

Influences of *Funneliformis mosseae* on the photosynthetic parameters and active secondary metabolites contents of *Astragalus membranaceus* and *Astragalus membranaceus* var. *mongholicus*

Jiquan Liu^{a,*}, Jin Cai^b, Runli He^a, Xichun Zhang^a

^a School of Chinese Drugs, Shanxi University of Chinese Medicine, Yuci 030619, Shanxi, China

^b Institute of Applied Chemistry, Shanxi University, Taiyuan 030006, Shanxi, China

*Corresponding author, e-mail: liujiquan2008@163.com

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ABSTRACT: *Astragalus membranaceus* and *Astragalus membranaceus* var. *mongholicus* are cultivated in large parts of Shanxi province in China for their high medicinal value in traditional Chinese medicine, and the quality of the medicinal materials is a problem worthy of attention. Traditional strategies to increase quality include use of chemical fertilizer and pesticides, but these may have negative impact on the quality of soil and medicinal materials. In this study, the potential of promoting the quality of the medicinal materials was investigated after inoculating *A. membranaceus* and *A. membranaceus* var. *mongholicus* with mycorrhizal fungus (*Funneliformis mosseae*). The photosynthetic parameters (net photosynthetic rate, stomatal conductance, transpiration rate and intercellular CO₂ concentration) and main active principle contents (astragaloside IV, calycosin-7-glucoside, astragalus polysaccharide and trace element Se) were determined in inoculated and non-inoculated plants. Results showed that there was a positive impact on photosynthetic process and the accumulation of secondary metabolites of the plants inoculated with *F. mosseae*. Compared with control plants, the photosynthetic parameters of *Astragalus* plants inoculated with *F. mosseae* were higher, more active substances (astragaloside IV, calycosin-7-glucoside, astragalus polysaccharide and Se) were found in the roots of inoculated plants. *F. mosseae* had similar promoting effects on both *A. membranaceus* and *A. membranaceus* var. *mongholicus*. The symbiosis of *F. mosseae* and *Astragalus* plant would be of benefit to improve the quality of the medicinal materials.

KEYWORDS: astragaloside IV, calycosin-7-glucoside, astragalus polysaccharide, Se

INTRODUCTION

The dried roots of *Astragalus membranaceus* (Fisch.) Bge. and *Astragalus membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao are commonly used in traditional Chinese medicine for various conditions, and it is reported that astragaloside IV, calycosin-7-glucoside, astragalus polysaccharide and trace element Se are the main pharmacodynamic material basis¹. The Chinese Pharmacopoeia stipulates that the contents of astragaloside IV and calycosin-7-glucoside shall not be less than 0.04% and 0.02% in both dried roots, respectively. Modern pharmacological research shows that *A. membranaceus* and *A. membranaceus* var. *mongholicus* have many functions such as regulating human immunity, anti-ageing, anti-fatigue and anti-tumour effects^{2,3}. The dried roots or preparation of these plants are used so frequently in traditional Chinese medicine, that

it is said that 8 out of 10 Chinese medicine prescriptions written contain them. There are as many as 200 kinds of Chinese patent drug containing *A. membranaceus* or *A. membranaceus* var. *mongholicus*⁴. The plants or herbals are also claimed to have high nutritional value. In 2018, the China Food and Drug Administration listed *A. membranaceus* and *A. membranaceus* var. *mongholicus* as the product of dual use of drug and food⁵. The Chinese market has great demand for *A. membranaceus* and *A. membranaceus* var. *mongholicus*. In international market, the medicinal materials of *A. membranaceus* and *A. membranaceus* var. *mongholicus* once topped the list of seven varieties of Chinese herbal medicines, which topped the list of more than \$10 million in Chinese herbal medicine exports⁶.

It is this strong demand that has nearly depleted the wild resources of *A. membranaceus* and *A. mem-*

branceus var. *mongholicus* in China. Hence the standardized cultivation of *A. membranaceus* and *A. membranaceus* var. *mongholicus* is an inevitable choice. At present, the plant product is mainly cultivated by traditional way. According to the theory and literature of Chinese medicine, Shanxi province is the birthplace of *A. membranaceus* and *A. membranaceus* var. *mongholicus*. In 2009, a GAP cultivation base for *A. membranaceus* var. *mongholicus* was established in Hunyuan county of Shanxi Province, China. By the end of 2017, the planting area of *A. membranaceus* and *A. membranaceus* var. *mongholicus* reached 35 000 ha. However, with the traditional or older cultivation method, the quality of the herbals is unstable due to the non-standard planting site selection and the blind use of chemical fertilizers and pesticides. The use of fertilizers and pesticides also leads to environmental pollution and serious damage to ecological balance⁷. Hence the introduction of new methods and techniques to improve the quality of these herbal products is a subject that needs to be studied in depth.

Arbuscular mycorrhizal fungi (AMF) are a kind of beneficial microorganisms widely distributed in soil. Studies have shown that AMF is a key microorganism in the terrestrial ecosystem and can form a reciprocal symbiosis with more than 90% of terrestrial plants⁸. Since AMF's mycelium is found in the cortex of the host plant's root system, it can expand the absorption area of the plant's root system, improve the utilization of nutrients and water, improve the photosynthesis of plants, and promote the growth and development of plants⁹. Inoculation of AMF can not only improve the absorption of nutrients in soil by plants, but also improve the resistance of plants to adversity, diseases and pests, and reduce the use of pesticides and the application of chemical fertilizers, which is beneficial to protect the ecological environment while improving the quality of cultivated medicinal materials¹⁰. It has been found that AMF can strongly stimulate the growth of leguminous plants and the formation of root nodules, and inoculation of AMF can effectively improve the growth potential and quality of cultivated crops, especially the yield and quality of leguminous plants¹¹. Inoculation of AMF can lead to abnormal accumulation of secondary metabolites in plants¹², which are most likely the effective material basis for medicinal plants to prevent and treat diseases. It has been reported that *F. mosseae* is a dominant fungus in the rhizosphere of *Astragalus* plants¹³.

In this article, by inoculating *F. mosseae* in the rhizosphere of *A. membranaceus* and *A. mem-*

branceus var. *mongholicus*, the influences of *F. mosseae* on the photosynthetic parameters (net photosynthetic rate, stomatal conductance, transpiration rate and intercellular CO₂ concentration) and main active principle contents (astragaloside IV, calycosin-7-glucoside, astragalus polysaccharide and trace element Se) were studied. We try to find a new cultivation method for improving the quality of the medicinal materials.

MATERIALS AND METHODS

Inoculum of *F. mosseae*

The inoculum of *F. mosseae* (labelled as BGC-HUB01A) was procured from the Institute of Plant Nutrition and Resources, Beijing Academy of Agriculture and Forestry Sciences, China. Every 20 ml of inoculation contains 405 spores.

Reagents

Reference products of astragaloside IV (110781-201616, 98.9%), calycosin-7-glucoside (111920-201606, 98.1%) and D-anhydrous glucose (110833-201707, 99.9%) were purchased from National Institutes for Food and Drug Control (Beijing, China). Se standard solutions containing (100 mg/ml) was supplied by Chinese National Standard Material Center. Acetonitrile, methanol, formic acid, H₂SO₄, phenol, ethanol, concentrated HNO₃ and perchloric acid (Trace metal grade) were purchased from Tianjin Kermel Chemical Testing Co., Ltd., China.

Seedling planting

The one year old seedlings of *A. membranaceus* (SXTCM-0001509) and *A. membranaceus* var. *mongholicus* (SXTCM-0001365) were purchased from Yingxian Qianbao Chinese Herbal Medicine Professional Cooperative, Shanxi province, China. All the seedlings in the trial have the same growth potential and the root length was about 10 cm of *A. membranaceus* and 15 cm of *A. membranaceus* var. *mongholicus*, respectively. The experimental site was located in the medicinal plant plantation of Shanxi University of Chinese Medicine.

Before planting, rectangular trenches (45 cm long, 15 cm wide and 15 cm deep) were dug in an east-west direction (The distance between every two trenches is 30 cm). Then a clean nylon cloth (There were round holes with a diameter of 6 mm on the nylon cloth every 3 cm) was spread in each rectangular trench. Every rectangular trench was filled with 10 cm thick aseptic soil (Soil used for the experiments was a mixture of 30 vol % sand,

30 vol % pine needle humus and 40 vol % field loamy soil, heated at 105 °C for 2 h and allowed to cool down before use). One *Astragalus* seedling was planted in each trench, and the seedling was laid flat along the east-west direction. The inoculum of *F. mosseae* was spread evenly around the root system at a volume of 20 ml, 40 ml or 60 ml per seedling. Finally, the seedlings were covered with sterile soil for 5 cm and routine managements were provided¹⁴. Seedlings that were not inoculated with inoculum of *F. mosseae* served as controls. Each treatment contains 10 seedlings. All the seedlings were randomly arranged under the same illumination condition. Six months later, the whole roots of two years old were harvested for determination of active substance content.

Determination of photosynthetic parameters

In July on a sunny day, the net photosynthetic rate, stomatal conductivity, transpiration rate and intercellular CO₂ concentration were determined by the LI-6400 portable photosynthesis determination system (LI-COR, USA) from 9:00–11:00. The light intensity was set to 1200 μmol m⁻²s⁻¹ and light source was set to red blue light. At the top of each plant, three fully expanded leaves with consistent light were selected for determination¹⁵. Plants that were not inoculated with inoculum of *F. mosseae* served as controls.

Determination of astragaloside IV

The roots of all tested plants harvested were dried at 50 °C and mechanically pulverized to a particle size of approximate 1 mm. Crushed sample (about 4 g) was accurately weighed and placed in a round bottom flask. 80 ml of 80% methanol was added. Sample was extracted by reflux for 4 h. After recovering the solvent, the extract was dissolved with 80% methanol and fixed in a 10 ml volumetric flask¹. Reference product of astragaloside IV was dissolved with an appropriate amount of 80% methanol. All the solutions were filtered with 0.22 μm microporous filter membrane for determination.

According to 2015 edition of Chinese Pharmacopoeia¹, an Agilent 1100 HPLC (Agilent Technologies, Germany), ELSD 2000 Evaporation Detector (Grace Co., Ltd., China) and Dikma C18 column (4.6 mm × 250 mm, 5 μm) were used for determination of astragaloside IV. The mobile phase was 32 vol % of acetonitrile + 68 vol % of water. The flow rate was 0.8 ml/min, the injection volume was 20 ml, and the column temperature was 30 °C.

Determination of calycosin-7-glucoside

The preparation method was the same as that of astragaloside IV test solution. Chromatographic method for calycosin-7-glucoside.

According to 2015 edition of Chinese Pharmacopoeia¹, DAD detector (Agilent Technologies, Germany) was used and the detector was set at the wavelength of 260 nm. The mobile phase was gradient elution (0–20 min, 20–40 vol % of acetonitrile + 80–60 vol % of 0.2% formic acid solution; 20–30 min, 40 vol % of acetonitrile + 60 vol % of 0.2% formic acid solution). The injection volume was 10 ml. Other conditions were the same as those for astragaloside IV determination.

Determination of astragalus polysaccharide

Crushed sample (about 2 g) was accurately weighed and placed in a round bottom flask. 200 ml distilled water was added. Sample was extracted by reflux for 2 h. The extract was fixed with distilled water in a 100 ml volumetric flask. 2 ml of the extraction solution was measured accurately and 10 ml of ethanol was added. The mixed solution was centrifuged for 10 min at 3000 rpm. The precipitate was dissolved in distilled water and fixed to 50 ml for determination⁶. Reference product of D-anhydrous glucose was dissolved with an appropriate amount of distilled water.

The UV-1601 spectrophotometer (Shimadzu Co., Ltd., Japan) and sulphuric acid-phenol method were used to determine the content of astragalus polysaccharide. The measurement wavelength was 490 nm.

Determination of Se

The roots were rinsed with deionized water to remove dirt and dried in an oven at 50 °C until constant mass was obtained. The dried samples were finely powdered for determination. All solutions were prepared using deionized water purified by a Milli-Q system (Millipore, Bedford, MA, USA). About 0.2 g sample was accurately weighed and placed in PTFE digestion vessel. 5 ml mixed acid (HNO₃:HClO₄ = 4:1) were added. The sample and solution were mixed fully and then allowed to stand for 12 h. The vessel was placed in a microwave oven (CEM, Matthews, NC, USA). The temperature was stepped up to 100 °C in 10 min and maintained for 1 h. Then the temperature was further stepped up to 130 °C in 5 min and maintained for 3 h. After cooling, the solution was transferred into a 10 ml polypropylene volumetric tube for determination⁶.

An ICP-AEMS (Leeman Labs, USA) instrument was used for Se analysis (operational parameters: output power 1.1 kW; frequency 40.68 MHz; cooling gas flow 19 l/min; auxiliary air flow 0.6 l/min; atomizer pressure 0.35 MPa; peristaltic pump lift rate 1.2 l/min; wavelength 196.026 nm; spectral line level 1; detection limit 0.3 µg/l)⁶.

Statistical analyses

Data were subjected to ANOVA using SPSS 18.0. Treatment means were tested with Duncan's Multiple Range. Test at the 5% level of probability.

RESULTS

Effects of *F. mosseae* on photosynthetic parameters

The results of Fig. 1 showed that inoculation of *F. mosseae* could improve the photosynthetic indexes of the cultivated plants. For *A. membranaceus*, compared with the control, the three inoculation amounts could significantly increase the values of net photosynthetic rate, stomatal conductance and intercellular CO₂ concentration, and the inoculation amounts of 40 ml/plant and 60 ml/plant could significantly promote transpiration rate. For *A. membranaceus* var. *mongholicus*, compared with the control, the three inoculation amounts had significant promotion effect on net photosynthetic rate and transpiration rate, and when the inoculation amount was 40 ml/plant and 60 ml/plant, it had significant promotion effect on stomatal conductance and intercellular CO₂ concentration.

Effects of *F. mosseae* on astragaloside IV

Fig. 2 showed that the content of astragaloside IV in roots of *A. membranaceus* and *A. membranaceus* var. *mongholicus* gradually increased with the increase of *F. mosseae* inoculation amount, and the higher the inoculation amount, the higher the content of astragaloside IV. At the same level, the content of astragaloside IV in the root of *A. membranaceus* var. *mongholicus* was higher than that in the root of *A. membranaceus*. For *A. membranaceus* var. *mongholicus*, compared with the control, the content of astragaloside IV in root was significantly increased when the inoculation amount was 40 ml/plant and 60 ml/plant. For *A. membranaceus*, the content of astragaloside IV in root was significantly increased when the inoculation amount was 60 ml/plant.

Effects of *F. mosseae* on calycosin-7-glycoside

It can be seen in Fig. 3 that the calycosin-7-glycoside content in the root of *A. membranaceus* var. *mongholicus* was higher than that in the root of *A. membranaceus* at the same level. Compared with the control, inoculation of *F. mosseae* can significantly increase the calycosin-7-glycoside content in the root of *A. membranaceus*. When the inoculation amount was 40 ml/plant or 60 ml/plant, the calycosin-7-glycoside content in the root of *A. membranaceus* var. *mongholicus* was significantly increased.

Effects of *F. mosseae* on astragalus polysaccharide

Compared with the control, all treatments can significantly increase the content of astragalus polysaccharide in the roots of *A. membranaceus* and *A. membranaceus* var. *mongholicus*. With the increase of inoculation amount, the content of polysaccharide in the roots also significantly increased. There were obvious differences between different inoculation amounts of *F. mosseae*. Inoculating *F. mosseae* had the greatest promotion effect on the content of polysaccharide. At the same level, the polysaccharide content in the root of *A. membranaceus* was higher than that in the root of *A. membranaceus* var. *mongholicus* (Fig. 4).

Effects of *F. mosseae* on Se

With the increase of inoculation amount, the content of trace element Se in the roots of *A. membranaceus* and *A. membranaceus* var. *mongholicus* was gradually increasing. For *A. membranaceus*, compared with the control, the content of Se in the root had no significant difference when the inoculation amount was 20 ml/plant, but when the inoculation amount was 40 ml/plant and 60 ml/plant, the enrichment of Se in the root was significantly promoted. For *A. membranaceus* var. *mongholicus*, the Se content of all treatments was significantly different from that of the control (Fig. 5).

DISCUSSION

The photosynthetic parameters (net photosynthetic rate, stomatal conductance, transpiration rate and intercellular CO₂ concentration) were important indicators to reflect the exuberance of plant photosynthetic metabolism. The higher the photosynthetic index value, the higher the photosynthetic efficiency of the plant, the more metabolic products will be synthesized¹⁶. It was reported that inoculation of

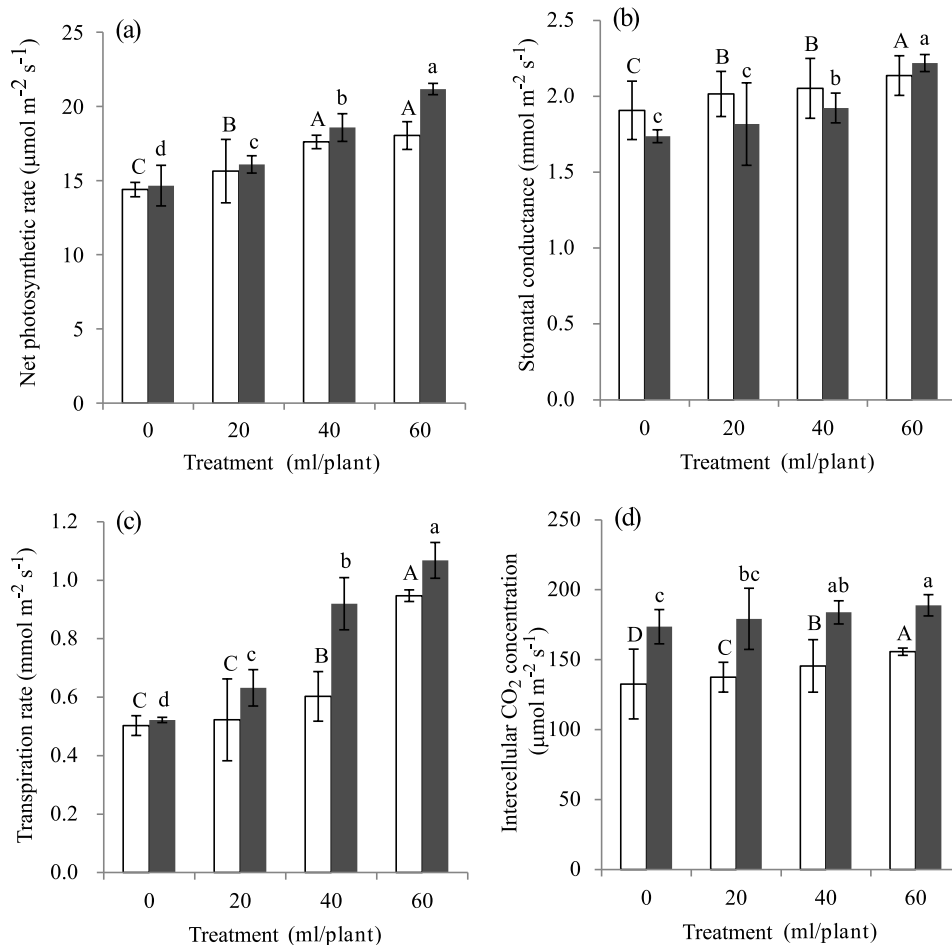


Fig. 1 Effects of *F. mosseae* on photosynthetic parameters of *A. membranaceus* and *A. membranaceus* var. *mongholicus*: (a) net photosynthetic rate; (b) stomatal conductance; (c) transpiration rate; (d) intercellular CO_2 concentration. Different letters indicate a significant difference between the treatment and control (Duncan's Multiple test) of each plant as distinguished by uppercase or lowercase letter.

Glomus mosseae can improve stomatal conductance, transpiration rate and intercellular CO_2 concentration of *Lolium perenne* L.¹⁷. *G. mosseae* inoculation can improve the photosynthetic efficiency and flavonoids accumulation of liquorice¹⁸. In this experiment, the inoculation of *F. mosseae* was beneficial to the photosynthetic parameters of *A. membranaceus* and *A. membranaceus* var. *mongholicus*. Inoculation amount is clearly positively correlated with promotion effect.

When photosynthetic efficiency increases, plants will synthesize more primary metabolites, such as sugars, lipids, nucleic acids, and proteins. Based on the accumulation of primary metabolites, some important secondary metabolites (such as saponins, flavonoids, and alkaloids) have been further synthesized by using primary metabolites

as raw materials or precursors through complex metabolic pathways (such as acetate-malonate pathway, mevalonic acid pathway, cinnamic acid pathway and amino acid pathway)¹⁹. *G. mosseae* inoculation can increase the accumulation of the major active components (hinesol, β -eudesmol and atractylodin) in the rhizome of *Atractylodes lancea* (Thunb.) DC.²⁰ and improved oil content in *Mentha arvensis* L.²¹. The results of this experiment showed that the inoculation of *A. membranaceus* and *A. membranaceus* var. *mongholicus* with *F. mosseae* promoted the accumulation of astragaloside IV, calycosin-7-glucoside, astragalus polysaccharide and Se in roots on the basis of increasing photosynthetic indexes.

AMF are the most widely distributed plant symbionts, and can infect the root systems of over

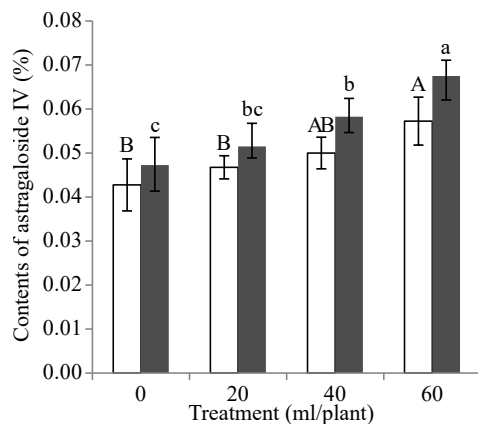


Fig. 2 Effects of *F. mosseae* on astragaloside IV in *A. membranaceus* and *A. membranaceus* var. *mongholicus*. Different letters indicate a significant difference between the treatment and control (Duncan's Multiple test) of each plant as distinguished by uppercase or lowercase letter.

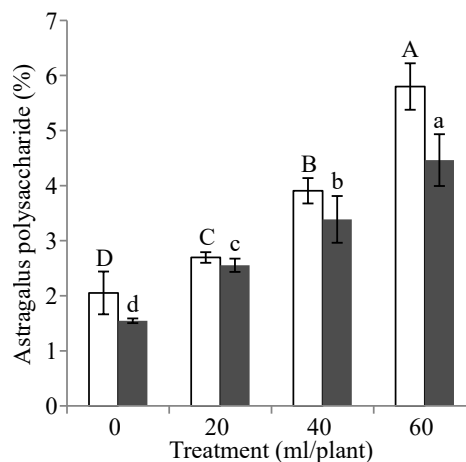


Fig. 4 Effects of *F. mosseae* on astragalus polysaccharide in *A. membranaceus* and *A. membranaceus* var. *mongholicus*. Different letters indicate a significant difference between the treatment and control (Duncan's Multiple test) of each plant as distinguished by uppercase or lowercase letter.

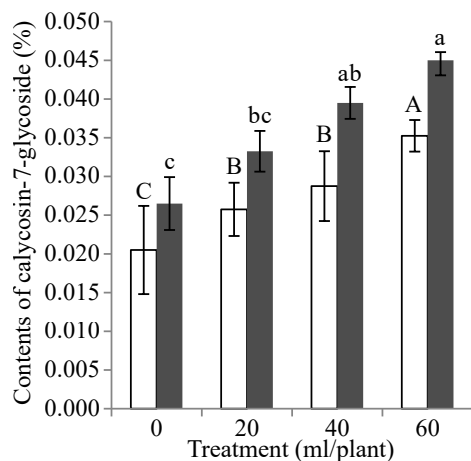


Fig. 3 Effects of *F. mosseae* on calycosin-7-glycoside in *A. membranaceus* and *A. membranaceus* var. *mongholicus*. Different letters indicate a significant difference between the treatment and control (Duncan's Multiple test) of each plant as distinguished by uppercase or lowercase letter.

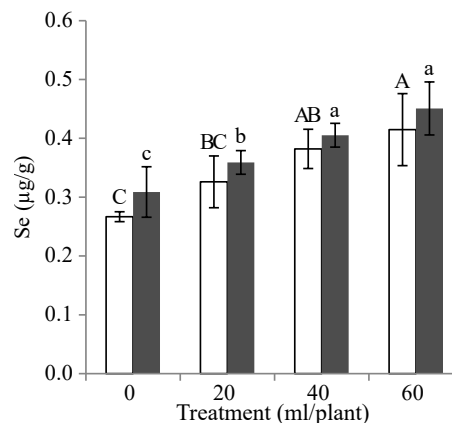


Fig. 5 Effects of *F. mosseae* on Se in *A. membranaceus* and *A. membranaceus* var. *mongholicus*. Different letters indicate a significant difference between the treatment and control (Duncan's Multiple test) of each plant as distinguished by uppercase or lowercase letter.

80% of vascular plants²². During the symbiotic period of AMF and plants, AMF obtained nutrients by infecting host plant root systems, but also hyphae of AMF can serve as an important channel for soil nutrients entering plants. This greatly improves the absorption of soil nutrients by plant roots^{23,24}. Water, minerals and other nutrients needed for plant growth and development can be effectively supplied, especially in the case of poor growth conditions. This channel is particularly important²⁵. AMF inoculation can improve the

ability of *Astragalus sinicus* L. and *Zea mays* L. to absorb soil nutrients^{26,27}. Inoculation of *G. mosseae* can promote the absorption of Cu, Zn and other mineral elements by white clover²⁸. The formation of plant arbuscular mycorrhizae can promote the absorption of nutrients with low mobility such as Cu, Zn and Fe in soil, and these three elements are often insufficient nutrients in the soil^{29,30}. Under drought stress, the relative water content in *Vigna radiata* L. leaves was 15.3% higher than that of the control after inoculation with AMF³¹.

Water and mineral elements are important factors affecting plant photosynthesis. When the water content of plant leaves is close to water saturation, the leaves can perform normal photosynthesis. When water deficit occurs in plant leaves, stomatal opening decreases or closes, which further affects the diffusion of CO₂ into leaf cells. When the water shortage of plant leaves reaches about 20%, photosynthesis is clearly inhibited¹⁶. The effects of mineral elements on plant photosynthesis are variable. Chlorine and manganese affect the photolysis of water. Iron, copper and phosphorus affect photosynthetic electron transport and photophosphorylation. Potassium, phosphorus and boron promote the transport and transformation of photosynthetic products. Within a certain range, the increase in the absorption of mineral elements will accelerate the photosynthetic rate of plants¹⁶.

In short, the symbiosis between AMF and host plants expands the channels for host plants to absorb nutrients needed for metabolism and promotes the effective absorption of soil nutrients by host plants. The effective absorption of soil nutrients by the host plant further improves its photosynthetic efficiency, enhances its adaptability to the environment, promotes its growth, and finally improves its yield and quality. This is a virtuous circle.

It was reported that in the pot experiment, when each plant of *A. membranaceus* was inoculated with 30 g of *G. mosseae* agent, the root infection rate reached 86.7%. Furthermore, it had a significant promoting effect on plant height, root length, aboveground dry weight and underground dry weight¹⁴. When a plant of *A. membranaceus* var. *mongholicus* was inoculated by 10 g of *G. mosseae*, the root infection rate reached 95%, plant height, root dry weight, stem dry weight, leaf dry weight and number of lateral roots were all higher than the control, the contents of chlorophyll, carotenoid, soluble sugar, N and P in the leaves increased, but the inoculation had no significant effect on the content of soluble protein in the leaves³².

In this experiment, with inoculating *F. mosseae*, the absorption area of the root system of *Astragalus* plant was increased, and the transportation of water and mineral elements by the root system of *Astragalus* plant was accelerated. The satisfaction of water and mineral elements in the metabolism of *Astragalus* plant will promote the improvement of photosynthesis efficiency and the accumulation of active substances to a certain extent. Within a certain range, the larger the amount of inoculation of *F. mosseae*, the larger the root absorption area

and the more mineral elements and water absorbed, the faster the physiological metabolism of *Astragalus* plant, and the more active substances accumulated. The physiological characteristics of *A. membranaceus* and *A. membranaceus* var. *mongholicus* are different and their light efficiency and light resistance are different during photosynthesis³³. The infection rates and influence mechanisms of *F. mosseae* on *A. membranaceus* and *A. membranaceus* var. *mongholicus* may be different. The specific mechanism of the effect of *F. mosseae* on *Astragalus* plant is still unclear and needs to be studied in depth.

In conclusion, *F. mosseae* could effectively promote the photosynthetic efficiency and the accumulation of active substances of *A. membranaceus* and *A. membranaceus* var. *mongholicus*. Intensified inoculation of *F. mosseae* will be an effective measure to improve the quality of *A. membranaceus* and *A. membranaceus* var. *mongholicus*.

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