITIONS

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Abstract

The study was to investigate the effects of combined inoculation of Glomus mosseae (arbusculr mycorrhizae fungi, AMF) and Sinorhizobium meliloti (nitrogen-fixing bacteria, i.e., an Rhizobium meliloti, RM) on yield, nutrient contents, nodulation and mycorrhizal colonization of different alfalfa cultivars under saline conditions. An experiment was conducted to test the efficacy of AMF and RM inoculation in development of salt tolerance in alfalfa cultivars (Zhaodong, Nongjing and Longmu) under different salinity levels (0, 60, 120 and 180 mM NaCl). We found that under non stress condition, double inoculation of alfalfa with rhizobium and AM increased the alfalfa yield, nodule weight and number, as well as shoot proline contents, the most when plants were double inoculated followed by AM and rhizobium inoculation, respectively. Whereas under salinity condition, double inoculation of alfalfa with rhizobium and AM increased alfalfa yield, mycorrhizal infection, nodule weight and number as well as increased in shoot proline content, the most followed by AM and rhizobium inoculation, respectively. The results suggest that growth of alfalfa may be improved by combined inoculation of alfalfa with AM and rhizobium under salt and non-stress conditions. Alleviation of alfalfa growth under saline condition was perhaps due to an increase in mycorrhizal infection and nodule weight and number as well as an increased in shoot proline content by dual inoculation.

Key words: Arbuscular mycorrhizal fungi (AMF), Rhizobium meliloti (RM), Alfalfa (*Medicago sativa* L.) yield, Salinity.

Introduction

All over the world, more than 77 million ha of the world's agricultural land were exposed to salt stress, which causes reduction in growth of crop plants through nutritional imbalance (Hamdia & Shaddad, 2010). Salt damage has become an important issue of world agriculture in the twenty-first century (Hamdia & Shaddad, 2010). Reclamation, drainage and improved irrigation practices, soil amendments, using salt-tolerant plants, conventional and modern plant breeding techniques may provide possible solutions to the salinity problems in the salt-affected areas (Hasegawa *et al.*, 2000). However, these methods are expensive and/ or very time consuming. Therefore, application of biological processes such as mycorrhizae may offer a cost-effective long term solution to improve productivity in saline lands. Plants are colonized both by external and internal microorganisms such as beneficial bacteria and fungi in their natural environment that can increase plant stress tolerance (Grant *et al.,* 2001; Midrarullah *et al.,* 2014, 2015). Arbuscular mycorrhizal fungi (AMF) are associated with the roots of over 80 % terrestrial plant species (Grant *et al.*, 2001). Mycorrhizal application has proved to be an appropriate alternative to alleviate salt stress. AMF improve salinity tolerance by employing various mechanisms, such as enhancing nutrient uptake, especially P nutrition (Giri *et al.*, 2002), producing plant growth hormone (Grant *et al.*, 2001) , improving rhizospheric and soil conditions (Giri *et al.*, 2002), altering the physiological and biochemical properties of the host plants (Grant *et al.*, 2001), defending roots against soil-borne pathogens (Marulanda *et al.*, 2003), improving host physiological processes (Asghari *et al.*, 2005) and enhancing nodulation and nitrogen fixation in legumes (Al-Karari *et al.*, 2001). In addition, AMF colonization can reverse the effect of salinity on K^+/Na^+ ratio by enhancing K^+ absorption under saline conditions (Sharifi *et al.*, 2007) and/ or preventing Na^+ translocation to shoot tissues. These benefits have prompted AMF to be a suitable candidate for bio-amelioration of saline soils.

Alfalfa is the most important forage crop in the world due to its high yield, nutritional quality, high crude protein (CP) content and its adaptability to a wide range of soil and climatic conditions (Howieson & Ballard, 2004). This plant is also widely cultivated in arid and semi-arid regions where salinity is a major limitation for crop production (Parvaiz & Satyawati, 2008). The gram-negative soil bacterium *Sinorhizobium meliloti* is able to interact with roots of alfalfa to form nitrogen-fixing nodules (Elboutahiri *et al.*, 2010; Biondi *et al.*, 2003). Soil salinity adversely affects the nodulation and nitrogen fixation capacities of rhizobia, results in lower productivity of the host legume (Scheublin & Vander Heijden, 2006). Alfalfa may benefit from symbiotic associations with nitrogen fixing bacteria (*Rhizobium meliloti* (RM)) and arbuscular mycorrhizae fungi (AMF) forming tripartite symbiosis. Plants benefit from this relationship in many ways including enhanced plant growth and nutrient content especially N and P (Tian *et al*., 2004). In addition, it has been shown that plant benefits derived from the tripartite symbiosis are superior to that of inoculated with either AMF or rhizobium alone (Giri *et al.*, 2002).

Rhizosphere / mycorrhizosphere system can therefore help plants to survive under stress conditions such as salinity stress (Evelin *et al*., 2009) and drought (Hildebrandt *et al*., 2001). Alleviation of salt stress in alfalfa by AMF has been reported (Evelin *et al*., 2009).

However, the role of combined inoculation of AMF and *Sinorhizobium meliloti* in alleviating salt stress in alfalfa cultivars has not been well investigated. Therefore, the objective of this study was to investigate the effects of combined inoculation of *Glomus mosseae* mycorrhizal fungi and *Sinorhizobium meliloti* on yield, nutrient contents, nodulation and mycorrhizal colonization of three alfalfa cultivars under saline conditions.

Materials and Methods

Experimental conditions: In this study, the experiment was laid out as factorial in a completely randomized block design with 3 replications. The treatments were included of four inoculations (mycorrhizae, rhizobium, mycorrhizae + rhizobium and control), three alfalfa cultivars (Zhaodong, Nongjing and Longmu) and four salt levels (0, 60, 120 and 180 mM NaCl). Soil texture are Loam-Clay with EC 1.8 $ds \cdot m^{-1}$, N 0.044%, P 16.0 $mg \cdot Kg^{-1}$, K 265.5 $mg \cdot Kg^{-1}$ and pH 7.8. Soil was passed through a 2 mm mesh sieve, mixed thoroughly and autoclaved (110 ˚C, 1 h, twice at 48 h intervals) to remove indigenous AM propagules and rhizobium. The carrier material for rhizobium inoculant was sterilized peat based containing minimum 2 × 109 viable cells of selected *Sinorhizobium meliloti* per gram.

Seeds lots were inoculated with 25 g of *S. meliloti* inoculum. The AMF inoculant of *Glomus mosseae* was provided by Heilongjiang Academy of Agricultural Sciences, Harbin. The pure cultures of *Glomus mosseae* was based on isolates from Harbin. Each pot was inoculated with 30 g inoculum for mycorrhizal treatment.

After 60 days of planting, plants were harvested and washed thoroughly and shoot and root dry weights, proline concentrations, nodules number and weight and root mycorrhizal infection were measured. The percentage of mycorrhizal root infection was estimated after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactophenol (v/v), according to Phillips & Hayman (1970). Nodule dry weight was measured after drying in a forced drought oven at 70 °C for 48 h. Proline content was estimated following the method of Bates *et al*. (1973). Fresh leaves (0.5 g) were extracted in 3% sulphosalicylic acid and the homogenates were centrifuged at 10,000 g for 10 min. After centrifugation, 2 ml of the supernatant was reacted with 2 ml of acid ninhydrin reagent and 2 ml of glacial acetic acid in a test tube for 1 h at 100°C and the reaction terminated in an ice bath. The reaction mixture was extracted with 4 ml of toluene and mixed vigorously with a vortex mixture for 15–20 s. The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and the absorbance was measured at 510 nm using toluene as blank. Proline concentration was calculated from a standard curve using 0–100 µg L-proline (Sigma).

Statistical analysis: Data were subjected to normal distribution tests and analysis of variance and least significant difference (LSD) for comparison of means were performed. All analyses were performed using SPSS 18.0 for Windows (SPSS, Chicago, Illinois, USA).

Results

Nodulation: The number of nodules per plant and nodule weight depended on the cultivar, salinity level, inoculation, cultivar × inoculation, cultivar × salinity level, inoculation × salinity level and cultivar × inoculation × salinity level (Table 1). Without salt treatment and in the absent of mycorrhizae, number of nodule per plant and nodule weight were 83 and 87 mg, while with double inoculation these values were 124 and 129 mg, respectively (Figs. 1, 2). The number and the weight of nodules were decreased with increasing salt level, however, the reductions were less when the plants were double inoculated followed by rhizobia inoculation (Figs. 1, 2) Generally, nodules per plant and nodule weight with or without the presence of salt and/ or mycorrhizae were highest in Zhaodong followed by Nongjing and Longmu, respectively (Figs. 6, 7). With or without salt treatment, the highest number of nodules per plant and nodule weight was obtained when Zhaodong cultivar was double inoculated (Figs. 6, 7).

Mycorrhizae infection: The mycorrhizae infection rate depended on the cultivar, salinity level, inoculation, cultivar × inoculation and inoculation × salinity level (Table 1).Without salt treatment and in the absent of rhizobium, mycorrhizae infection rate was 40 while with double inoculation this number was 43 (Fig. 4). In the presence of salt, the rate of mycorrhizae infection was reduced with increasing salt level, however, the reduction was least when the plants were double inoculated followed by mycorrhizae inoculation. Furthermore, mycorrhizae infection rate with or without the presence of salt was highest with double inoculation followed by mycorrhizae inoculation in Fig. 4. In general, mycorrhizae infection rate were highest in Zhaodong followed by Nongjing and Longmu, respectively (Table 2).

Table 1. Analysis of variance for nodule number and weight, mycorrhiza infection, proline, and forage yield in four inoculation treatments, four salinity levels and with three cultivars of alfalfa.

Variable	df	Nodule number		Nodule weight		Mycorrhiza infection		Proline		forage yield	
		F	P	F	P	F	P	F	P	F	P
C	2	486.72	▪ 0.001	557.63	▪ 0.001	48.75	▪ 0.001	82.92	▪ 0.001	83.45	▪ 0.001
I	3	827.33	▪ 0.001	4522.81	▪ 0.001	236.87	▪ 0.001	75.33	▪ 0.001	122.37	▪ 0.001
S	3	166.72	▪ 0.001	2433.52	▪ 0.001	116.25	▪ 0.001	657.58	▪ 0.001	1092.66	▪ 0.001
C×I	6	183.75	▪ 0.001	237.17	▪ 0.001	17.6	▪ 0.001	0.26	0.334	0.16	0.228
C×S	6	6.22	▪ 0.001	8.03	▪ 0.001	0.64	0.129	2.49	0.033	3.82	0.003
I×S	9	66.67	▪ 0.001	106.83	▪ 0.001	37.6	▪ 0.001	1.33	0.021	4.05	0.015
C×I×S	18	3.68	▪ 0.001	7.85	▪ 0.001	0.22	0.755	0.16	0.532	0.16	0.337

Notes: C, I and S were cultivar, inoculation and salinity, respectively. Significant at 0.05, 0.01 and 0.001 levels of probability, respectively

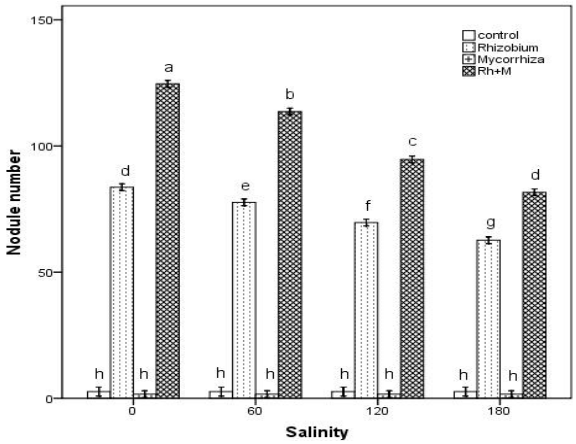

Fig. 1. The effects of inoculations (mycorrhizae, rhizobium, mycorrhizae + rhizobium and control) and salinity (0, 60, 120 and 180 mM NaCl) on alfalfa nodule number. Data are shown as means (±2 SE, n = 3). Letters indicate result of Tukey's HSD posthoc test of significant different between treatments; bars that share the same letter are not significant different ($p<0.05$).

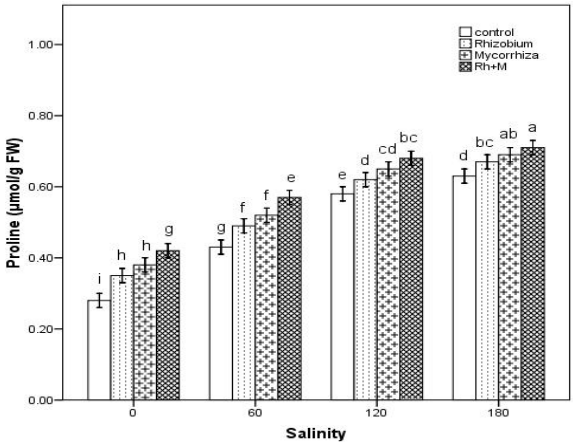

Fig. 3. The effects of inoculations (mycorrhizae, rhizobium, mycorrhizae + rhizobium and control) and salinity (0, 60, 120 and 180 mM NaCl) on proline content. Data are shown as means(±2SE, n = 3). Letters indicate result of Tukey's HSD posthoc test of significant different between treatments; bars that share the same letter are not significant different ($p<0.05$).

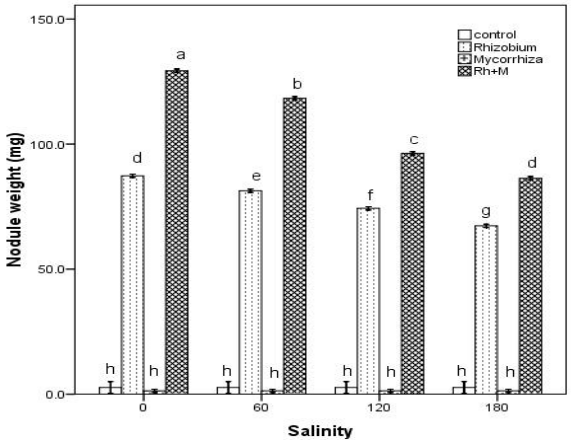

Fig. 2. The effects of inoculations (mycorrhizae, rhizobium, mycorrhizae + rhizobium and control) and salinity (0, 60, 120 and 180 mM NaCl) on alfalfa nodule weight. Data are shown as means(±2 SE, n = 3). Letters indicate result of Tukey's HSD posthoc test of significant different between treatments; bars that share the same letter are not significant different ($p<0.05$).

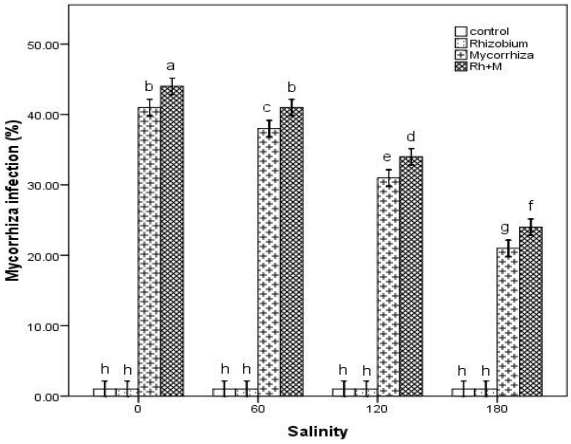

Fig. 4. The effects of inoculations (mycorrhizae, rhizobium, mycorrhizae + rhizobium and control) and salinity (0, 60, 120 and 180 mM NaCl) on Mycorrhizae infection. Data are shown as means(±2SE, n = 3). Letters indicate result of Tukey's HSD posthoc test of significant different between treatments; bars that share the same letter are not significant different ($p<0.05$).

Proline content: Proline content was affected by the cultivar, inoculation, salinity level, cultivar × salinity level and inoculation × salinity level (Table 1). Plants treated with rhizobia and mycorrhizae (R+M) contained highest proline content followed by mycorrhizae, rhizobia and control treatments, respectively (Fig. 3). Regardless of salinity level and inoculation treatment, highest proline content was measured in Zhaodong, followed by Nongjing and Longmu, respectively (Table 2). In contrast to other measured parameters, proline content was significantly increased with increasing salinity level.

Forage yield: Cultivar, salinity level, inoculation, cultivar × salinity level, and inoculation × salinity level affected forage yield (Table 1). Forage yield reduced as salinity level increased, but reduction was less when plant treated AMF or Rhizobium especially with double inoculation (Fig. 5). With or without salt treatment, plants treated with rhizobia and mycorrhizae (R+M) produced highest forage yield followed by mycorrhiza, rhizobia and control treatments, respectively in Fig. 5. With or without salt treatment, highest yield was measured in Zhaodong, followed by Nongjing and Longmu, respectively (Table 3).

Discussion (do not use headings in discussion)

In the present investigation inoculation of alfalfa with Rhizobium especially with AMF and Rhizobium (Rh+M) significantly enhanced nodulation (nodules per plant and nodule weight). Similar results have been reported by other scientists for different crops (Hildebrandt *et al*., 2001). Mizukami & Yamamoto (1991) suggested that

infection with AMF interacts with nodules leading to change in hormonal balance of the plants. This was suggested to be due to improved P nutrition by AMF (Hildebrandt *et al*., 2001).

In the presence of salt, the number and weight of nodules was decreased with increasing salt level. The results agreed with the findings reported by others (Sharifi *et al*., 2007). Reduction in nodulation under salt stress could be due to a reduction in survival and multiplication of rhizobia cells (Elboutahiri *et al*., 2010). The reduction in the number and weight of nodules was less when the plants were double inoculation, compared to inoculation with rhizobium alone. This indicated that the harmful effect of salinity could be reduced by dual inoculation. That was perhaps due to the presence of extra-matrical hyphae of AMF which increased the absorption of immobile elements such as phosphorus and nitrogen fixed by Rhizobium as indicated in Figs. 1, 2 and in Table 3. AMF has thick-walled inter or intracellular vesicles that are believed to function as endophytic storage organs (e.g. storage water in case of salinity which is an important problem in salt-affected soil). In addition, increased in NaCl tolerance was related to increased proline production as indicated in Fig. 3 and reported by Elboutahiri *et al*. (2010).

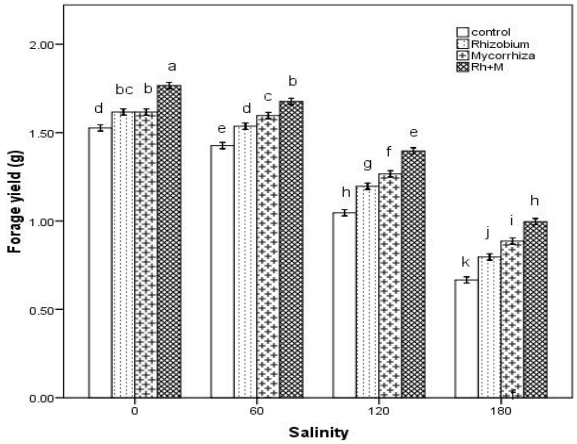

Fig. 5. The effects of inoculations (mycorrhizae, rhizobium, mycorrhizae + rhizobium and control) and salinity (0, 60, 120 and 180 mM NaCl) on forage yield. Data are shown as means(±2SE, n = 3). Letters indicate result of Tukey's HSD posthoc test of significant different between treatments; bars that share the same letter are not significant different ($p<0.05$).

The results showed that the non-rhizobium or AMF inoculated treatments remained free from nodules. Scheublin & Vander Heijden (2006) reported that inoculation of several legumes with AMF resulted in nodule colonization, however, AMF-colonized nodules never fixed nitrogen. Increased nodulation (nodules per plant and nodule weight) in this experiment under both stress and non-stress conditions was perhaps due to inoculation of alfalfa seeds with Rhizobium and especially with double inoculation as indicated in Figs. 1, 2 and in Table 3.

With or without presence of salt, root colonization of alfalfa was enhanced when plants were double inoculated with mycorrhizae and rhizobia (Rh+M), compared to inoculation with mycorrhizae alone. The rate of mycorrhizal infection was reduced with increasing salt level, however, the reduction was less when plants were double inoculated followed by mycorrhizae inoculation. Atul-Nayyar *et al*. (2008) reported that inoculation of Russian wild rye with three species of Glomus caused significant AM colonization. Nourinia *et al*. (2007) reported that root inoculation of barley with Glomus mosseae produced significant colonization, but colonization percentage was reduced as salinity level was increased and this was due to a direct effect of NaCl on the fungi. Al-Karari *et al*. (2001) reported that mycorrhizal colonization was negatively affected by salinity stress possibly due to the effects of salt on initial colonization. Several other researchers have also shown that salinity reduces mycorrhizal colonization by inhibiting the germination of spores (Evelin *et al*., 2009), inhibiting growth of hyphae in soil and hyphal spreading after initial infection has occurred (Evelin *et al*., 2009), and reducing the number of arbuscules (Tian *et al*., 2004).

Enhancement of AMF colonization by Rhizobium (double inoculation) was also reported by others (Rao & Tak, 2002). The results showed that the non AMF-inoculated treatments remained free from AMF colonization. These results were in line with results reported by Scheublin & Vander Heijden, (2006) in Lotus corniculatus, Trifolium repens and Ononis repens.

It has been reported that AMF colonization of roots could be species dependant (Evelin *et al*., 2009). Scheublin & Vander Heijden (2006) showed that all plant roots in several legume species inoculated with AMF colonized by AMF, however, Lotus corniculatus had the highest colonization followed by Trifolium repens and Ononis repens, respectively. Our results showed that AMF colonization of alfalfa roots was also affected by cultivars. AMF infection in Zhaodong was highest followed by Nongjing and Longmu, respectively that was perhaps due to genetic differences between the cultivars. Increased mycorrhizal colonization in our experiment under both stress and non-stress conditions was perhaps due to inoculation of alfalfa plants with AMF and especially with double inoculation as indicated in Fig. 4.

With or without salt stress, proline concentrations in the leaves was increased the most with double inoculation followed by mycorrhizae, Rhizobium and control treatments, respectively (Fig. 3). Elboutahiri *et al*. (2010) reported that under well watered condition, Rhizobium-inoculated alfalfa had the highest leaf proline levels followed by double inoculation, AMF inoculation and control, respectively. In line with our results, Sharifi *et al*. (2007) in soybean showed that plants inoculated with AMF had higher proline than control under saline conditions. In contrast, Al-Karaki *et al*. (2001) showed that non-AMF Vicia faba plants accumulated more proline than AMF plants under various salt levels. Elboutahiri *et al*. (2010) showed that Rhizobium meliloti can use proline betaine which synthesizes by alfalfa as a carbon, nitrogen and energy sources as well as an osmoprotectant. Our results showed that proline in alfalfa leaf tissues were increased with inoculation of rhizobia or AMF, especially with double inoculation under salt stress and non-saline conditions.

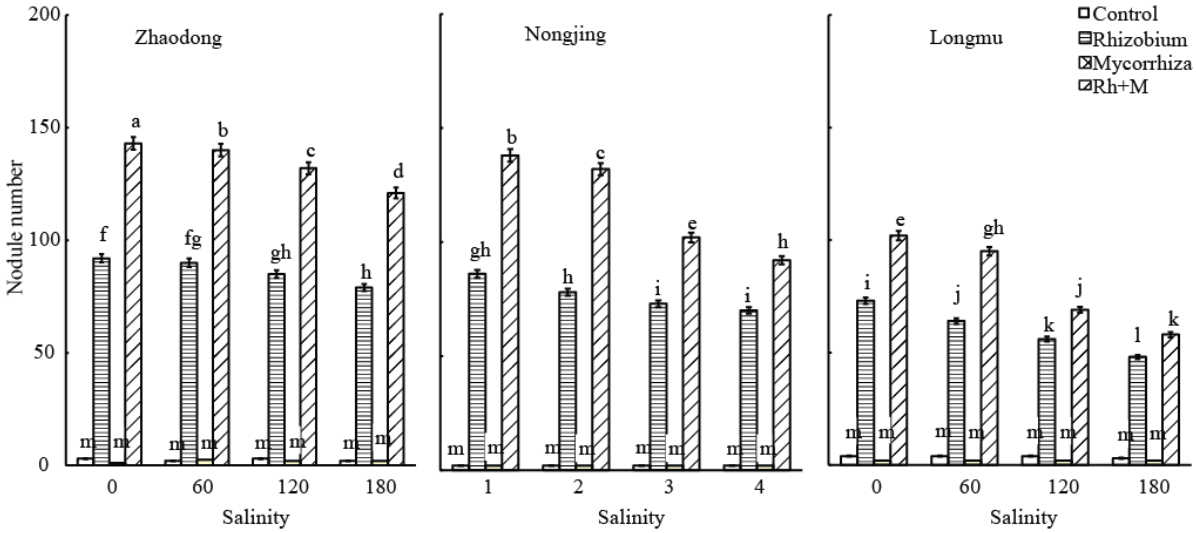

Fig. 6. The effects of inoculations (mycorrhizae, rhizobium, mycorrhizae + rhizobium and control) and salinity (0, 60, 120 and 180 mM NaCl) on of nodule number of three alfalfa cultivars (Zhaodong, Nongjing and Longmu) . Data are shown as means(±2SE, n = 3). Letters indicate result of Tukey's HSD posthoc test of significant different between treatments; bars that share the same letter are not significant different ($p<0.05$).

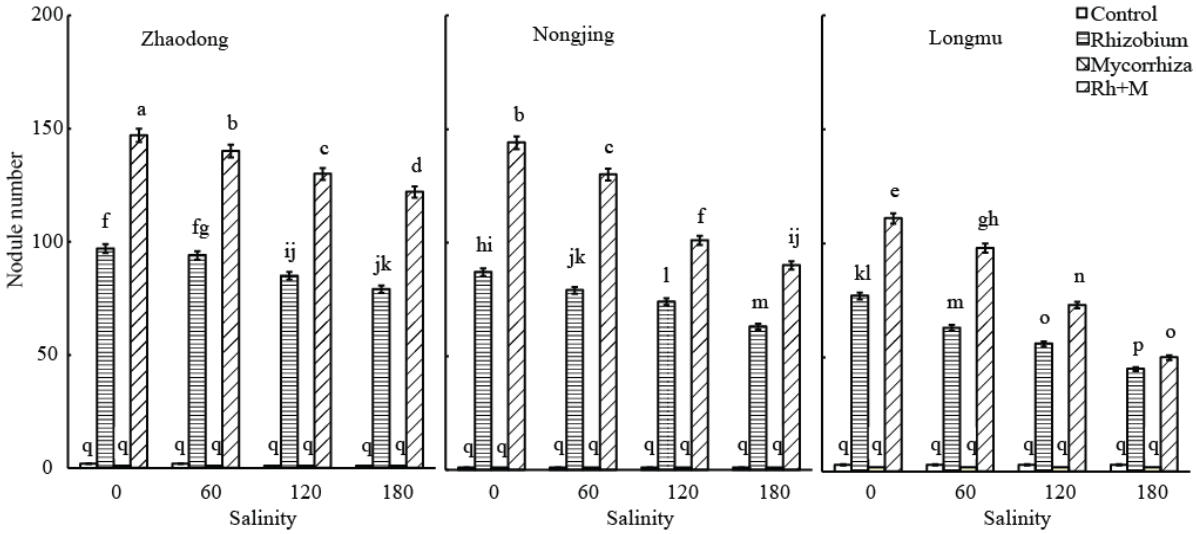

Fig. 7. The effects of inoculations (mycorrhizae, rhizobium, mycorrhizae + rhizobium and control) and salinity (0, 60, 120 and 180 mM NaCl) on nodule weight of three alfalfa cultivars (Zhaodong, Nongjing and Longmu). Data are shown as means(±2SE, n = 3). Letters indicate result of Tukey's HSD posthoc test of significant different between treatments; bars that share the same letter are not significant different ($p<0.05$).

With or without salt treatment, plants treated with both rhizobia and mycorrhizae (Rh+M) produced highest forage yield followed by mycorrhizae (M), rhizobia (Rh) and control treatments, respectively (Fig. 5). The results agree with Safapour *et al*. (2011) who reported that alfalfa plants inoculated with both Rhizobiun and Glomus (Rh+M) had the highest shoot yield followed by mycorrhizae, rhizobium and control treatments and concluded that Rhizobium may affect fungal metabolism. Increased yield of barely by Rhizobium japonica and Glomus mosseae was also reported by Nourinia *et al*. (2007). In line with our results, Mycorrhizal alfalfa (Khan, 2001) have been shown to produce higher yield as compared to control under saline conditions. Increased forage yield of subterranean clover inoculated with mycorrhizae under saline and non-saline conditions was also reported by Asghari (2005) and it was concluded that inoculation could increase mycorrhizae effectiveness and enhance root colonization under both conditions. The results were also in line with other studies that have shown that inoculation of legumes with both rhizobium and AMF increases the plant growth to a greater extent than with either inoculum when added singly (Mizukami & Yamamoto, 1991). An increase in N and P nutrition supply resulting in an increase in nitrogen fixation have been suggested for the benefits of the synergistic relationship (Giri *et al*., 2002).

Table 2. Proline, mycorrhiza infection, nodule number and weight and forage yield as affected by salinity level, inoculation treatment and cultivar.

Variable source	Nodule number (No./Plot)	Nodule weight (mg/Plot)	Mycorrhiza infection (%)	Proline (µmol/gFW)	Forage yield (g)
Cultivar					
Zhaodong	53.5a	54.9a	19.2a	0.58a	1.40a
Nongjing	46.4b	47.3b	17.2b	0.52b	1.28b
Longmu	32.3c	36.5c	14.3c	0.49c	1.23c
LSD0.05	1.3	1.1	0.9	0.01	0.02
Inoculation					
Control	0c	0c	0c	0.47d	1.15d
Rhizobium	73.2b	77.7b	0c	0.52c	1.27c
Mycorrhiza	0c	0c	32.3b	0.55b	1.34b
Rh+M	103.2a	107.3a	35.3a	0.58a	1.44a
LSD0.05	1.5	1.2	1.1	0.01	0.03
Salinity					
0	52a	54.1a	20.9a	0.35d	1.63a
60	47b	50.0b	19.5b	0.49c	1.54b
120	40c	42.5c	16.0c	0.62b	1.21c
180	35d	38.4d	11.0d	0.66a	0.82d
LSD0.05	2	1.2	1.1	0.01	0.03

†Means within the column for each treatment with the same letters are not significantly different at 5% level

Table 3. Forage yield, proline, nodule number and weight of alfalfa cultivars as affected by salinity levels.

Cultivar	Salinity	Nodule number (No./Plot)	Nodule weight (mg/Plot)	Proline (µmol/gFW)	Forage yield (g)
Zhaodong	0	58.77a†	59.8a	0.40g	1.68a†
Nongjing	0	53.95bc	55.5b	0.33h	1.63ab
Longmu	0	43.58f	47.0e	0.30i	1.58b
Zhaodong	60	56.20ab	57.7a	0.56d	1.62b
Nongjing	60	50.33de	51.7c	0.47e	1.53c
Longmu	60	36.58g	40.3g	0.44f	1.48c
Zhaodong	120	51.33cd	52.4c	0.66a	1.33d
Nongjing	120	43.83f	43.0f	0.61b	1.18e
Longmu	120	27.58h	31.6h	0.58c	1.13e
Zhaodong	180	47.83e	49.3d	0.69a	0.96f
Nongjing	180	37.83g	38.8g	0.67a	0.77g
Longmu	180	21.82i	27.0i	0.64b	0.72g
LSD0.05		2.75	2.20	0.02	0.05

†Means within rows and column for each item with the same letters are not significantly different at 5% level

Conclusions

Under non stress condition, double inoculation of alfalfa with rhizobium and AM increased the alfalfa yield, nodule weight and number, as well as shoot proline contents, the most when plants were double inoculated followed by AM and rhizobium inoculation, respectively. Whereas under salinity condition, double inoculation of alfalfa with rhizobium and AM increased alfalfa yield, mycorrhizal infection, nodule weight and number as well as increased in shoot proline content, the most followed by AM and rhizobium inoculation, respectively. The results suggest that growth of alfalfa may be improved by combined inoculation of alfalfa with AM and rhizobium under salt and non-stress conditions. Alleviation of alfalfa growth under saline condition was perhaps due to an increase in mycorrhizal infection and nodule weight and number as well as an increased in shoot proline content by dual inoculation.

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