



Arbuscular mycorrhizal symbiosis regulates hormone and osmotic equilibrium of *Lycium barbarum* L. under salt stress

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Abstract

Arbuscular mycorrhizal fungi (AMF) enhance salt tolerance of host plants via multiple mechanisms, but their effect on hormone mediated tolerance is unclear. The influence of AMF on hormone regulation and osmotic adjustment of *Lycium barbarum* L. (Goji) under salt stress was evaluated. AMF enhanced indol-3-acetic acid (IAA) in leaves and roots and abscisic acid (ABA) in leaves of Goji growing in saline soils. These changes resulted in enhanced osmotic adjustment in mycorrhizal plants, leading to higher leaf water potential despite saline conditions. AM symbiosis improved photosynthesis under 100 mM salt level and stimulated the growth of Goji plants, especially for roots under salt stress. These results enhance our understanding on how AMF contribute to salt tolerance of host plants via hormone regulation and highlight its promising role for sustaining crop production as bio-ameliorator.

Key words – ABA – arbuscular mycorrhiza – IAA – photosynthesis – salt tolerance

Introduction

Soil salinization is a serious problem worldwide, primarily caused by excessive irrigation and fertilization (Wang 2004). This is particularly severe in arid and semi-arid areas with intensive evaporation, like Northwest China (Chen et al. 2010). Plants in such situations are affected by high soil salinity through osmotic stress and ion toxicity (Evelin et al. 2009). This leads to physiological drought and metabolic disorders which ultimately decreases growth and results in plant death. Therefore, it is essential to seek for integrated agronomic practices to sustain the agricultural yield in salinized soils.

Arbuscular mycorrhizal fungi (AMF) are common in saline soil (Evelin et al. 2009) and have shown to be particularly good at enhancing host salt tolerance (Chandrasekaran et al. 2014). Mechanisms employed by AMF to alleviate salt stress include increasing nutrient uptake (Garg & Pandey 2015), balancing ion homeostasis (Estrada et al. 2013), improving antioxidant systems (Evelin & Kapoor 2014), elevating photosynthesis ability (Sheng et al. 2008) and enhancing osmotic adjustment in plants (Aroca et al. 2007). Recently, AMF have been utilized in field production for potato (Hijri 2016), yam (Lu et al. 2015) and maize (Sabia et al. 2015), and the

results confirmed great potential of AMF for improving yield of crops. However, less is known on the effects of AMF inoculation for the host plants in salinized fields (Liu et al. 2016). In particular, little is known about how AMF influence the hormonal regulation of host plants under salt stress (Evelin et al. 2009, Pozo et al. 2015).

Phytohormones help to regulate plant growth and development in response to stressful environments, such as salinity (Pozo et al. 2015). AMF are well known to influence plant growth by affecting plant hormone production (Nadeem et al. 2014), particularly in response to salt stress. For example, lettuce inoculated with AMF decreased abscisic acid (ABA) but increased strigolactone production to acclimate salt stress (Aroca et al. 2013). Similarly, AMF inoculated *Sesbania sesban* maintained higher levels of indole-3-acetic acid (IAA) and gibberellic acid (GA) and allayed the negative effects of salinity (Allah et al. 2015). Clearly, AMF influence hormones associated with salt stress (Pozo et al. 2015). However, whether or not inoculation by AMF can ameliorate the growth of commercial crops through hormone regulation under salt stress is mostly unclear. The altered levels of cytokinin and auxin in mycorrhizal plants synergistically regulate root architecture (Fusconi 2014). AM symbiosis can also regulate ABA to alter root hydraulic properties, enhancing water uptake in unfavorable conditions (Ruiz-Lozano et al. 2012). GA enhanced salt tolerance of wheat through ionic partitioning and photosynthesis (Iqbal & Ashraf 2013). Therefore, IAA, ABA, ZR and GAs are critical for plants to acclimate salt stress and might be altered by AM symbiosis.

Photosynthesis is usually associated with plant growth in long-term experiment (Iqbal & Ashraf 2013). Salt stress hinders water uptake of plants to inhibit photosynthesis (Kumar et al. 2015). However, AMF can alleviate the photosynthesis inhibition caused by salt stress through augmenting osmotic adjustment (Ruiz-Lozano et al. 2012). Photosynthesis is also closely related with ABA which controls the stomatal conductance (Zörb et al. 2013). The higher photosynthesis rate increases sugar content (Sheng et al. 2011), which subsequently ensure sufficient water absorption of mycorrhizal plants under salt stress (Evelin et al. 2009). Thus, it is essential to estimate the influence of AMF on the photosynthesis and sugar metabolism of plants under salt stress.

Lycium barbarum L. (Goji) is an important medicinal tree species with economic value in Northwest China. Goji berries and leaves are rich in flavonoids, polysaccharides and amino acids, which are important nutritionally and medicinally (Wang et al. 2015). However, salinity is restricting the sustainable production of Goji in Northwest China. To maintain current and future demands for this crop, it is crucial to develop tools to produce crops utilizing increasingly marginal soils. Our previous research confirmed that Goji can establish AM symbiosis (Zhang et al. 2010). Thus, this study addresses the question of how AMF-induced physiological changes affect salt tolerance in Goji. In particular, we asked: 1) Dose AMF inoculation improve growth of Goji under salt stress? 2) Dose AMF inoculation affect hormone levels of Goji under salt stress? 3) Dose AMF inoculation enhance photosynthesis and sugar metabolism of Goji under salt stress?

Materials & Methods

Experimental design

The experiment consisted of a randomized complete block design with two factors: 1) AMF inoculation: (Plants inoculated with (+AM) or without (-AM) *Glomus versiforme*), and 2) Salt stress: 0 mM, 100 mM (moderate) and 200 mM (severe) NaCl. Fifteen replicates per treatment gave $2 \times 3 \times 15 = 90$ pots.

Plants, soil and fungal inoculum

Goji (*L. barbarum*) cv. Ningqi No. 1 is a cultivated variety and widely distributed in saline arable field of arid and semi-arid regions of Northwest China, primarily in Ningxia province (Gu et al. 2007, Zheng et al. 2010). Soil was collected from the campus of Northwest A & F University, Yangling City, Shaanxi province, China, selecting only the top layer (0–20 cm, pH 8.39, cation

exchange capacity $6.30 \text{ cmol} + \text{kg}^{-1}$), which contained 7.98 g kg^{-1} organic matter, 51.59 mg kg^{-1} available nitrogen, 2.98 mg kg^{-1} available phosphorus, and 40 mg kg^{-1} available potassium measured according to the method described by Bao (2000). Soil was sieved through a 2-mm sieve, and thoroughly mixed with fine sand and vermiculite (soil/sand/vermiculite, 1:1:1 v/v/v). The mixture was sterilized at $121 \text{ }^\circ\text{C}$ for 2 h. *G. versiforme* (Karsten) Berch (BGC GD01C) was used as AMF inoculant since it had been previously identified as the most effective arbuscular mycorrhizal fungus for alleviating salt stress of trifoliolate orange (Zou & Wu 2011). This isolate was provided by the Bank of Glomales in China (BGC, Beijing, China) in the form of whole inoculum (soil, root fragments and spores). Trap culture of mycorrhizal inoculum was produced in pots with maize.

Inoculation treatment, salt treatment and growth conditions

Goji seeds (provided by Ningxia Wolfberry Engineering and Technology Research Center) were surface sterilized with 10 % (v/v) H_2O_2 for 10 min, followed by a gentle wash with deionized water. Sterilized seeds were sown on a vermiculite:sand mixture (1:1, v/v) for germination. Four weeks after germination, one Goji seedling was transferred into each pot (upper diameter: 22.5 cm ; bottom diameter: 14.5 cm; height: 22.5 cm). One hundred grams AMF inoculum (containing 527 spores) was placed at 2 cm pot depth, prior to transplanting, to facilitate fungal colonization of Goji seedlings roots. Autoclaved AMF inoculum (100 g pot^{-1}) was added to the control plants with a 10 ml of inoculum filtrate ($1 \mu\text{m}$). Seedlings were grown for 26 weeks in the greenhouse of Northwest A&F University under natural light. The temperature ranged from 22 to $35 \text{ }^\circ\text{C}$ and the humidity ranged from 70% to 75%.

Plants were grown for 10 weeks prior to salinization to allow adequate plant growth and symbiotic establishment. After confirming successful AM establishment, Goji plants were watered with gradually elevated salt solution to avoid salt shock. NaCl was introduced by successively adding 300 ml of the prescribed NaCl solutions (as per the saturation level of the soil) to reach 0, 100 and 200 mM salt levels for each salt treatment for 10 weeks. The electrical conductivities at the end to 20 weeks in the substrate were 0.19, 10.52 and 20.81 dS m^{-1} for the salt levels of 0, 100 and 200 mM NaCl, respectively. Plants were maintained under these conditions for additional 6 weeks before harvesting.

AMF colonization

Three Goji plants per treatment were harvested (26 weeks) to estimate AMF colonization. Two hundred of 1-cm-length root fragments per treatment were washed with tap water, and dried. The fresh roots were cleared for 15 min in 10% (w/v) KOH at $90 \text{ }^\circ\text{C}$, bleached in alkaline hydrogen peroxide for 20 min, acidified in 1% (v/v) HCl, and stained in trypan blue (Phillips & Hayman 1970). Colonization rate was measured using the gridline intercept method (Giovannetti & Mosse 1980).

Growth performance

Plant height, stem base diameter and leaf number of five plants per treatment were recorded at harvest. Three shoots and washed roots were dried in oven at $70 \text{ }^\circ\text{C}$ for 90 h to measure biomass.

Root areas of five plants per treatment were determined using methylene-blue colorimetric method described by (Liu et al. 2014). Roots were immersed in sequence into three beakers (marked 1, 2, 3) for 90 s, which contained $200 \mu\text{M}$ methylene-blue of the known volume. After that 1-ml liquid was pipetted from each beaker into test tube and diluted ten-fold. The absorbance value was measured with UV-Vis spectrophotometer (UV mini 1240, Shimadzu, Kyoto, Japan) at a wavelength of 660 nm to calculate the concentration of methylene-blue in each beaker by consulting the established standard curve. The root areas were calculated by the following equations:

$$\text{Total root area (m}^2\text{)}=[(\text{C}-\text{C}_1)\times\text{V}_1]+[(\text{C}-\text{C}_2)\times\text{V}_2]\times 1.1$$

$$\text{Active root area (m}^2\text{)}=[(\text{C}-\text{C}_3)\times\text{V}_3]\times 1.1$$

C (mg ml^{-1}): initial concentration of methylene-blue solution;

C_1, C_2, C_3 (mg ml^{-1}): the concentration of methylene-blue solution after root soaking;
 V_1, V_2, V_3 (ml): volume of methylene-blue solution in each beaker.

Phytohormone analysis

Hormone contents of three Goji plants per treatment were determined by an indirect enzyme-linked immunosorbent assay (ELISA). ELISA has been widely utilized for determining plant hormone for its high sensitivity (Bai et al. 2010, Cheng et al. 2013, Liu et al. 2015). The ELISA kit was produced by China Agriculture University, and the reliability was confirmed by HPLC and GC-MS (Yang et al. 2001, Teng et al. 2006, Zhao et al. 2006).

The extraction, purification endogenous levels of free indole-3-acetic acid (IAA), zeatin riboside (ZR), gibberellic acid (GAs: GA1+GA3) and abscisic acid (ABA) were performed as described by He (1993). Briefly, 0.5 g of fresh root and leaf tissue were homogenised on ice and extracted in 4 ml of pre-chilled methanol 80% (v/v) with butylated hydroxytoluene (1 mM) for 4 h at 4 °C. The extracts were collected after centrifugation at $2000\times g$ (4 °C) for 8 min. One millilitre of pre-cold methanol 80% (v/v) with butylated hydroxytoluene (1 mM) was added to the precipitate and stirred until mixed. Samples were centrifuged at $2000\times g$ (4 °C) for 1 h. The total supernatant was passed through a C_{18} Sep-Pak cartridge (Waters, Milford, MA, USA) and dried in N_2 . The residues were dissolved in PBS (phosphate buffered saline 0.01 M, pH 7.5) with 0.1 % Tween-20 (v/v) and 0.1 % gelatine (w/v) in order to determine the levels of free IAA, GAs, ZR and ABA.

Microtitration plates (96 wells) were coated with standard IAA, ZR, GAs, ABA, and samples. The maximum concentration of standard IAA, ABA and ZR was 100 ng ml^{-1} , while for GAs was 50 ng ml^{-1} . Standards were used to create a 2×dilution series (including 0 ng ml^{-1}) in duplicate. Fifty microliter of IAA, ABA, ZR and GAs antibodies (diluted according to manufacturer's instructions) to microtitration plates which were then incubated for 0.5 h at 37 °C. After incubation, the liquid was discarded and the plates were washed using PBS (0.01 M, pH 7.5) with 0.1 % Tween-20 (v/v) (four times). Each well was then loaded with 100 μl diluted samples with enzyme-labeled second antibody (1000:1 v/v), and the microtitration plates were incubated for 0.5 h at 37 °C. The microtitration plates were washed as described above. Finally, the buffered enzyme substrate (ortho-phenylenediamine) was added, and the enzyme reaction was carried out in the dark at 37 °C for 15 min, then terminated using 2 M H_2SO_4 . Absorbance was recorded at 490 nm and concentrations of hormones were calculated according the method described by Weiler et al. (1981). The percentage recoveries of the four hormones were all above 90 % and all sample extract dilution curves paralleled the standard curves, suggesting the absence of non-specific inhibitors in the extracts.

Photosynthesis parameters

Nine plants per treatment were selected to determine photosynthesis parameters. Net photosynthesis rate ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), intercellular CO_2 concentration ($\mu\text{mol CO}_2 \text{ mol}^{-1}$), stomatal conductance ($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$) and transpiration rate ($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$) were determined from 8:30 to 11:30 A.M. using a portable open flow gas exchange system LI-6400 (LI-COR, USA) following manufacturer's instructions. Measurements were carried out in the second youngest leaf of each plant after 25 weeks growth.

Soluble sugar, reducing sugar and starch determination

Soluble sugar and starch content in Goji leaves was determined by anthrone colorimetry method at 620 nm with a UV-Vis spectrophotometer (UV mini 1240, Shimadzu, Kyoto, Japan). The reducing sugars of Goji leaves were determined using salicylic colorimetry method at 540 nm with a UV-Vis spectrophotometer (UV mini 1240, Shimadzu, Kyoto, Japan) according to Gao (2000).

Leaf water potential measurement

Leaf water potential was determined with a C-52 thermo-couple psychrometer chamber and

a PSYPRO dew point microvoltmeter (Wescor Inc., Logan, UT, USA) (Porcel & Ruiz-Lozano 2004). Leaf discs corresponding to the second youngest leaves were cut and placed inside the psychrometer chamber, allowed to reach temperature and water vapor equilibrium for 30 min before measurements were made by the dew point method.

Statistical analysis

One-way ANOVA, followed by Duncan's test, was conducted to determine the significant differences in mycorrhizal and morphological parameters (shoot height, stem base diameter and leaf number), growth and photosynthesis parameters, soluble and reducing sugars and starch, leaf water potential and hormones across all treatments. Prior to the analysis, the distribution normality was verified using Shapiro-wilk test. Levene's test was performed to assess the equality of variances.

Two-way ANOVA followed by Duncan's test was analyzed to assess the effect of AMF and salinity on morphological parameters, biomass, root area, soluble and reducing sugars and starch, leaf water potential.

For multivariate responses of photosynthesis parameters (Net photosynthesis rate, Intercellular CO₂ concentration, Stomatal conductance and Transpiration rate), data were analyzed using PERMANOVA in a two-way design (with salinity, inoculation treatment as fixed factors, Euclidean distance, type III sums of squares, 9999 unrestricted permutations) (PrimerE, Lutton, Ivybridge, UK).

ANCOVA with AMF inoculation as factor and shoot (leaf hormone) or root (root hormone) biomass as covariate was conducted to dissect the effect of AMF and plant biomass on the hormone levels of Goji. Two-way ANOVA followed by Duncan's test was conducted to assess the effect of AMF and salinity on hormone.

All ANOVA and ANCOVA were carried out using SPSS 19.0 statistical program (SPSS Inc., Chicago, IL, USA). All figures were created using Origin Pro v8.0 (Origin Lab, Hampton, USA).

Results

Symbiotic development and growth performance

The colonization rates of AM Goji for 0, 100 and 200 mM NaCl treatments were 60.91±2.91%, 52.50±3.15% and 57.27±3.90%, respectively. No sign of AM colonization was found for non-inoculated Goji plants. Salt addition did not influence AMF colonization on Goji plants ($F_{2, 57}=2.26$, $p=0.1138$).

At harvest, mycorrhizal Goji plants had higher shoot height than non-mycorrhizal Goji under non-salt stress (Table 1, $F_{5, 24}=22.84$, $p<0.0001$). Although the shoot height of mycorrhizal Goji plants decreased at moderate salt level, it was still higher than that of non-mycorrhizal Goji plants ($F_{5, 24}=22.84$, $p<0.0001$). Under severe salt level, the shoot height of mycorrhizal Goji was higher than non-mycorrhizal plants ($F_{5, 24}=22.84$, $p<0.0001$). Salt stress decreased stem base diameter (Table 1, $F_{2, 24}=10.56$, $p=0.0005$). However, AMF inoculation enhanced stem base diameter ($F_{5, 24}=62.07$, $p<0.0001$) of Goji plants under moderate and severe salt levels compared with control. Mycorrhizal Goji plants had higher leaf number compared with non-mycorrhizal plants with and without salt stress (Table 1, $F_{5, 24}=35.17$, $p<0.0001$). No significant interaction of AMF and salt stress on these attributes was found ($p>0.05$). Overall, AMF promoted plant growth, for all parameters, at all salt levels.

Salinity negatively affected shoot (Fig. 1a, $F_{2, 12}=7.75$, $p=0.0069$) and root biomass (Fig. 1b, $F_{2, 12}=14.21$, $p=0.0007$). Shoot biomass of Goji plants was strongly improved by AMF inoculation (Fig. 1a, $F_{1, 12}=82.96$, $p<0.0001$). Under non-saline conditions, mycorrhizal plants had more than 2-fold of shoot biomass than non-mycorrhizal plants. The shoot biomass of mycorrhizal Goji plants was higher compared with that of non-mycorrhizal plants under salt stress.

The root biomass was also significantly increased by AMF inoculation (Fig. 1b, $F_{1, 12}=88.54$, $p<0.0001$). The interaction of AMF and salt significantly affected root biomass of Goji plants ($F_{2, 12}=13.98$, $p=0.0007$). Mycorrhizal Goji plants had 3-fold more root biomass relative to non-mycorrhizal plants. But under moderate salt stress, no difference of root biomass between mycorrhizal and non-mycorrhizal Goji plants was found. While under severe salt level, mycorrhizal Goji plants had higher root biomass compared with non-mycorrhizal Goji.

Table 1 Effect of AMF on morphological parameters of Goji plants under salinity stress.

Salt level	AMF inoculation	Shoot height (cm)	Stem base diameter (cm)	Leaf number per plant
0 mM	-AM	64.60±7.19c	2.83±0.14c	31.40±0.93b
	+AM	141.20±9.62a	4.53±0.29a	54.80±2.85a
100 mM	-AM	57.80±3.88c	2.33±0.04c	25.40±2.71b
	+AM	114.60±3.29b	3.72±0.06b	50.80±2.78a
200 mM	-AM	76.00±12.39c	2.44±0.19c	29.60±1.94b
	+AM	128.40±2.46ab	3.74±0.14b	52.00±1.38a
Significance				
Salt		ns	**	ns
AMF		**	**	**
Salt×AMF		ns	ns	ns

Note: * $p < 0.05$; ** $p < 0.01$; ns, non-significant. Values followed by the same letter in columns do not differ significantly at $p=0.05$ according to Duncan's multiple range test ($n=5$).

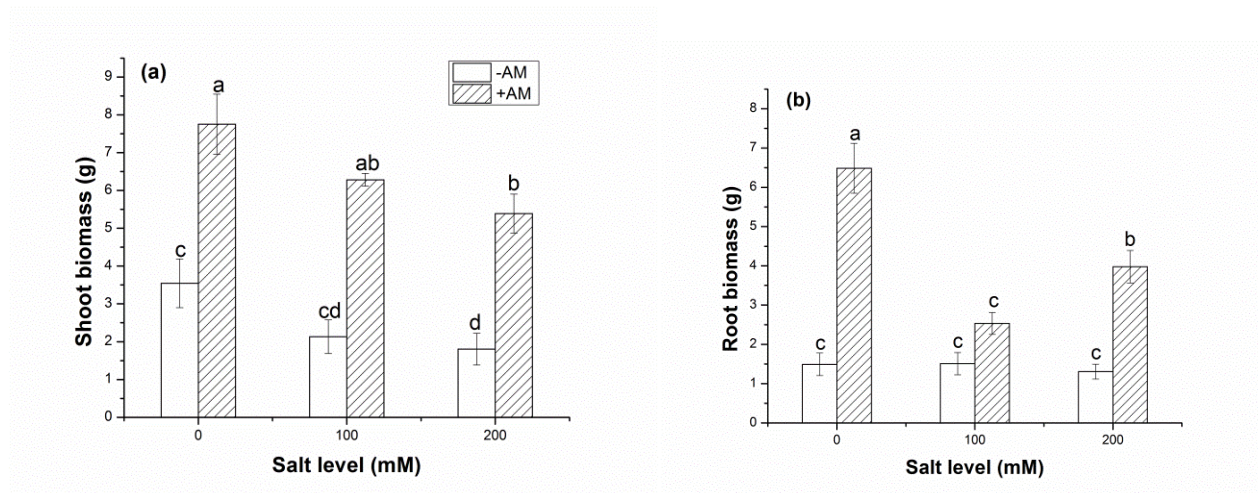


Fig. 1 – Effect of AMF inoculation on (a) shoot and (b) root biomass of Goji plants under salinity stress. Data are expressed as means±SE ($n=3$). Bars sharing the same letter are not significantly different at $P<0.05$ according to Duncan's multiple test.

While salinity decreased total (Fig. 2a, $F_{2, 24}=75.32$, $p<0.0001$) and active (Fig. 2b, $F_{2, 24}=71.28$, $p<0.0001$) root area of Goji, inoculation by AMF greatly elevated total root areas (Fig. 2a, $F_{1, 24}=186.7$, $p<0.0001$) and active root areas (Fig. 2b, $F_{1, 24}=164.1$, $p<0.0001$) in all salt levels. Mycorrhizal plants had more than three times of root areas compared with non-mycorrhizal plants with and without salt stress. The interaction of AMF and salt stress significantly affected total ($F_{2, 24}=20.76$, $p<0.0001$) and active ($F_{2, 24}=26.21$, $p<0.0001$) root areas of Goji.

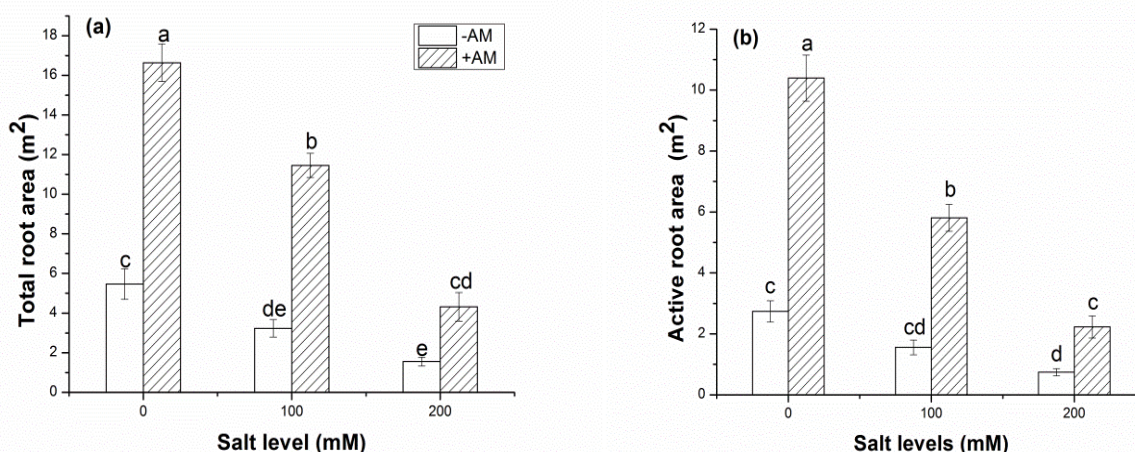


Fig. 2 – Effect of AMF inoculation on (a) total root area and (b) active root area of Goji plants under salinity stress. Data are expressed as means±SE (n=5). Bars sharing the same letter are not significantly different at $P<0.05$ according to Duncan’s multiple test.

Hormone regulation

Both salt stress and AMF inoculation influenced the hormone profile of Goji plants (Table 2). AMF directly affected IAA in leaves ($F_{1, 15}=4.793$, $p=0.018$) and roots ($F_{1, 15}=3.989$, $p=0.032$) of Goji plants, regardless of plant biomass (shoot: $F_{1, 15}=0.933$, $p=0.352$; root: $F_{1, 15}=0.515$, $p=0.486$) (Table 3). In contrast, while AMF inoculation significantly affected leaf ABA, ZR and GAs, the effect varied according to shoot biomass (Table 3).

Specifically, under no salt stress, mycorrhizal plants had higher IAA content in Goji leaves (Table 2). Salt stress decreased IAA content in Goji leaves ($F_{2, 12}=143.71$, $p<0.0001$), especially at the severe salt level. However, the IAA content in mycorrhizal Goji leaves was higher than that of non-mycorrhizal Goji under salt stress. Salt stress decreased root IAA content of Goji plants ($F_{2, 12}=26.81$, $p<0.0001$). The IAA content in roots of mycorrhizal Goji was higher than non-mycorrhizal plants in non-saline and moderate salt conditions. However, the severe salt level decreased IAA content in mycorrhizal Goji roots, to the same level of non-mycorrhizal plants.

No difference of leaf ABA between mycorrhizal and non-mycorrhizal Goji was found under non-saline condition (Table 2). The moderate salt stress decreased leaf ABA content of non-mycorrhizal Goji plants, but had no effect on that of mycorrhizal Goji. Namely, the leaf ABA content in mycorrhizal Goji plants was higher than that of non-mycorrhizal plants in moderate salt treatment. But under the severe salt level, mycorrhizal Goji leaves had the same ABA level as non-mycorrhizal plants. In contrast to leaves, root ABA showed no difference across salt levels and AMF treatments.

Salt stress and AMF inoculation had less impact on ZR levels in Goji plants. ZR content showed no difference between mycorrhizal and non-mycorrhizal Goji leaves under no and severe salt stress (Table 2). But at moderate salt level, mycorrhizal Goji leaves had higher ZR content than non-mycorrhizal Goji plants. Similar to root ABA, no difference was detected among all treatments for root ZR level.

Mycorrhizal Goji plants had higher root GAs (GA1+GA3) content without salt stress. But at moderate and severe salt level, no difference of GAs was found in leaves and roots between mycorrhizal and non-mycorrhizal plants (Table 2).

Table 2 Effect of AMF on the hormone content in the leaves and roots of Goji plants under salinity stress.

Salt level (mM)	AMF	IAA (ng/g FW)		ABA (ng/g FW)		ZR (ng/g FW)		GAs (ng/g FW)	
		Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
0	–	85.74±3.2	64.50±1.7	192.00±14.	114.44±10.	16.81±1.9	10.46±1.	8.36±1.	4.50±0.
	AM	9c	7b	88a	65a	3ab	89a	08a	47b
	+A	173.45±5.	96.94±6.2	193.51±13.	106.68±7.5	15.62±3.0	13.22±2.	9.67±1.	5.95±0.
100	–	80.12±4.1	60.95±2.6	105.80±8.3	115.36±5.8	11.57±2.3	11.45±1.	6.65±0.	3.74±0.
	AM	1c	2bc	8c	1a	7b	44a	91a	42b
	+A	105.45±3.	88.34±4.2	162.00±10.	120.72±10.	19.06±1.5	13.20±1.	8.19±0.	4.41±0.
200	–	56.58±3.1	50.19±2.7	147.08±5.7	122.37±8.2	12.59±1.7	8.00±0.8	7.11±0.	4.10±0.
	AM	2d	7c	7b	9a	7ab	8a	54a	31b
	+A	74.75±2.2	59.06±2.7	150.86±10.	95.56±7.54	14.34±0.8	10.59±1.	7.65±0.	4.31±0.
	M	7c	9bc	80b	a	3ab	50a	66a	35b
Significance									
Salt		***	***	**	ns	ns	ns	ns	ns
AMF		***	***	*	ns	ns	ns	ns	ns
Salt×AMF		***	*	*	ns	ns	ns	ns	ns

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$; ns, non-significant. Values followed by the same letter in one column do not differ significantly at $p=0.05$ according to Duncan's multiple range test ($n=3$).

Table 3 Results of ANCOVA for the effects of AMF colonization rate and shoot or root biomass on hormone content of Goji plants under salinity stress.

Source of variation		IAA		ABA		ZR		GAs	
		Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
Factor	AMF	*	*	***	ns	***	ns	***	ns
Co-variate	SB	ns	ns	***	ns	***	ns	***	ns
Co-variate	RB	ns	ns	ns	ns	ns	ns	ns	ns

SB–Shoot biomass; RB–Root biomass * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$; ns, non-significant.

Photosynthesis

Salt addition had significant influence on the photosynthesis performance of Goji plants (Fig. 3, Pseudo $F_{2, 48}=88.657$, $p=0.0001$), but AMF inoculation did not affect photosynthesis parameters (Pseudo $F_{1, 48}=2.8171$, $p=0.0829$). The interaction of AMF and salt stress showed significant effect on photosynthesis performance of Goji (Pseudo $F_{2, 48}=5.8192$, $p=0.0021$). In pairwise tests, AMF significantly improved photosynthesis for plants at 100 mM salt level (p (perm)=0.0038), but had no effect at 0 and 200 mM salt levels (0 mM p (perm)=0.138; 200 mM p (perm)=0.3724).

When variables were analyzed individually (ANOVA), AMF inoculation had no effect on intercellular CO_2 concentration, stomatal conductance and transpiration rate under no salt stress. AMF even decreased net photosynthesis rate of Goji plants in non-saline soils (Fig. 3). But at moderate salt level, inoculation by AMF significantly increased net photosynthesis rate ($F_{5, 48}=21.51$, $p<0.0001$) and transpiration rate ($F_{5, 48}=58.20$, $p<0.0001$), but decreased intercellular CO_2 concentration ($F_{5, 48}=37.91$, $p<0.0001$). At severe salt level, no difference for these parameters was found between mycorrhizal and non-mycorrhizal Goji plants. AMF×salt interaction was significant for net photosynthesis rate ($F_{2, 48}=8.55$, $p=0.0007$) and intercellular CO_2 concentration ($F_{2, 48}=7.60$, $p=0.0014$), but had no effect on stomatal conductance ($F_{2, 48}=1.09$, $p=0.3437$) and transpiration rate ($F_{2, 48}=2.34$, $p=0.1072$).

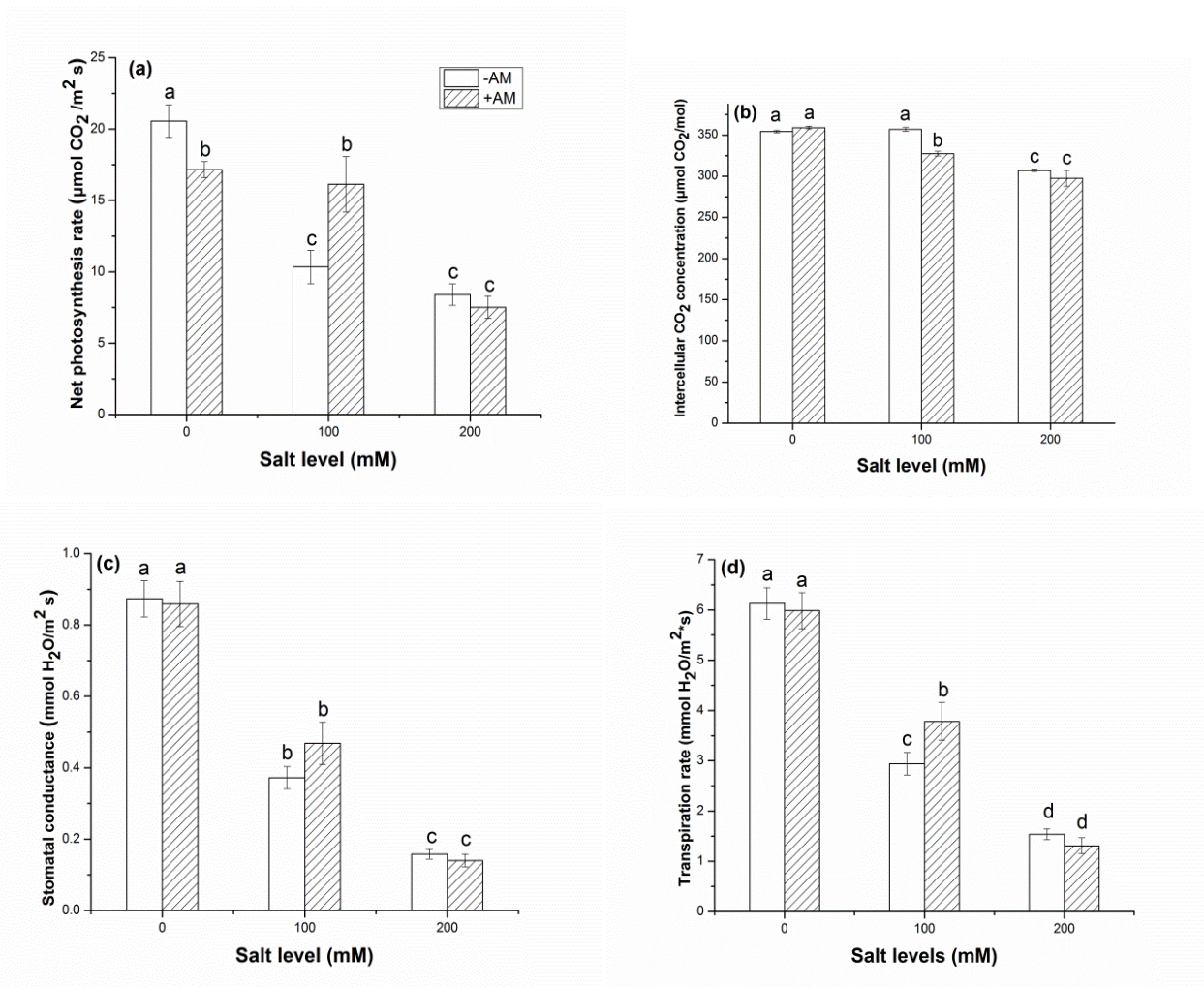


Fig. 3 – Effect of AMF inoculation on photosynthesis performance of Goji plants: (a) net photosynthesis rate, (b) intercellular CO₂ concentration, (c) stomatal conductance and (d) transpiration rate under salinity stress. Data are expressed as means±SE (n=9). Bars sharing the same letter are not significantly different at $P<0.05$ according to Duncan’s multiple test.

Soluble sugar, reducing sugar and starch

The soluble sugar in Goji leaves was increased by salinity stress (Fig. 4a, $F_{2, 12}=95.57$, $p<0.0001$). However, AMF inoculation further enhanced soluble sugar content in the leaves of Goji plants with and without salt stress (Fig. 4a, $F_{1, 12}=72.85$, $p<0.0001$). Salinity stress showed no effect on reducing sugar content in Goji leaves (Fig. 4b, $F_{2, 12}=0.05$, $p=0.9561$). AMF showed no effect on reducing sugars in Goji leaves under control and moderate salt stress, but it increased reducing sugars in Goji leaves compared with non-mycorrhizal plants (more than 2-fold) under severe salt stress (Fig. 4b). The starch content in mycorrhizal Goji leaves was lower compared with non-mycorrhizal plants under no and moderate salt stress (Fig. 4c, $F_{2, 12}=4.88$, $p=0.0282$). But at severe salt stress, mycorrhizal plants had similar leaf starch level as non-mycorrhizal plants.

Leaf water potential

Salt stress decreased leaf water potential of Goji plants (Fig. 5, $F_{2, 12}=21.25$, $p=0.0001$). No difference of leaf water potential between mycorrhizal and non-mycorrhizal Goji was found under no salt stress condition. But at moderate and severe salt levels, mycorrhizal Goji plants had higher leaf water potential relative to non-mycorrhizal plants (Fig. 5, $F_{1, 12}=60.81$, $p<0.0001$).

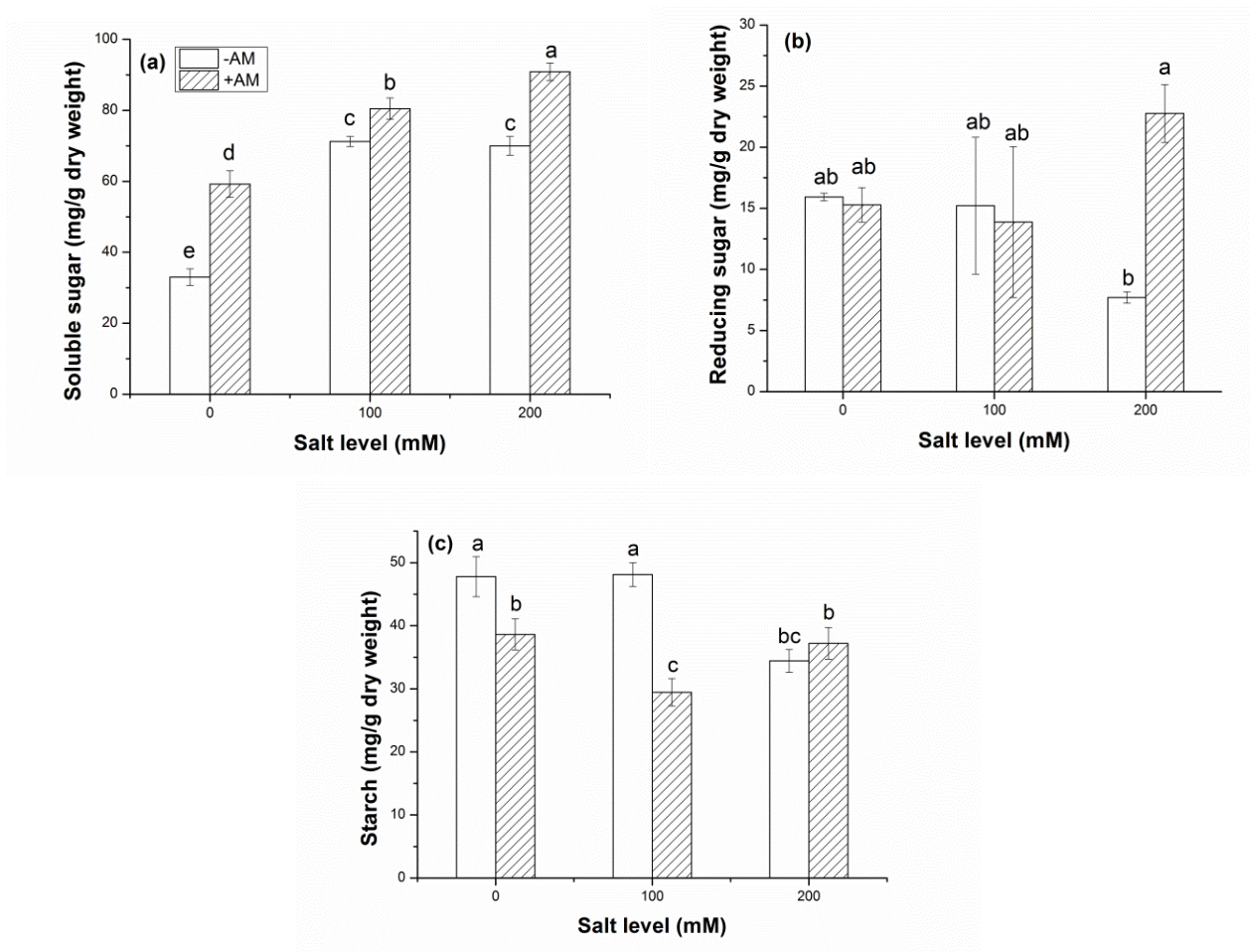


Fig. 4 – Effect of AMF inoculation on (a) soluble sugars, (b) reducing sugars and (c) starch content of Goji leaves under salinity stress. Data are expressed as means±SE (n=3). Bars sharing the same letter are not significantly different at $P<0.05$ according to Duncan’s multiple test.

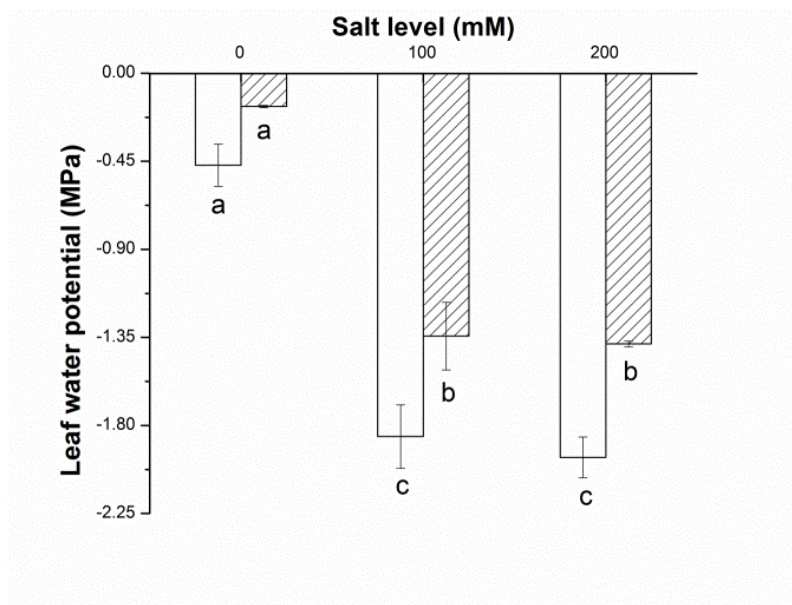


Fig. 5 – Effect of AMF inoculation on leaf water potential of Goji plants under salinity stress. Data are expressed as means±SE (n=3). Bars sharing the same letter are not significantly different at $P<0.05$ according to Duncan’s multiple test.

Discussion

The aim of the present study was to illuminate how AMF inoculation induced physiological changes affect the plant salt tolerance. AMF are corroborated to be most effective in alleviating the detrimental effect of salt stress on IAA, ABA and ZR content and photosynthesis of Goji plants under moderate salt level.

The positive effect of AMF on plant growth under salt stress is widely accepted (Chandrasekaran et al. 2014). The present study suggests that while salt stress has limited effect on morphological traits, the biomass of Goji plants is sensitive to salt stress, which is considered as crucial index for deleterious effect of salt stress (Porcel et al. 2012). However, this detrimental effect was mitigated by AMF inoculation, which supports previous findings (Ruiz-Lozano et al. 2012). Accordingly, AMF significantly increased Goji plant growth in non-saline and saline conditions.

Hormonal regulation is important for plant adaptation to stressful growing conditions (Pozo et al. 2015). Salt stress affects hormone homeostasis in plants, typically decreasing IAA and increasing ABA (Albacete et al. 2008). Our results show that while salt stress decreased IAA in both leaves and roots of Goji plants, AMF inoculation attenuated IAA decrease in both leaves and roots. This supports previous research on *Sesbania sesban* (Allah et al. 2015) and tomato (Abeer et al. 2015). Salt stress suppresses plant growth via decreasing IAA content due to the close relationship between IAA and biomass accumulation (Shao et al. 2016). As a result, the AMF induced amelioratory effect on biomass under salt stress might be attributed to higher IAA in Goji plants, which is consistent with *Sesbania sesban* and Jerusalem artichoke (Allah et al. 2015, Shao et al. 2016).

ABA is required for plant acclimation to salt stress as it controls the stomatal closure to reduce water loss (Suzuki et al. 2016). It is also responsible for dry matter accumulation and root growth (Sharp & LeNoble 2002, Shao et al. 2016). AM symbiosis has been shown to increase the ABA levels of host plants as it is essential for mycorrhizal symbiosis establishment (Ludwig-Müller 2010). In the present study, ABA in Goji leaves decreased in response to salinity stress, unlike other research reported increased ABA under salt stress (Allah et al. 2015). However, the literature shows salinity having multiple effects on ABA in plants. For example, salinity has been shown to decrease leaf ABA for *Atriplex halimus*, while other plants showed no influence on leaf ABA (*Suaeda fruticosa*) (Bankaji et al. 2014). Consequently, plant identity may have an effect on ABA regulation under salt stress.

ABA converts environmental signals into gene expression changes (Suzuki et al. 2016). Mycorrhizal Goji had higher leaf ABA content compared with non-mycorrhizal plants under moderate salt stress, which may be related to priming for enhanced salt tolerance (Aroca et al. 2013). ABA content in Goji roots was not affected by salt stress, which is in agreement with a salt-resistant cultivar of maize (Zörb et al. 2013). They suggest that ABA is mainly synthesized in roots and the higher leaf ABA is related to higher salt tolerance (Zörb et al. 2013). Therefore, the higher ABA content in Goji leaves under moderate salt level possibly represent higher salt tolerance of mycorrhizal Goji plants.

Zeatin riboside (ZR) is the most abundant, biologically active cytokinin in plants (Ludwig-Müller 2000). Mycorrhizal Goji plants had higher ZR in leaves under moderate salt stress. This is in accordance with previous study (Hause et al. 2007). Cytokinin has been reported to improve salt tolerance of eggplant via increasing photosynthesis and antioxidant enzymes (Wu et al. 2012). Thus, the improved ZR content in mycorrhizal Goji leaves might be associated with enhanced photosynthesis under moderate salt stress.

The role of GAs in plants responding to salt stress remains elusive. In this study, mycorrhizal plants had higher root GAs under non-saline salt stress. This is consistent with *Sesbania sesban* (Allah et al. 2015). However, GAs seems to be less important compared with IAA and ABA of Goji plants in symbiosis with AMF under salt stress.

Salt stress has been reported to disrupt photosynthesis of plants (Porcel et al. 2012), such as maize (Sheng et al. 2008), lettuce (Jahromi et al. 2008) and citrus (Wu et al. 2010). However,

inoculation by AMF alleviated the detrimental effect of salt stress on photosynthesis on these plants. And it is thought to occur via improved water supply (Sheng et al. 2008) and balanced ionic status (Evelin et al. 2012). Our data supports this as mycorrhizal Goji had higher photosynthesis, particularly under moderate salt stress. And this may be involved with hormonal and sugar regulation. Higher IAA, ABA and ZR content in mycorrhizal Goji leaves might promote photosynthesis of Goji plants under moderate salt stress. Enhanced accumulation of soluble sugars in mycorrhizal Goji leaves augments water uptake for photosynthesis. As AM symbiosis can also be affected by salinity (Pozo et al. 2015), the severe salt level likely exceeded the tolerance of both AMF and Goji plants (Wei et al. 2006), resulting in photosynthesis inhibition for inoculated and non-inoculated treatments.

The results in this study showed an increase of soluble sugars in Goji leaves in response to salt stress. Over-accumulation of salts in soil leads to water potential decline, thus, plants must decrease their water potential to maintain a favorable gradient for water flow from the soil into the roots and to avoid cell dehydration (Ruiz-Lozano et al. 2012). The buildup of organic solutes including soluble sugars is considered effective for osmotic adjustment (Sheng et al. 2011) and plants have been reported to accumulate more soluble sugars to counteract the osmotic stress induced by salinity (Kumar et al. 2015).

AMF inoculation further enhanced soluble sugars accumulation in Goji leaves, which supports previous research on *Aeluropus littoralis* (Hajiboland et al. 2015) and *Acacia ampliceps* (Theerawitaya et al. 2015). The reinforced osmotic adjustment of mycorrhizal plants allows cells to maintain turgor and related processes, such as cellular expansion and growth, stomatal opening, and photosynthesis (Ruiz-Lozano et al. 2012). The higher soluble sugar content in mycorrhizal Goji leaves under salt stress is likely due to the hydrolysis of starch (Ruiz-Lozano et al. 2012). This demonstrates AMF are associated with sugar metabolism to confer host plants osmotic tolerance in salinized soils, which supports previous work (Liu et al. 2016). The augmented soluble sugars lead to higher leaf water potential, indicating better water status of AM plant leaves. The increased reducing sugar is advantageous for protection of mesophyll cell from oxidative stress (Sheng et al. 2011).

In conclusion, this study shows AMF influence plant stress tolerance on multiple levels, particularly IAA, ABA and ZR regulation. AMF enhance sugar accumulation in mycorrhizal plants to facilitate water uptake under salt stress. The photosynthesis of Goji plants was strengthened by AMF under moderate salt stress, which subsequently augments plant growth, with emphasis on root development. The root development in turn maintains the water uptake to alleviate the detrimental effect from salt stress. Taken together, AMF can enhance salt tolerance of plants through regulating hormone and osmotic equilibrium.

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