The Effects of Arbuscular Mycorrhizal Fungi Inoculation on Reactive Oxyradical Scavenging System of Soybean (*Glycine max*) Nodules under Salt Stress Condition

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Summary

The effects of arbuscular mycorrhizal fungi (AMF), *Glomus mosseae*, on oxygen radical scavenging system (including superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), ascorbate peroxidase (APX) and peroxidase (POX)) in nodules of soybean (*Glycine max*) plants under salt stress condition were studied in potted culture experiment. The experiment was arranged as a factorial in Randomized Complete Block Design (RCBD) with four replications in greenhouse of College of Agriculture, Tehran University, Iran. Results indicated that AMF colonization notably increased the activities of SOD, CAT, POX and GR in the nodules, whereas it had little effect on APX. The results indicate that the AM fungus is capable of alleviating the damage caused by salt stress on symbiotic nitrogen fixation of soybean plants by increasing antioxidant enzyme activity. In conclusion, AMF could enhance the salinity tolerance of soybean plant, and thereby play a very important role in improving symbiotic nitrogen fixation and promoted plant growth.

Key words

arbuscular mycorrhizal fungi (AMF), oxygen radical scavenging system, nodules, salinity, soybean

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Introduction

Legumes such as soybean (*Glycine max*) have been suggested as appropriate crops for the enhancement of bioproductivity and the reclamation of marginal lands, because these plants not only yield nutritious fodder, protein-rich seeds and fruits, but they also enrich soil nitrogen in symbiotic association with *Rhizobium* (Alexander, 1984).

Nodulation and nitrogen fixation in legume-*Rhizobium* associations are adversely affected by salinity, which can preclude legume establishment and growth, or reduce crop yield (Mohammad et al., 1991). Salinity is one of the major agricultural limitations in the arid and semi-arid regions, especially in the Mediterranean basin. Soybean is classified as a salt-sensitive crop (Läuchli, 1984) and the limitation in their productivity is associated with lower growth of soybean, poor symbiotic development of root-nodule bacteria (Georgiev and Atkins, 1993) and as a consequence a reduction in the nitrogen- fixation capacity (Delgado et al., 1994).

The mechanisms of N_2-fixation inhibition by salinity are well documented for initiation, development and function of nodules (Bekki et al., 1987; Serraj et al., 1998; Ramos et al., 1999; Garg and Gupta, 2000; Sadallah et al., 2001). Unsuccessful nodulation under salt-stress may be due to failure in the infection process because of the effect of salinity on the establishment of rhizobia (Singleton and Bohlool, 1984). Sprent and Zahran (1988) reported that NaCl inhibits the expansion and curling of root-hairs and reduces the number of nodules in faba bean. The process of nodule initiation and legume plants are both more sensitive to osmotic stress than are rhizobia (Russell, 1976; Tu, 1981; Velagaleti et al., 1990). Cordovilla et al., (1994) and Soussi et al., (1999) found that the tolerance of the host plant to salt stress could be a determinant factor.

It seems, the sensitivity of the symbiotic nitrogen fixation is not always associated with a high Na^+ accumulation in nodules. Salinity causes oxidative damage and hence affects nitrogen fixation and assimilation in nodules of legums. Some studies have implicated reactive oxygen species (ROS) in nodule senescence (Becana et al., 2000; Garg and Manchanda, 2008). But plants are not defenseless; under salt stress they initiate some defense mechanism to protect themselves from harmful effects of oxidative stress. Reactive oxygen species (ROS) scavenging is one such common defense response against abiotic stress (Vranova et al., 2002). The major ROS scavenging system includes a complex enzymatic group such as catalase (CAT), peroxidase (POX), superoxide dismutase (SOD), glutathione reductase (GR), ascorbate peroxidase (APX) and non-enzymatic molecules such as proline, glycine betain, sorbitol and manitol (Prochazkova et al., 2001).

Arbuscular mycorrhizal fungi (AMF) demonstrated a variety of benefits to many plant species (Jayachandran and Shetty, 2003; Al-Karaki et al., 2004). These fungi exploit water and mineral salts from soils more effectively than plant roots and transfer them to host (Kaya et al., 2003). Arbuscular mycorrhizal fungi (AMF) widely occur in saline soils (Aliasgharzadeh et al., 2001). Many studies have demonstrated that AMF protected the host plants to improve the growth of plants under salt stress condition (Trimble and Knowles, 1995; Tian et al., 2004; Sharifi et al., 2007). Moreover, additive and sometimes synergistic effects on legume performance are frequently seen when both rhizobia and AMF are present (Goss and de Varennes, 2002; Sanginga et al., 1999; Fitter and Garbaye, 1995). Reports on the response of antioxidant defense system to stress factors in mycorrhizal plants are contradictory; increase, no change, or even decrease in the activity of SOD, CAT, POX and APX were reported in mycorrhizal soybean (Porcel et al., 2003) subjected to drought and tomato subjected to salinity (He et al., 2007; Hajiboland et al., 2010). Thus, the aim of this study was to evaluate the effect of *Glomus mosseae* on reactive oxyradical scavenging system of soybean nodules under salt stress condition, in order to further understand salt tolerance mechanisms in AM plants.

Materials and methods

The experiment was conducted in a greenhouse of the College of Agriculture, University of Tehran, Iran. The experimental treatments consisted of three levels of salinity (0 - control, 6 and 12 dsm^{-1}) and two AMF inoculations (AMF and non-AMF) and were arranged as a factorial in completely randomized design. Each treatment was replicated four times.

The soybean seeds were rinsed with water and surface sterilized by dipping in 0.1% sodium hypochlorite for 2 min and then washed three times with distilled water. Seeds were pretreated with a standard rhizobial inoculum of *Bradyrhizobium japonicum*. The AMF spores were applied with 10 spores per seed (approximately 1500 spores/100 g of media). Seeds were inoculated by placing the AMF inoculum in the hole under the seeds and covering with the soil.

The soil used for pots was collected from the uncultivated site located in Qom province, Iran. The basic soil properties were as follows: organic matter content 1.08%, total N 0.062%, total K 740.8 mg kg^{-1}, total P 10.90 mg kg^{-1}, available P ($NaHCO_3$-extractable) 2.78 mg kg^{-1}, water-soluble K 13.43 mg kg^{-1} and electrical conductivity 8.1 dSm^{-1}.

Five seeds were sown in each pot containing 2 kg of soil mixture. After 21 days, thinning was carried out to leave three uniform seedlings in each pot. When the seedlings were established (30 days after sowing), the plants were treated with saline solution with electrical conductivities 6 and 12 dSm^{-1}. The control plants were treated with distilled water only. Pots were irrigated according to their weight at 80% field capacity moisture. The desired soil salinity levels were regularly maintained after monitoring the conductivity levels of the soils, using EC meter at weekly intervals till the end of the experiments. Parameters such as mycorrhizal infection, nodule number and weight, and antioxidant enzymes activities were studied after 180 days of sowing. The plants and the adhering soil were transferred to the sieve and roots and nodules were collected from the sieve and combined with the rest of the plant material. For dry weight measurements, the samples were dried in an oven at 70 °C for 72 h.

Mycorrhizal colonization

Mycorrhizal infection was estimated by the method of Phillips and Hayman (1970). The roots were cut and dipped in 8% KOH solution for 24 h and then kept in 2% HCl solution for 15 to 30 min. Staining solution containing cotton blue dye was

added. The samples were kept for 24 to 36 h. The roots were cut in to small pieces of 2.5 cm approximately and observed under compound light microscope. Root pieces that contained even a single vesicle or arbuscules were considered as infected. The percentage of AM infection was calculated from the following equation: percentage of AM colonization = (root length infected/root length observed) ×100.

Superoxide dismutase (SOD) activity

The activity of superoxide dismutase (SOD) was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT), according to Stewart and Bewley (1980). The reaction mixture (3 ml) contained 13 mM methionine, 75 mM NBT, 100 mM EDTA, 50 ml of enzyme extract within 50 mM phosphate buffer (pH 7.8). The reaction was started with 2 mM riboflavin by exposing the cuvette to a 15-W fluorescent tube for 10 min. The absorbance of each reaction mixture was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme that causes 50% inhibition of the photochemical reduction of NBT.

Catalase (CAT) activity

The activity of catalase (CAT) was determined as a decrease in absorbance at 240 nm for 1 min following the decomposition of H_2O_2 (Chance and Meahly, 1955). The reaction mixture contained 50 mM phosphate buffer (pH 7.0) and 15 mM H_2O_2.

Peroxidase (POX) activity

Peroxidase (POX) activity was measured by following the change of absorption at 470 nm due to guaiacol oxidation. The activity was assayed for 1 min in a reaction solution (3 ml final volume) composed of 100 mM potassium phosphate buffer (pH 7.0), 20 m Mguaiacol, 10 m MH_2O_2 and 0.15 ml enzyme extract (Polle et al., 1994).

Glutathione reductase (GR) activity

For determination of GR activity, nodulated root sample was homogenized with 5 mL of 0.1M Tricine-NaOH buffer (pH 7.8) and centrifuged at 15,000 g at 4°C for 15 min. The supernatant was collected and diluted properly for determination of GR activity according to the method of Chen and Wang (2002). The reaction mixture (1 mL) contained 1 mM NADPH, 0.1 M Tricine-NaOH buffer (pH 7.8), 5 mM oxidized glutathione and 0.2 mL enzyme extract. Absorbance decrease of 0.01 at 340 nm per 1 min mg^{-1} protein, was considered 1 unit of GR activity.

Ascorbate peroxidase (APX) activity

For determination of APX activity, nodulated root sample was extracted with 5 mL of ice-cold 100 mM phosphate buffer (pH 7.0) containing 1mM ASC and 1mM EDTA, centrifuged at 15,000g at 4°C for 15 min, and determined as described by Chen and Wang (2002). The assay mixture (3mL) contained 100 mM phosphate buffer (pH 7.0), 5mM ASC, 0.3 mM H_2O_2 and 0.1 mL enzyme extract. Absorbance decreased 0.01 at 290 nm was defined as an activity unit during 1 min mg^{-1} protein.

All data were subjected to analysis of variance using two-way ANOVA and means were compared by Duncan's multiple range test (Duncan, 1955).

Results

The results pointed out that high level of salt stress had inhibitory effects on mycorrhizal infection. The highest mycorrhizal infection was observed in the plants with moderate level of salinity stress and in the control plants (Table 1).

Salinity stress significantly reduced the root and shoot dry matter compared with the control treatment (Table 1). However, AM colonization mostly improved dry matter in the salt-stressed plants. This effect of AM on dry matter was more pronounced in shoot biomass than in root biomass.

Nodule number and dry mass of the nodules decreased under all saline treatments (Figure 1). AM inoculation further boosted the nodulation under saline stress and the nodule number showed a significant increase in unstressed as well as in stressed conditions.

Exposure of the plants to salt stress resulted in general increment in the antioxidant enzyme activities of the nodules (Table 2, 3). Mycorrhizal inoculations further increased the antioxidant enzyme activities. However, AMF plants had not significantly higher APX activity than non- AM plants grown under stress and non - stress condition (Table 3). Saline stress led to enhanced SOD activity in nodules of all the plants. SOD activity was higher in mycorrhizal plants than in nonmycorrhizal-stressed plants at 6 and 12 dSm, respectively. CAT activity increased in the nodules at 12 dSm, while the salt levels of 6 dSm did not bring a significant increase in CAT activity where it was almost at par with the control. Symbiosis with the mycorrhizal fungi significantly increased the CAT activity at 6 and 12 dSm and it was higher than corresponding nodules of stressed

Table 1. Effect of salinity on shoot length, root length, shoot DM, root DM and colonization in AM and non-AM soybean plants under salt stress

		Shoot length (cm plant $^{-1}$± SD)	Root length (cm plant $^{-1}$± SD)	Shoot DM (g plant $^{-1}$± SD)	Root DM (g plant $^{-1}$± SD)	AMF colonization (%)
Control (0)	Non-AMF	54.6±1.43b	34.8±1.64a	11.3±0.64b	4.6±0.7b	-
	AMF	61.8±1.16a	36.6±1.65a	13.6±0.6a	5.8±0.51a	28.8± 1.05a
6 dsm^{-1}	Non-AMF	44.3±1.82c	31.2±0.48b	7.9±1.1c	3.8±0.67c	-
	AMF	58.8±0.68ab	35.3±0.73a	10.6±0.75b	5.3±1.37a	26.3±1.63a
12 dsm^{-1}	Non-AMF	32.5±1.46d	30.7±1.33b	6.3±0.65c	2.1±0.28d	-
	AMF	42.3±1.07c	34.5±1.02a	9.4±1.13bc	3.6±0.7c	18.8±0.23b

Results represent the average of three experiments ± SD. Different letters represent significant differences ($p < 0.05$) between treatments in each column.

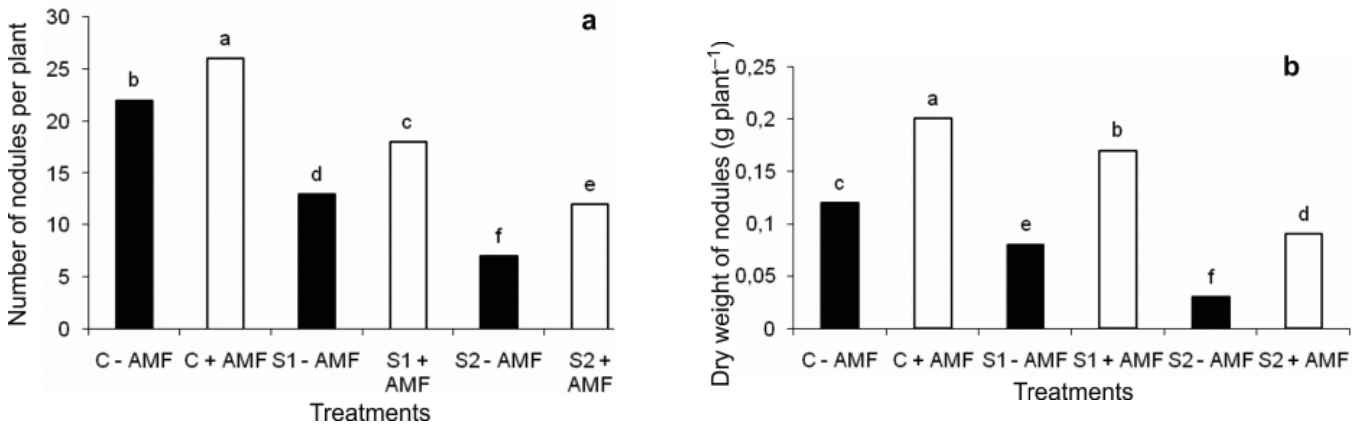

Figure 1. Effect of AM inoculation on number of nodules per plant (a) and dry weights of nodules per plant (b) of soybean under salt stress. Treatments are designed as uninoculated controls, saline stress (S1 = 6 and S2 = 12 dSm^{-1}) and arbuscular mycorrhiza (AM). Means followed by the same letter are not significantly different ($p<0.05$) as determined by Duncan's Multiple Range test

Table 2. Effect of salt stress on SOD, CAT, POX activities in nodules of AM and non-AM soybean plants under salt stress

		SOD (units mg^{-1} protein min^{-1} ± SD)	CAT (s mol H_2O_2 red. mg^{-1} protein min^{-1}± SD)	POX (n mol tetra- guaiacol formed min^{-1} g^{-1} f.wt. ± SD)
Control (0)	Non-AMF	16.05± 0.48e	7.86±0.23e	204.23±3.22f
	AMF	20.4± 1.11d	9.84±0.85d	217.54±1.78e
6 dsm^{-1}	Non-AMF	27.87±2.3c	8.03±0.45e	257.44±2.1d
	AMF	47.04±0.78b	15.11±1.33c	321.02±0.76b
12 dsm^{-1}	Non-AMF	29.85±1.3c	22.12±0.65b	279.6±1.28c
	AMF	53.87±1.42a	36.7±1.37a	349.77±1.48a

Results represent the average of three experiments ± SD. Different letters represent significant differences ($p < 0.05$) between treatments in each column.

Table 3. Effect of salt stress on APX and GR activities in nodules of AM and non-AM soybean plants under salt stress

		APX (units mg^{-1} protein min^{-1} ± SD)	GR (units mg^{-1} protein min^{-1}± SD)
Control (0)	Non-AMF	4.88± 0.35c	11.32± 0.82e
	AMF	5.1± 1.23c	11.8±0.52e
6 dsm^{-1}	Non-AMF	12. 35 ±0.67b	16.03±1.17d
	AMF	12.17±0.43b	21.34± 0.94c
12 dsm^{-1}	Non-AMF	23.62± 1.28a	27.28±0.73 b
	AMF	24.47±1.11a	33.17±0.66 a

Results represent the average of three experiments ± SD. Different letters represent significant differences ($p < 0.05$) between treatments in each column.

non- AM plants. POX activity increased with application of saline doses of 6 and 12 dSm. Salinity 6 dSm induced increase in POX activity of nonmycorrhizal nodules. A higher increase was observed in nodules of mycorrhizal plants at 6 and 12 dSm, respectively. Salinity led to enhanced APX and GR activity in nodules of soybean plants. Colonization with AMF further increased the GR activity at 6 and 12 dSm, while APX activity was similar in AMF and non-AMF plants at three levels of salinity.

Discussion

Mycorrhizal symbiosis plays an important role in enhancing the growth and health of the host plant. The beneficial effects of different mycorrhizal fungi had been demonstrated in various plant species (Torres-Barragan et al., 1996; Brown et al., 1997). It has been reported that addition of various salts to soil inhibits hyphal growth with a subsequent decrease in the spread of mycorrhizal colonization (Ruiz-Lozano and Azcon, 1996; Hajiboland et al., 2010).

The data from the present experiment indicated the positive effect of AMF on growth of soybean under salt stress condition. Similar results had been reported for other plant species (Ruiz-Lozano and Azcon, 1996; Al-Karaki et al., 2001). Enhanced growth of mycorrhizal plants grown in saline environments has been related partly to mycorrhiza-mediated enhancement

of host plant nutrition (Kaya et al., 2003). Also, it seems that the presence of higher amounts of antioxidant production in AM plants could be related to plant growth. In this experiment, the effect of AM on dry matter was more pronounced in aerial biomass than root biomass that may be because of arbuscular mycorrhizal colonization caused a proportionally greater allocation of carbohydrates to the shoot than root tissues (Shokri and Maadi, 2009).

A constitutively high antioxidant capacity under stress conditions can prevent damages due to ROS formation (Harinasut et al. 2003). In this study, though AM symbiosis affected reactive oxygen metabolism and antioxidant production, but the exact mechanisms involved are still unclear. The results made clear that, AM seedlings were bigger than non-AM seedlings. At the same time, the antioxidant productions were higher in AM seedlings. And it is well known that plant size has surely an effect on overall plant physiology.

Our results showed that moderate and high salinity caused a significant increase in SOD activity in nodules of both mycorrhizal and non-mycorrhizal soybean plants. These results are similar in part to results obtained by Garratt et al., (2002) who found enhanced SOD activity under salinity condition in cotton. Based on the induced SOD activity in the nodules of soybean plants grown under salinity, it could be concluded that SOD is important for soybean to tolerate salinity. Furthermore, enhanced SOD activity in mycorrhizal plants as compared to non-mycorrhizal plants supports the view that increased antioxidative enzyme activities could be involved in the beneficial effects of mycorrhizal colonization on the performance of plants grown under semi-arid conditions (Alguacil et al., 2003). Gradual exposure of the AM fungus to salinity enhanced its ability to increase SOD activity in the host plants. The great SOD activity in mycorrhizal plants could increase the capacity of nodules to scavenge superoxide radicals.

Plant possesses hydrogen peroxide scavenging enzymes POX and CAT. Detoxifications of the reactive oxygen protect cells against harmful concentration of hydroperoxides (Castillo, 1992). The increased POX in response to salinity has been reported (Harinasut et al., 2003). In tolerant plants, POX activity was found to be higher to protect plants against the oxidative stresses (Sreenivasulu et al., 1999). Pacovsky et al. (1989) studied POX activity in *Phaseolus vulgaris* infected by *Glomus etunicatum* and found that peroxidase activity increased in the mycorrhizal plants. Alguacil et al. (2003) reported that mycorrhizal inoculation increased CAT activity in *Olea europaea* grown under semi-arid conditions. On the other hand, since CAT is involved in decomposition of H_2O_2 in peroxisomes, similar increases in CAT activity of non-mycorrhizal and mycorrhizal plant at moderate and high NaCl indicate that under these conditions H_2O_2 is probably produced in higher concentrations in the peroxisome. Both GR and APX are two pivotal enzymes in the cycle ASC–GSH (Polle, 2001) and this cycle functions to remove H_2O_2. However, in the present investigation, AM colonization did not increase the activity of GR in AMF plants.

In conclusion, AMF-inoculation had a positive effect on reactive oxygen metabolism in nodules of salt-stressed soybean, increasing antioxidant enzymes (e.g. SOD, POX , CAT and APX).

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