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Influence of *Rhizoglyphus irregularis* on nutraceutical quality and regeneration of *Lycium barbarum* leaves under salt stress

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Abstract

Whether arbuscular mycorrhizal fungi (AMF) augment the nutraceutical quality of crops under salt stress is critical as a potential agronomic practice in salinized farmland. To evaluate the effect of *Rhizoglyphus irregularis* on the nutraceutical quality of *Lycium barbarum* leaves under salt stress, growth parameters, rutin, polysaccharide, acidic polysaccharide and amino acids of two harvests were analyzed. Inoculation of *R. irregularis* significantly increased the regenerated bud number (PES=0.577, $P<0.0001$) and rutin concentration (PES=0.544, $P<0.001$) of *L. barbarum* leaves, with and without salt stress. The biomass of the 2nd harvest (PES=0.355, $P=0.0091$) and acidic polysaccharide (PES=0.518, $P=0.001$) of *L. barbarum* leaves were notably enhanced by *R. irregularis* under 200 mM salt level. *R. irregularis* had insignificant effect on polysaccharide (PES=0.092, $P=0.221$) and amino acids levels (PES=0.263, $P=0.130$) in the leaves of *L. barbarum*. However, inoculation by *R. irregularis* decreased proline level (PES=0.761, $P=0.001$) in the leaves of *L. barbarum* when subjected to salt stress. Taken together, *R. irregularis* significantly improved the nutraceutical quality and facilitated the sustainable production of *L. barbarum* leaves exposed to salt stress.

Keywords: *Lycium barbarum*, arbuscular mycorrhizal fungi, rutin, acidic polysaccharide, bud regeneration

Introduction

The nutraceutical value of crops refers to the health-promoting ingredients or natural components that provide potential health benefits to humans (Dev et al. 2011; Leoncini et

al. 2012). Thus, the nutraceutical value of crops is considered as a crucial component to build the health of humans as well as an evaluation of crop quality (Rahal et al. 2014). However, soil salinization induced by anthropogenic activities and climate changes are challenging the nutraceutical quality of crops (Wheeler and von Braun 2013). In particular, salt stress triggers numerous physiological and biochemical reactions in plants that can alter the chemical composition of crops and thus impair the nutraceutical quality of crops (Wang and Frei 2011). How to improve the nutraceutical quality of crops becomes significant to nourish human beings.

It is well known that arbuscular mycorrhizal fungi (AMF) can establish the symbiosis with more than 80% of terrestrial plants (Smith and Read 2008). AMF are reported to enhance salt tolerance of host plants through improving nutrient uptake (Garg and Pandey 2015), maintaining ion homeostasis (Estrada et al. 2013; Wu et al. 2013), enhancing antioxidant system (Evelin and Kapoor 2014), elevating photosynthesis (Sheng et al. 2008) and augmenting osmotic adjustment (Auge et al. 2014). Besides, AMF have been shown to increase the quality of tomato, yam and strawberry through regulating the secondary metabolites (Bona et al. 2015; Hart et al. 2015; Lu et al. 2015). Inoculation with *Rhizophagus intraradices* (Schenck & Smith) and *Funneliformis mosseae* [(Nicol. & Gerd.) Walker & Schüßler comb. nov.] improved the medicinal component (glucosinolate) in the leaves of *Moringa oleifera* (Cosme et al. 2014). Although inoculation with AMF is effective in augmenting the nutraceutical quality of crops (Giovannetti et al. 2013), the beneficial effect may be affected by environmental

factors, such as soil salinization (Nadeem et al. 2014). However, whether the positive effect of AMF on the nutraceutical value of crops persists under salt stress is mostly unclear.

Lycium barbarum is an important crop with medicinal and economic value in Northwest China. The fruits of *L. barbarum* are usually consumed for dietetic and medicinal purpose. The leaves of *L. barbarum* have potential benefits for human, but they are less exploited compared with the fruits. The leaves of *L. barbarum* are rich in flavonoid, polysaccharide and amino acid (Wang et al. 2015). *L. barbarum* leaves even have higher flavonoid level than fruits (Liu et al. 2012). Besides, the most abundant flavonoid in the leaves of *L. barbarum* is rutin (Dong et al. 2009) which has anti-inflammatory and antioxidative actions, and has been described as neuroprotective and able to reduce damage in central nervous system diseases (Rodrigues et al. 2013). Recently, the leaves of *L. barbarum* are increasingly processed as vegetable and tea which are popular in China. Meanwhile, the exploitation and utilization of *L. barbarum* leaves can increase the income of farmers (Wei et al. 2006). The total area of saline soil in China is about 3.6×10^7 ha, accounting for 4.88 % of the country's total available land (Li et al. 2014). *L. barbarum* is proposed as a potential pioneer plant to reclaim salinized soils, but the growth and photosynthesis of *L. barbarum* were negatively affected by high level salt stress (e.g. 200 mM NaCl) (Wei et al. 2006). The techniques for improving the fitness of *L. barbarum* under high level salt conditions are needed for reclaiming the salinized areas in China. Our previous studies proved that *L. barbarum* could establish

mutual symbiosis with AMF in nature (Zhang et al. 2010). Furthermore, AMF enhanced the salt tolerance of *L. barbarum* through physiological and ultrastructural protection (unpublished data). But the influence of AMF on the nutraceutical quality of *L. barbarum* is still unclear. We hypothesized that AMF can enhance the nutraceutical quality regarding to rutin, polysaccharide and amino acids of *L. barbarum* exposed to salt stress.

Materials and methods

Experimental design and biological materials

The experiment was based on a completely randomized blocked design with two factors: mycorrhizal treatments [*Rhizoglyphus irregularis* and non-AMF control] and salt levels of 0 and 200 mM NaCl (Fig. S1). The concentration of NaCl stress was based on the previous study of Wei et al. (2006). The variety Ningcai No. 1 (*L. barbarum*) was chosen in the current study due to its prevalence as vegetable *L. barbarum* cultivar in Northwest China. *L. barbarum* propagated by cutting were provided by National Wolfberry Engineering Research Center of Ningxia Academy of Agriculture and Forestry Sciences. A widely used commercially available AM fungus *R. irregularis* DAOM 197198 (Premier Tech Inc., Canada, containing 60 spores per gram inoculum) was employed as inoculum. The soils used in the experiment were collected from the campus of Northwest A&F University, Yangling city, Shaanxi province. The sieved (1 mm) soils and silica sand were sterilized (121 °C, 0.1 MPa for 2 h) and mixed (1:1, v/v) thoroughly. Each pot was filled with 1.5 kg of the culture substrate. Thirty *L. barbarum* plants were inoculated with 15 g

R. irregularis inoculum substrate (vermiculite) (+AM), the other 30 plants were inoculated with 15 g sterilized inoculum substrate (-AM). To maintain the same soil microbial community except for *R. irregularis*, the control treatments (-AM) received 10 mL filtration of *R. irregularis* inoculum ($1 \mu\text{m}$).

L. barbarum plants were grown in a greenhouse of Northwest A&F University with solar light from May to July 2015. The mean temperatures during the experiments in the greenhouse were 30/22 °C day/night and the mean relative humidity was 70-75%. After four weeks, half of the plants of each treatment (+AM or -AM) were irrigated with distilled water (0 mM) or NaCl solution (200 mM). The 50 mM NaCl solution per day was applied to the salt treatment to avoid salt shock of *L. barbarum*. The final soil electric conductivity (EC) for 0 and 200 mM treatments were 0.13 ± 0.03 and 7.50 ± 1.61 mS cm^{-1} , respectively. The *L. barbarum* leaves were harvested 4 weeks after application of salt stress as the 1st harvest. Then the buds of *L. barbarum* regenerated and grew into leaves. The regenerated buds number was recorded for 4 sequential weeks. Four weeks after the 1st harvest, all *L. barbarum* plants were harvested and the regenerated leaves were regarded as the 2nd harvest.

Determination of AMF colonization

Root samples of *L. barbarum* plants were cleared and stained according to the method of Phillips and Hayman (1970). Mycorrhizal structures of arbuscule, vesicule, hyphae and spore were examined under compound microscope (Olympus U-TV0.63XC, Japan). Colonization rate was measured using the gridline intersect method (Giovannetti

and Mosse 1980). Two hundreds cm of roots per treatment were used to assess mycorrhizal colonization.

Determination of growth parameters

Leaf biomass of *L. barbarum* of the 1st harvest was measured after drying at 70 °C for 72 hours. After the 2nd harvest, the leaves, shoots and roots were separated to determine the biomass as described above. The number of regenerated buds was recorded for 4 sequential weeks after the 1st harvest.

Determination of rutin

L. barbarum leaves of the two harvests were dried at 60 °C for 72 hours and homogenized into fine powders. The leaf powder was placed in a centrifuge tube with 70% ethanol (1:30 w/v). Rutin extraction was conducted by sonicating for 50 min at 40 °C at 250 W after incubating at room temperature overnight (Shumei KQ-500DE, Kunshan, China). Ethanol (70%) was used to supplement the weight loss. The extract was centrifuged at 3250×g (Eppendorf 5810 R, Germany) for 10 min. The supernatant passing through 0.22-µm filter was rutin extract.

Rutin concentration in *L. barbarum* leaves was determined using HPLC (Shimadzu, Kyoto, Japan) equipped with an Apollo C18 column (5µm, 4.6 mm×250mm, Alltech, Deerfield, IL, USA). Twenty µL rutin extract was injected into the column at room temperature. The wavelength for detector was set at 330 nm. The separation was conducted with 0.1% acetic acid (solvent A) and acetonitrile (solvent B) using the following procedure: 0.01-10:00 min 25% B, 10:00-18:00 min 70% B, 18:00-18:01 min

70% B, 18:01-23:00 min 100% B, 23:00-25:00 min 100% B, 25:00-30:00 min 25% B.

Determination of polysaccharide and acidic polysaccharide

The leaf powder obtained as described above was used for polysaccharide and acidic polysaccharide extraction. Leaf powder was decolored in ethanol (1:20 w/v) for 5 min twice. The dried precipitate was weighed and dissolved in distilled water (1:20 w/v). Polysaccharide was extracted at 80 °C for 2 hours in water bath and cooled for 5 min at room temperature. After passing through 0.22- μ m filter, the polysaccharide extract was diluted 1:10 using distilled water for determination.

Polysaccharide concentration was determined using phenol-sulfuric acid method (Cuesta et al. 2003). Two mL of sample was added into a test tube with 1 mL 6% phenol. Then 5 mL concentrated sulfuric acid was added into the test tube. After incubating at room temperature for 30 min, the absorbance of 490 nm was determined on UV-Vis spectrophotometer (UV mini 1240, Shimadzu, Kyoto, Japan). The polysaccharide content was calculated according to the standard curve.

Acidic polysaccharide concentration in *L. barbarum* leaves was determined using sulfate-3-phenylphenylol method (Zhang et al. 2004). One test tube with 0.5 mL extracted polysaccharide was added with 4.5 mL sodium tetraborate-sulfuric acid, cooled on ice and thoroughly shaken. The test tube was heated in water bath at 100 °C for 10 min, and subsequently cooled in water-ice bath. Fifty μ L of 0.15% m-hydroxydiphenyl was added to the test tube for showing color. Absorbance of 525 nm was determined using UV-Vis spectrophotometer (UV mini 1240, Shimadzu, Kyoto, Japan). The acidic

polysaccharide was calculated according to the standard curve.

Determination of amino acids

Leaf powder (0.1 g) of *L. barbarum* prepared as described above was used to determine amino acids. Ten mL of 6 M hydrochloric acid was injected into a digestion vessel and sealed on the burner, incubated in the oven at 110 °C for 22 h, then cooled at room temperature. The hydrolysate was filtered and diluted to a final volume of 50 mL. One mL of diluted filtrate was evaporated to dry on a water bath. The residue was dissolved in 1 mL deionized water and evaporated till dry. This procedure was repeated for three times. The residue was dissolved in 25 mL 0.1 M hydrochloric acid. The dissolved samples were filtered through a 0.45- μ m PTFE membrane before analysis.

Amino acid content was determined on amino acid analyzer (L-8900, Hitachi, Japan) fitted with a 2.6 mm $\times$ 105 mm column packaged with 2619 sulfonic acid strong-acid anion exchange resin (Hitachi Co., Japan). The optimal analytical time was 35 min, with buffer flow rate at 0.225 mL/min and ninhydrin at 0.3 mL/min. The pump pressure was set at 15-35 kg/cm², column pressure at 80-130 kg/cm², column temperature at 53 °C. The injection volume was 50 μ L. In addition, 3 nmol per 50 μ L of the reference solution was employed and the nitrogen pressure was 0.28 kg/cm².

Statistical analysis

Prior to data analysis, the Kolmogorov-Smirnov test and Levene test were used to check the data normality and the homogeneity of variance. In the present study, all the original datasets conformed to a normal distribution. When necessary, dependent

variables were transformed using the natural logarithmic, arcsine or Box-Cox functions to achieve requirements of homogeneity of variance. Repeated measure ANOVA was applied to evaluate the effect of *R. irregularare* and salt stress on bud regeneration number, rutin, polysaccharide, acidic polysaccharide and amino acids content. One way ANOVA followed by Tukey's HSD at $P < 0.05$ was used to compare the differences of these parameters among the treatments. Partial Eta squared (PES) was used to evaluate the effect size. All statistical analyses were carried out on SPSS software package (SPSS Inc., Chicago, IL, USA). Graphics were prepared on OriginPro 8.5 (OriginLab, Northampton, MA, USA).

Results

Mycorrhizal colonization, bud regeneration and plant growth parameters

Typical structures of AM fungi were observed in the roots of *L. barbarum* inoculated with *R. irregularare*. In particular, the hyphal colonization was prominent in mycorrhizal *L. barbarum* roots (Table 1). No sign of mycorrhizal colonization was observed on non-AM plants (Fig. S2). Mycorrhizal colonization rates of *L. barbarum* decreased under salt stress (Table 1).

Salt stress significantly decreased the regenerated bud number of *L. barbarum* inoculated or not with *R. irregularare* after the 1st harvest (Fig. 1, PES=0.878, $P < 0.0001$). However, inoculation with *R. irregularare* alleviated the decrease in bud regeneration of *L. barbarum* under salt stress (PES=0.577, $P < 0.0001$). Compared with control, inoculation with *R. irregularare* significantly increased the number regenerated buds for the 4

respective weeks by 89.5%, 121.5%, 31.3% and 79.2% under non-salt stress condition. Under 200 mM NaCl stress, the regenerated bud number of control plants was drastically decreased for the first two weeks after the 1st harvest (Fig. 1), but the *L. barbarum* inoculated with *R. irregularare* had 9 and 24 folds higher regenerated bud number than control for the 1st and 2nd week. Four weeks after the 1st harvest, mycorrhizal plants had up to 11.3-fold higher regenerated bud number than control under salt stress condition. Moreover, at the 4th week, the regenerated bud number of +AM *L. barbarum* under salt stress was similar with that of control under no salt stress. Consequently, *R. irregularare* notably increased the number of regenerated bud of *L. barbarum* with and without salt stress.

Salt stress significantly decreased the leaf biomass of *L. barbarum* of the two harvests (PES=0.608, $P<0.01$). Inoculation with *R. irregularare* notably increased leaf biomass of the 2nd harvest (PES=0.355, $P<0.01$) by 1.1 folds under non-salt stress (Table 2). The stem and root biomass of *L. barbarum* were not affected by salt stress and inoculation with *R. irregularare*.

Rutin content

Inoculation with *R. irregularare* significantly elevated rutin level in *L. barbarum* leaves for both harvests (Table 3, PES=0.544, $P<0.001$). Under non-salt stress, the rutin level of mycorrhizal *L. barbarum* leaves increased 96.3% and 134.2% compared with control plants for the two harvests, respectively. In the presence of salt stress, the rutin content of mycorrhizal *L. barbarum* was increased by 96.1% and 77.5% relative to

control for the two respective harvests. Although the promotion effect of *R. irregularis* on rutin content fluctuated in different harvests and salt conditions, overall, the inoculation of *R. irregularis* efficiently augmented rutin levels of *L. barbarum* leaves.

Polysaccharide and acidic polysaccharide

Inoculation with *R. irregularis* showed insignificant influence on polysaccharide concentration in the leaves of *L. barbarum* with or without salt stress (Table 3, PES=0.092, $P=0.221$). However, under salt stress, inoculation with *R. irregularis* increased the polysaccharide content in the leaves of *L. barbarum* for the 2nd harvest by 64.2% compared with control.

In the absence of salt stress, the acidic polysaccharide content in *L. barbarum* inoculated with *R. irregularis* was similar with that of control for both harvests (Table 3). However, under salt stress, the acidic polysaccharide of mycorrhizal *L. barbarum* leaves was notably higher compared with control plants (Table 3, PES=0.518, $P=0.001$). Inoculation with *R. irregularis* increased the acidic polysaccharide by 66.7% and 103.1% for the two respective harvests under salt stress.

Amino acids

Under salt stress, *R. irregularis* inoculation decreased proline content by 57.7% and 65.6% compared with control for two harvested leaves (Fig. 2, PES=0.761, $P<0.05$). The most abundant amino acid in *L. barbarum* leaves was proline and the least abundant was cysteine. On the other hand, the content of total amino acid in *L. barbarum* remained unchanged between mycorrhizal and non-mycorrhizal treatments regardless of salt stress

(PES=0.263, $P=0.130$). Meanwhile, the total amino acid content of mycorrhizal *L. barbarum* decreased for both harvested leaves under salt stress compared with non-salt stress condition, but remained unchanged for control plants (Fig. 3).

Discussion

The aim of this study was to explore the impact of AM fungus *R. irregulare* on the nutraceutical quality of *L. barbarum* leaves under salt stress. Specifically, the nutraceutical quality of the leaves of *L. barbarum* includes rutin, polysaccharide, acidic polysaccharide and amino acids. The present results accepted our hypothesis that AM symbiosis improved the nutraceutical quality of *L. barbarum* leaves through elevating rutin and acidic polysaccharide content under salt stress. Moreover, *R. irregulare* inoculation had positive impact on the regeneration of *L. barbarum* buds, which can potentially facilitate the sustainable production of *L. barbarum* leaves.

The present results showed that *L. barbarum* can establish symbiosis with *R. irregulare*, showing abundant hypha in roots (Table 1, Fig. S2). The aseptate hyphae of AMF connecting roots and soil, can explore soil pores inaccessible to plants due to their 10-fold smaller diameter than root hairs (Smith et al. 2010); they can transport 375-760 nL of water per hour (Faber et al. 1991), taking up 20% of the total water absorbed by plant roots (Ruth et al. 2011). Moreover, the nutrient uptake along with water transfer by AMF hyphae has been widely accepted (Hodge et al. 2010). The AMF mycelia are thereby complementary to root system for absorbing nutrients and water, in order to ameliorate the detrimental effect of salt stress. The decreased colonization rate in the

roots of *L. barbarum* under salt stress might be attributed to direct deleterious impact on *R. irregulare* and indirect effect through decreased plant growth (Evelin et al. 2009).

As the buds can regenerate after *L. barbarum* leaf harvest, the cost for producing *L. barbarum* vegetables would decline. As a result, the regenerative ability of *L. barbarum* buds is critical for sustainable production of *L. barbarum* leaves. In this study, salinity inhibited recovery of *L. barbarum* buds. But *R. irregulare* inoculation promoted bud regeneration of *L. barbarum* with and without salt stress, therefore increasing the additional yield for the successional harvest of *L. barbarum* leaves. This might be explained by the fact that AMF can increase cytokinin content in plant which plays a key role in inducing plant bud regeneration (Ludwig-Müller 2010; Premkumar et al. 2011). Another reason may be the promotion of plant growth mediated by AMF mycelium facilitating the uptake of water and nutrients in salinized soils (Aroca et al. 2012; Koide 2010). To the best of our knowledge, this is the first report on the effect of AMF on plant bud regeneration under salt stress. The regeneration promotion effect induced by AMF inoculation might have implication on the leaf regeneration of tissue-cultured plants.

Inoculation by *R. irregulare* increased the leaf biomass of *L. barbarum* compared with control for the 2nd harvest under no salt stress, but showed insignificant impact on the biomass of leaves of *L. barbarum* of the 1st harvest with and without salt stress. The biomass of stem and root of *L. barbarum* were not significantly affected by salt stress and inoculation with *R. irregulare*. This is not surprising as the insignificant effect of AMF on biomass has been reported on tomato and *Viola tricolor* L. (Hart et al. 2015; Zubek et al.

2015). Therefore, the influence of AMF on the biomass of plants may depend on the plant species.

The leaves of *L. barbarum* are rich in flavonoid, and the most abundant flavonoid therein is rutin (Dong et al. 2009). In this study, *R. irregularare* inoculation significantly enhanced rutin content in *L. barbarum* leaves regardless of salt stress. This was in accordance with the observation on *V. tricolor* L. inoculated with *R. irregularare* (Zubek et al. 2015). The positive effect of AM symbiosis on the phenolic content has also been reported on artichoke, *Moringa oleifera*, lettuce and onion (Baslam et al. 2011; Ceccarelli et al. 2010; Cosme et al. 2014; Mollavali et al. 2016). Moreover, the enhanced flavonoid has been illustrated in mycorrhizal *Rose geranium* under drought stress and in cucumber under cold stress (Amiri et al. 2015; Chen et al. 2013). Flavonoid can directly scavenge active oxygen molecular species to protect the membrane system of plant cells in adversities (Abbaspour et al. 2012). Therefore, increased flavonoid content may be a general response to AMF colonization since flavonoids are signal molecules in plant-fungal interactions (Abdel-Lateif et al. 2012). Meanwhile, flavonoids are also beneficial to human health with antioxidative activity, free-radical scavenging capacity, coronary heart disease prevention and anti-cancer activity (Yao et al. 2004). Consequently, the higher flavonoid in *L. barbarum* leaves induced by *R. irregularare* represents enhanced salt tolerance as well as nutraceutical quality.

Polysaccharides derived from *L. barbarum* leaves have been shown to possess a range of biological activities, including effects on aging, neuroprotection, increased

metabolism, glucose control in diabetics, glaucoma, immunomodulations and anti-tumor activity (Amagase and Farnsworth 2011). Salt stress increased polysaccharide of aloe, and inoculation with *Glomus intraradices* and *Glomus mosseae* further increased the polysaccharide levels in aloe under salt stress (Cardarelli et al. 2013). However, *R. irregulare* had no significant impact on polysaccharide levels in *L. barbarum* leaves in this study. In the pattern of intensive agriculture, chemical fertilizer is increasingly used in *L. barbarum* orchards, but reported to reduce polysaccharide content in *L. barbarum* plants (Chung et al. 2010). The utilization of AMF can replace part of chemical fertilizer (Oliveira et al. 2016), which might avoid the polysaccharide decrease in *L. barbarum* production.

Acidic polysaccharide generally connects with uronic acid residues which can modify the solubility of polysaccharide and thus affect the activity of polysaccharide (Chen et al. 2004). Besides, acidic polysaccharide of *L. barbarum* has been shown to have higher efficiency to scavenge free radicals and inhibit tumor cells compared with neutral polysaccharide (Liu et al. 2016; Zhang et al. 2013; Zhang et al. 2015). Thus, the higher content of acidic polysaccharide in *L. barbarum* leaves represents higher nutraceutical quality under salt stress. Meanwhile, acidic polysaccharide is more effective to prevent humans from oxidative damage (He et al. 2012). Consequently, *R. irregulare* is effective in enhancing the nutraceutical quality of *L. barbarum* leaves through increasing acidic polysaccharide content under salt stress. Although the biomass of *L. barbarum* leaves remained low under salt stress relative to non-salt stress condition, inoculation

with *R. irregulare* had higher regenerated bud number compared with control. Besides, the regenerated bud number of +AM *L. barbarum* was similar with that of –AM plants under no salt stress. If the growing period extends, the leaf biomass of +AM *L. barbarum* may potentially increase to a higher level to develop a viable business.

The amino acids of mycorrhizal *L. barbarum* leaves decreased in response to salt stress, but remained unchanged for non-mycorrhizal plants. AMF enhance sink strength in plants by requiring carbon fixed by plants, which is considered as ‘cost’ of symbiosis (Lerat et al. 2003). This mycorrhizal enhanced sink strength may accelerate the outflow of amino acids from leaves, resulting in lower amino acids in mycorrhizal *L. barbarum* leaves (Wright et al. 1998). Salinity may further promote this amino acid outflow as both AMF and plant roots were stressed. Free proline is an osmoprotectant in plants under stress (Kumar et al. 2015). However, the influence of AMF on the total proline in plants has been less studied. In this study, the total proline content in mycorrhizal plants was lower than that of non-mycorrhizal *L. barbarum* under salt stress. The decreased proline in mycorrhizal plants may imply that AM *L. barbarum* were less stressed in salty soils (Ruiz-Lozano et al. 2012).

In conclusion, *R. irregulare* significantly improved nutraceutical quality of *L. barbarum* leaves in absence and presence of salt stress, including increased rutin and acidic polysaccharide levels. Inoculation with *R. irregulare* notably enhanced the regeneration of *L. barbarum* buds under salt stress, which is preferable for sustainable production of *L. barbarum* leaves.

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Table 1. The colonization rate (%) of *Rhizogloinus irregulare* in the roots of *Lycium barbarum* with and without salt stress

Salt (mM)	Arbuscule	Vesicule	Hyphae	Spore	Total mycorrhizal structures
0	9.04±1.18a	7.22±1.71a	39.00±2.03a	16.07±4.25a	71.33±5.19a
200	1.05±0.79b	2.30±0.84b	14.26±7.07b	1.15±0.16b	18.76±8.39b

Means with different letters in each column are significantly different at $P<0.05$. Data are mean±SE (n=3)

Draft

Table 2. Effect of *Rhizoglyphus irregularis* on the biomass of two harvested leaves, shoot and root of *Lycium barbarum* with and without salt stress

Salt (mM)	AMF	Biomass (g)			
		1 st harvested leaf	2 nd harvested leaf	Shoot	Root
0	-AMF	0.56±0.12a	0.20±0.00b	2.10±0.31a	1.27±0.23a
	+AMF	0.46±0.05a	0.42±0.10a	3.63±0.74a	3.40±1.60a
200	-AMF	0.14±0.09b	0.10±0.01b	1.67±0.97a	1.20±0.65a
	+AMF	0.14±0.02b	0.20±0.04b	1.50±0.60a	1.13±0.33a

Means with different letters in each column are significantly different at $P<0.05$. Data are mean±SE (n=3)

Draft

Table 3. Effect of *Rhizoglosum irregulare* on the rutin, polysaccharide, acidic polysaccharide (mg/g) of *Lycium barbarum* leaves with and without salt stress

Harvest	Salt (mM)	AMF	Rutin	Polysaccharide	Acidic polysaccharide
1 st	0	-AM	1.07±0.12b	12.94±2.44a	1.72±0.11ab
		+AM	2.10±0.37ab	14.67±1.77a	1.58±0.12ab
	200	-AM	1.28±0.26b	11.60±2.99a	1.23±0.18b
		+AM	2.51±0.56a	12.31±1.84a	2.05±0.22a
2 nd	0	-AM	0.76±0.06b	21.41±4.40a	1.68±0.16a
		+AM	1.78±0.42a	24.42±7.89a	1.76±0.17a
	200	-AM	0.71±0.11b	12.41±0.95a	0.98±0.11b
		+AM	1.26±0.18ab	20.38±0.91a	1.99±0.10a

Means with different letters for each harvest in each column are significantly different at $P<0.05$. Data are mean±SE (n=5).

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Figure captions

Figure 1. Effect of *Rhizoglyphus irregularis* on the number of regenerated bud of *Lycium barbarum* with and without salt stress. Data sharing the same letter are not significantly different at $P < 0.05$. Data are mean $\pm$ SE (n=5).

Figure 2. Effect of *Rhizoglyphus irregularis* on the amino acid composition of *Lycium barbarum* leaves with and without salt stress. (a) 1st harvest; (b) 2nd harvest. Bars sharing the same letter are not significantly different at $P < 0.05$. Data are mean $\pm$ SE (n=3).

Figure 3. Effect of *Rhizoglyphus irregularis* on the total amino acids of *Lycium barbarum* leaves with and without salt stress. (a) 1st harvest; (b) 2nd harvest. Data points sharing the same letter are not significantly different at $P < 0.05$. (For interpretation of the references to color in this figure legend, the readers are referred to the online version of this article.)

Figure 1. Effect of *Rhizoglossus irregularis* on the number of regenerated bud of *Lycium barbarum* with and without salt stress. Data sharing the same letter are not significantly different at $P < 0.05$. Data are mean $\pm$ SE (n=5).

Fig. 1
296x209mm (300 x 300 DPI)

Figure 2. Effect of *Rhizoglomus irregulare* on the amino acid composition of *Lycium barbarum* leaves with and without salt stress. (a) 1st harvest; (b) 2nd harvest. Bars sharing the same letter are not significantly different at $P < 0.05$. Data are mean $\pm$ SE ($n = 3$).

Fig. 2

287x201mm (300 x 300 DPI)

Figure 3. Effect of *Rhizoglyphus irregularis* on the total amino acids of *Lycium barbarum* leaves with and without salt stress. (a) 1st harvest; (b) 2nd harvest. Data points sharing the same letter are not significantly different at $P < 0.05$. (For interpretation of the references to color in this figure legend, the readers are referred to the online version of this article.)

Fig. 3
202x141mm (300 x 300 DPI)