Influence of *Rhizoglomus irregulare* on nutraceutical quality and regeneration of *Lycium barbarum* leaves under salt stress

Hongguang Liu, Yajun Wang, Hui Chen, and Ming Tang

**Abstract:** Whether arbuscular mycorrhizal fungi augment the nutraceutical quality of crops under salt stress is critical as a potential agronomic practice in salinized farmland. To evaluate the effect of *Rhizoglomus irregulare* on the nutraceutical quality of *Lycium barbarum* leaves under salt stress, we analyzed growth parameters and the rutin, polysaccharide, acidic polysaccharide, and amino acids contents of 2 harvests. Inoculation of *R. irregulare* significantly increased the regenerated bud number (partial eta squared \(PES = 0.577, P < 0.0001\)) 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. irregulare* under 200 mmol/L salt level. *Rhizoglomus irregulare* 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. irregulare* decreased proline level \(PES = 0.761, P = 0.001\) in the leaves of *L. barbarum* when subjected to salt stress. Taken together, these results indicate that *R. irregulare* significantly improved the nutraceutical quality and facilitated the sustainable production of *L. barbarum* leaves exposed to salt stress.

**Key words:** *Lycium barbarum*, arbuscular mycorrhizal fungi, saline stress, rutin, acidic polysaccharide, bud regeneration.

**Résumé :** Il est important de savoir si les champignons arbusculaires mycorhiziens augmentent la qualité nutraceutique des récoltes soumises à un stress salin dans le cadre d’une possible pratique agronomique sur les terres agricoles salinisées. Afin d’évaluer l’effet de *Rhizoglomus irregulare* sur la qualité nutraceutique des feuilles de *Lycium barbarum* soumises à un stress salin, les paramètres de croissance, la rutine, les polysaccharides, les polysaccharides acides et les acides aminés de 2 récoltes ont été analysés. L’inoculation de *R. irregulare* augmentait significativement le nombre de bourgeons régénérés \(PES = 0.577, P < 0.0001\) et la concentration de rutine \(PES = 0.544, P < 0.001\) des feuilles de *L. barbarum*, avec ou sans stress salin. La biomasse de la deuxième récolte \(PES = 0.355, P = 0.0091\) et les polysaccharides acides \(PES = 0.518, P = 0.001\) des feuilles de *L. barbarum* étaient particulièrement accrus par *R. irregulare* en présence de 200 mmol/L de sel. *Rhizoglomus irregulare* avait un effet non significatif sur les niveaux de polysaccharides \(PES = 0.092, P = 0.221\) et d’acides aminés \(PES = 0.263, P = 0.130\) dans les feuilles de *L. barbarum*. Cependant, l’inoculation de *R. irregulare* diminuait les niveaux de proline \(PES = 0.761, P = 0.001\) dans les feuilles de *L. barbarum* soumises à un stress salin. En somme, *R. irregulare* améliorait significativement la qualité nutraceutique et facilitait la production durable des feuilles de *L. barbarum* exposées à un stress salin. [Traduit par la Rédaction]

**Mots-clés :** *Lycium barbarum*, champignons arbusculaires mycorhiziens, stress salin, rutine, polysaccharides acides, régénération des bourgeons.

**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 a crucial component to build the health of humans and a means of evaluating crop quality (Rahal et al. 2014). However, soil salinization induced by anthropogenic activities and climate change 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...
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 symbiosis with more than 80% of terrestrial plants (Smith and Read 2008). AMF are reported to enhance salt tolerance of host plants by improving nutrient uptake (Garg and Pandey 2008), maintaining ion homeostasis (Estrada et al. 2013; Wu et al. 2013), enhancing antioxidant systems (Evelin and Kapoor 2014), elevating photosynthesis (Sheng et al. 2008), and augmenting osmotic adjustment (Augé 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 purposes. The leaves of *L. barbarum* have potential benefits for humans, but they are less exploited than the fruits. The leaves of *L. barbarum* are rich in flavonoids, polysaccharides, and amino acids (Wang et al. 2015). *Lycium barbarum* leaves even have higher flavonoid levels than the fruits do (H. 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 a vegetable and tea, both of 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). *Lycium barbarum* is proposed as a potential pioneer plant to reclaim salinized soils, but the growth and photosynthesis of *L. barbarum* were shown to be negatively affected by high levels of salt stress (e.g., 200 mmol/L NaCl) (Wei et al. 2006). Techniques for improving the fitness of *L. barbarum* under high 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 (H. Zhang et al. 2010). Furthermore, AMF enhanced the salt tolerance of *L. barbarum* through physiological and ultrastructural protection (H. Liu, Y. Wang, H. Chen, M. Tang, 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 rutin, polysaccharide, and amino acids content 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 2 factors: mycorrhizal treatments (*Rhizoglomus irregularare* and non-AMF control) and salt levels of 0 and 200 mmol/L 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 owing to its prevalence as vegetable *L. barbarum* cultivar in northwest China. *Lycium barbarum* propagated by cutting were provided by the National Wolfberry Engineering Research Center of Ningxia Academy of Agriculture and Forestry Sciences. The widely used commercially available AM fungus *R. irregularare* 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 of *R. irregularare* inoculum substrate (vermiculite) (+AM); the other 30 plants were inoculated with 15 g of sterilized inoculum substrate (−AM). To maintain the same soil microbial community except for *R. irregularare*, the control treatments (−AM) received 10 mL filtration of *R. irregularare* inoculum (1 μm).

*Lycium 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 °C (day) and 22 °C (night), and the mean relative humidity was 70%–75%. After 4 weeks, half of the plants of each treatment (+AM or −AM) were irrigated with distilled water (0 mmol/L) or NaCl solution (200 mmol/L). The 50 mmol/L NaCl solution per day was applied to the salt treatment to avoid salt shock of *L. barbarum*. The final soil electric conductivity for the 0 and 200 mmol/L treatments were 0.13 ± 0.03 and 7.50 ± 1.61 mS/cm, 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 number of regenerated buds...
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, vesicle, 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 hundred centimetres 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 h. 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**

*Lycium barbarum* leaves of the 2 harvests were dried at 60 °C for 72 h and homogenized into fine powders. The leaf powder was placed in a centrifuge tube with 70% ethanol (1:30 m/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 3250g (Eppendorf 5810 R, Germany) for 10 min. The supernatant passing through 0.22 μm pore size filter was rutin extract.

Rutin concentration in *L. barbarum* leaves was determined using high-performance liquid chromatography (Shimadzu, Kyoto, Japan) equipped with an Apollo C18 column (5 μm particle size, 4.6 mm inside diameter × 250 mm length, Alltech, Deerfield, Illinois, USA). Twenty microlitres of 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: 0:00–10:00 25% B, 10:00–18:01 70% B, 18:01–25:00 100% B, 25:00–30:00 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 m/v) for 5 min, twice. The dried precipitate was weighed and dissolved in distilled water (1:20 m/v). Polysaccharide was extracted at 80 °C for 2 h in water bath and cooled for 5 min at room temperature. After passing through a 0.22 μm pore size filter, the polysaccharide extract was diluted 1:10 (v/v) using distilled water for determination.

Polysaccharide concentration was determined using the phenol – sulfuric acid method (Cuesta et al. 2003). Two millilitres of sample was added to a test tube with 1 mL of 6% phenol. Then 5 mL of concentrated sulfuric acid was added to the test tube. After incubating at room temperature for 30 min, the absorbance at 490 nm was determined on a 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 the sulfate-3-phenylphenol method (Z. Zhang et al. 2004). One test tube with 0.5 mL of extracted polysaccharide was added with 4.5 mL of sodium tetaborate-sulfuric acid, cooled on ice, and thoroughly shaken. The test tube was heated in a water bath at 100 °C for 10 min and subsequently cooled in a ice water bath. Fifty microlitres of 0.15% m-hydroxydiphenyl was added to the test tube for showing color. The absorbance at 525 nm was determined using a 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 millilitres of 6 mol/L hydrochloric acid was injected into a digestion vessel and sealed on the burner, incubated in the oven at 110 °C for 22 h, and then cooled at room temperature. The hydrolysate was filtered and diluted to a final volume of 50 mL. One millilitre of diluted filtrate was evaporated to dry on a water bath. The residue was dissolved in 1 mL of deionized water and evaporated till dry. This procedure was repeated 3 times. The residue was dissolved in 25 mL of 0.1 mol/L hydrochloric acid. The dissolved samples were filtered through a 0.45 μm pore size PTFE membrane before analysis.

Amino acid content was determined on an amino acid analyzer (L-8900, Hitachi, Japan) fitted with a 2.6 mm inside diameter × 105 mm length column packaged with sulfonate acid strong-anion exchange resin (product No. 2619, Hitachi Co., Japan). The optimal analytical time was 35 min, with flow rates of 0.225 mL/min for buffer and 0.3 mL/min for ninhydrin. The pump pressure was set at 15–35 kg/cm², column pressure at 80–130 kg/cm², and column temperature at 53 °C. The injection volume was 50 μL. In addition, 3 nmol per 50 μL 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, respectively. In the present study, all the original data sets 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. A repeated-measure analysis of variance (ANOVA) was applied to evaluate the effect of *R. irregulare* and salt stress on bud regeneration number and rutin, polysaccharide, acidic polysaccharide, and amino acids content. One-way ANOVA followed by Tukey’s honestly significant difference test 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 version 19.0 (IBM Corp., Armonk, New York, USA). Graphics were prepared on OriginPro version 8.5 (OriginLab, Northampton, Massachusetts, USA).

**Results**

**Mycorrhizal colonization, bud regeneration, and plant growth parameters**

Typical structures of AMF were observed in the roots of *L. barbarum* inoculated with *R. irregulare*. 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. irregulare* after the 1st harvest (Fig. 1, PES = 0.878, \( P < 0.0001 \)). However, inoculation with *R. irregulare* alleviated the decrease in bud regeneration of *L. barbarum* under salt stress (PES = 0.577, \( P < 0.0001 \)). Compared with the control, inoculation with *R. irregulare* significantly increased the number of regenerated buds for 4 respective weeks by 89.5%, 121.5%, 31.3%, and 79.2% under non-salt stress conditions. Under 200 mmol/L NaCl stress, the regenerated bud number of control plants was drastically decreased for the first 2 weeks after the 1st harvest (Fig. 1), but the *L. barbarum* inoculated with *R. irregulare* had 9- and 24-fold higher regenerated bud number than the 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 the control under salt stress condition. Moreover, at the 4th week, the regenerated bud number of +AM L. barbarum under salt stress was similar to that of the control under no salt stress. Consequently, R. irregulare notably increased the number of regenerated buds of L. barbarum with and without salt stress. Salt stress significantly decreased the leaf biomass of L. barbarum of the 2 harvests (PES = 0.608, P < 0.01). Inoculation with R. irregulare notably increased leaf biomass of the 2nd harvest (PES = 0.355, P < 0.01) by 1.1-fold under non-salt stress (Table 2). The stem and root biomasses of L. barbarum were not affected by salt stress and inoculation with R. irregulare.

**Rutin content**

Inoculation with R. irregulare 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 2 respective harvests. In the presence of salt stress, the rutin content of mycorrhizal L. barbarum was increased by 96.1% and 77.5% relative to the control, for the 2 respective harvests. Although the promotion effect of R. irregulare on rutin content fluctuated in different harvests and salt conditions, overall, the inoculation of R. irregulare efficiently augmented rutin level of L. barbarum leaves.

**Polysaccharide and acidic polysaccharide contents**

Inoculation with R. irregulare showed no significant 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. irregulare increased the polysaccharide content in the leaves of L. barbarum for the 2nd harvest by 64.2% compared with the control.

In the absence of salt stress, the acidic polysaccharide content in L. barbarum inoculated with R. irregulare was similar to that of the control, for both harvests (Table 3). However, under salt stress, the acidic polysaccharide of mycorrhizal L. barbarum leaves was notably higher than that of the control plants (Table 3, PES = 0.518, P = 0.001). Inoculation with R. irregulare increased the acidic polysaccharide content by 66.7% and 103.1% for the 2 respective harvests under salt stress.

**Amino acids content**

Under salt stress, R. irregulare inoculation decreased proline content by 57.7% and 65.6% compared with the control, for 2 leaf harvests (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 total amino acid content 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 leaf harvests under salt stress.

**Table 2.** Effect of Rhizoglomus irregulare on the biomass of leaves after 2 harvests, shoots, and roots of Lycium barbarum with and without salt stress.

<table>
<thead>
<tr>
<th>Salt concn.</th>
<th>AMF</th>
<th>1st harvested leaf</th>
<th>2nd harvested leaf</th>
<th>Shoot</th>
<th>Root</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mmol/L</td>
<td>–AM</td>
<td>0.56±0.12a</td>
<td>0.20±0.00b</td>
<td>2.10±0.31a</td>
<td>1.27±0.23a</td>
</tr>
<tr>
<td></td>
<td>+AM</td>
<td>0.46±0.05a</td>
<td>0.42±0.10a</td>
<td>3.63±0.74a</td>
<td>3.40±1.60a</td>
</tr>
<tr>
<td>200 mmol/L</td>
<td>–AM</td>
<td>0.14±0.09b</td>
<td>0.10±0.01b</td>
<td>1.67±0.97a</td>
<td>1.20±0.65a</td>
</tr>
<tr>
<td></td>
<td>+AM</td>
<td>0.14±0.02b</td>
<td>0.20±0.04b</td>
<td>1.50±0.60a</td>
<td>1.13±0.33a</td>
</tr>
</tbody>
</table>

**Note:** Within each column, means with different letters are significantly different at P < 0.05. Data are the mean ± SE (n = 3).

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

<table>
<thead>
<tr>
<th>Harvest</th>
<th>Salt concn.</th>
<th>AMF</th>
<th>Rutin</th>
<th>Polysaccharide</th>
<th>Acidic polysaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>0 mmol/L</td>
<td>–AM</td>
<td>1.07±0.12b</td>
<td>12.94±2.44a</td>
<td>1.72±0.11ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+AM</td>
<td>2.10±0.37ab</td>
<td>14.67±1.77a</td>
<td>1.58±0.12ab</td>
</tr>
<tr>
<td></td>
<td>200 mmol/L</td>
<td>–AM</td>
<td>1.28±0.26b</td>
<td>11.60±2.99a</td>
<td>1.23±0.18b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+AM</td>
<td>2.51±0.56a</td>
<td>12.31±1.84a</td>
<td>2.05±0.22a</td>
</tr>
<tr>
<td>2nd</td>
<td>0 mmol/L</td>
<td>–AM</td>
<td>0.76±0.06b</td>
<td>21.41±0.95a</td>
<td>0.98±0.11b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+AM</td>
<td>1.78±0.42a</td>
<td>24.42±7.89a</td>
<td>1.76±0.17a</td>
</tr>
<tr>
<td></td>
<td>200 mmol/L</td>
<td>–AM</td>
<td>0.71±0.11b</td>
<td>12.41±0.95a</td>
<td>0.98±0.11b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+AM</td>
<td>1.26±0.18ab</td>
<td>20.38±0.91a</td>
<td>1.99±0.10a</td>
</tr>
</tbody>
</table>

**Note:** Within each column, means with different letters for each harvest are significantly different at P < 0.05. Data are the mean ± SE (n = 5).
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 the AMF *R. irregulare* on the nutraceutical quality of *L. barbarum* leaves under salt stress. Specifically, the nutraceutical quality of the leaves of *L. barbarum* includes the content of rutin, polysaccharide, acidic polysaccharide, and amino acids. The present results supported our hypothesis that AM symbiosis improves the nutraceutical quality of *L. barbarum* leaves by elevating rutin and acidic polysaccharide content under salt stress. Moreover, *R. irregulare* inoculation had a 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 \textit{L. barbarum} can establish symbiosis with \textit{R. irregulare}, showing abundant hyphae 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 the root system for absorbing nutrients and water, to ameliorate the detrimental effect of salt stress. The decreased colonization rate in the roots of \textit{L. barbarum} under salt stress might be attributed to a direct deleterious impact on \textit{R. irregulare} and indirect effect through decreased plant growth (Evelin et al. 2009).

Since the buds can regenerate after \textit{L. barbarum} leaf harvest, the cost for producing \textit{L. barbarum} vegetables would decline. As a result, the regenerative ability of \textit{L. barbarum} buds is critical for sustainable production of \textit{L. barbarum} leaves. In this study, salinity inhibited recovery of \textit{L. barbarum} buds. But \textit{R. irregulare} inoculation promoted bud regeneration of \textit{L. barbarum} with and without salt stress, thus, increasing the additional yield for the successional harvest of \textit{L. barbarum} leaves. This might be explained by the fact that AMF can increase cytokinin content in plants, 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 (Koido 2010; Aroca et al. 2012). 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 \textit{R. irregulare} increased the leaf biomass of \textit{L. barbarum} compared with the control for the 2nd harvest under no salt stress, but showed no significant impact on the biomass of leaves of \textit{L. barbarum} of the 1st harvest with and without salt stress. The biomasses of stems and roots of \textit{L. barbarum} were not significantly affected by salt stress and inoculation with \textit{R. irregulare}. This is not surprising, as the insignificant effect of AMF on biomass has been reported on tomato and \textit{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 \textit{L. barbarum} are rich in flavonoids, and the most abundant flavonoid therein is rutin (Dong et al. 2009). In this study, \textit{R. irregulare} inoculation significantly enhanced rutin content in \textit{L. barbarum} leaves regardless of salt stress. This was in accordance with the observation on \textit{V. tricolor} L. inoculated with \textit{R. irregulare} (Zubek et al. 2015). The positive effect of AM symbiosis on the phenolic content has also been reported on artichoke, \textit{Moringa oleifera}, lettuce, and onion (Ceccarelli et al. 2010; Baslam et al. 2011; 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 (S. Chen et al. 2013; Amiri et al. 2015). 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 because of their antioxidative activity, free-radical scavenging capacity, coronary heart disease prevention ability, and anti-cancer activity (Yao et al. 2004). Consequently, the higher flavonoid level in \textit{L. barbarum} leaves induced by \textit{R. irregulare} represents enhanced salt tolerance as well as nutraceutical quality.

Polysaccharides derived from \textit{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 levels of Aloe, and inoculation with \textit{Glomus intraradices} and \textit{Glomus mosseae} further increased the polysaccharide levels in Aloe under salt stress (Cardarelli et al. 2013). However, \textit{R. irregulare} had no significant impact on polysaccharide levels in \textit{L. barbarum} leaves in this study. In the pattern of intensive agriculture, chemical fertilizer is increasingly used in \textit{L. barbarum} orchards but is reported to reduce polysaccharide content in \textit{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 \textit{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 (H. Chen et al. 2004). Besides, acidic polysaccharide of \textit{L. barbarum} has been shown to have higher efficiency to scavenge free radicals and inhibit tumor cells compared with neutral polysaccharide (H. Zhang et al. 2013; Z. Zhang et al. 2015; W. Liu et al. 2016). Thus, the higher content of acidic polysaccharide in \textit{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, \textit{R. irregulare} is effective in enhancing the nutraceutical quality of \textit{L. barbarum} leaves through increasing acidic polysaccharide content under salt stress. Although the biomass of \textit{L. barbarum} leaves remained low under salt stress relative to non-salt stress condition, inoculation with \textit{R. irregulare} had a higher regenerated bud number compared with the control. Besides, the regenerated bud number of +AM \textit{L. barbarum} was similar to...
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 nonmycorrhizal plants. AMF enhance sink strength in plants by requiring carbon fixed by plants, which is considered a “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 nonmycorrhizal 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. irregularre significantly improved nutraceutical quality of L. barbarum leaves in the absence and presence of salt stress, including increased rutin and acidic polysaccharide levels. Inoculation with R. irregularre 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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