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Arbuscular Mycorrhizal Fungi Regulate the Growth and Phyto-Active Compound of *Salvia miltiorrhiza* Seedlings

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Abstract: Roots and rhizomes of *Salvia miltiorrhiza* (*S. miltiorrhiza*) are widely used for the treatment of cardiovascular diseases. Arbuscular mycorrhizal fungi (AMFs) have been shown to enhance plant growth and increase secondary metabolites concentration in many plant species. However, effects of AMFs on *S. miltiorrhiza* have not been explored. A pot culture was designed as one control (non-AMF) treatment and four AMFs (*G.m*, *Glomus mosseae*; *G.a*, *Glomus aggregatum*; *G.v*, *Glomus versiforme*; *G.i*, *Glomus intraradices*) treatments were performed in order to evaluate the effects of AMFs on plant growth, as well as phyto-active compounds' concentration of *S. miltiorrhiza* seedlings. Plants were harvested after 90 days: agronomic traits and concentration; and an accumulation of mineral elements, as well as phyto-active compounds were detected. All AMFs inoculated plants formed mycorrhizal structures, and an infection ratio; also, the intensity of inoculated roots was higher than 84.61% and 23.86%, respectively. Mycorrhizal dependency was above 144.62%. Seedlings with AMFs inoculation had significantly higher plant height, leather leaf length, top leaflet size, base leaflet length, taproot length, taproot diameter and biomass than those with non-AMF inoculation. In addition, inoculation with AMFs increased N, P, and K accumulation significantly, but barely had any effect on mineral elements' concentrations. AMFs inoculation also significantly improved tanshinones concentrations and stimulation in order to accumulate salvianolic acid B. *G.v* and *G.i* were effective for seedlings growth; *G.m* and *G.i* were also effective for phyto-active compounds. In total, *S. miltiorrhiza* inoculation with AMFs had positive effects on growth and active components, especially inoculation with *G.v*.

Keywords: *Salvia miltiorrhiza*; AMFs; mineral elements; tanshinone; salvianolic acid B

1. Introduction

Dried roots of *Salvia miltiorrhiza* Bunge (*S. miltiorrhiza*), or Danshen in Chinese, are among the most popular herbs; they have been widely used for medicinal purposes to improve blood circulation and remove blood stasis. The main active ingredients of *S. miltiorrhiza* are tanshinones and salvilic acid; both are products of the secondary metabolism. With the development of the modern Chinese medicine industry, demands for *S. miltorrhiza* raw materials increase each year due to the important role in maintaining human health; thus resulting in a shortage of wild resources. Improving the quality and yield of cultivated, raw materials is increasingly important, since *S. miltorrhiza* is cultivated by most pharmaceutical companies.

Arbuscular mycorrhizae fungi (AMFs) can form symbiotic relationships with the vast majority of land plants, enhance biomass and growth rates, improve stress resistance, stimulate mineral nutrients uptake and raise photosynthesis of the infected plants [1–3]. For instance, P uptake and growth parameters of cowpea plants were positively influenced by AMFs inoculation under a medium or low P fertilization treatment [4]. Genes that encode the transporters of the macro/micro elements (N, P, K, Cu, Fe, Zn) also have been identified from mycorrhizas [5,6]. Stimulation of AMFs in an agro-ecosystem suppresses some aggressive weeds [7]. Inoculation with AMFs has an effective and persistent impact on improving crop productivity and quality in low input agricultural systems [8]. This is because mutual benefits are particularly reinforced under extreme environmental conditions [9]. These symbioses also increase production of secondary metabolites in medicinal plants [3]. *Stevia rebaudiana* inoculated with AMF can produce higher concentrations of steviol glycosides compared to non-AMF inoculated plants; it can stimulate an uptake of nutrients, as well as increase chlorophyll and carbohydrate concentrations that directly result in biomass improvement [10]. Cucumber inoculated with AMF increases secondary metabolites' concentration and up-regulates transcription that would alleviate chilling stress [11].

AMFs improve the host plants' absorption of water and nutrients from the soil; in return, 20% of the carbon that the plants fixed on their own is transferred to the fungi [12,13]. If carbon acquired from the plant is reduced, N may have a negative impact on arbuscular mycorrhizae (AM) abundance [14]. In addition, N transported in the symbiosis is stimulated only when the C is delivered by the host across the mycorrhizal interface; not when C is supplied directly to the fungal extraradical mycelium in the form of acetate [15]. Therefore, *S. miltiorrhiza* inoculation with AMFs may improve the nutrients uptake from the soil, thereby reducing fertilizer input in the cultivation.

AMFs can strengthen the drought resistance of *S. miltiorrhiza* by improving water and mineral uptake, as well as the physiological metabolic activity of mycorrhizal plants [16]. Secondary metabolites of *S. miltiorrhiza,* such as tanshinones compounds and phenolic acid, are the main medicinal components. Improvement of phyto-active compound content is an effective way to increase the production per plant. *S. miltiorrhiza* usually is continuously cropped for at least 2 years, which will consume many soil nutrients and cause an imbalance in soil micro-organisms. AMFs can increase resistance, as well as the uptake of mineral nutrients and secondary metabolites of host plants. Nevertheless, effects of AMF inoculation on *S. miltorrhiza* growth, nutrient uptake, physiological metabolism and phyto-active compounds have not been thoroughly investigated. Therefore, a pot culture with four different AMFs inoculation was conducted in a greenhouse, aimed to study the effects of AMFs inoculation on seedling growth, nutrient uptake and secondary metabolites of *S. miltiorrhiza*.

2. Materials and Methods

2.1. Experimental Design

Soil in this study was collected from the topsoil of the Botanical Garden of the Beijing Academy of Agriculture and Forestry Sciences. The chemical and physical characteristics were as follows: pH, 7.82; organic matter, 1.40%; total N, P and K were 0.076%, 0.083%, 2.33%, respectively; and available N, P, K, Ca, Mg, Cu, Zn, Fe and Mn were 60.90 mg/kg, 30.92 mg/kg, 95.60 mg/kg, 3800 mg/kg, 237 mg/kg,

2.54 mg/kg, 2.38 mg/kg, 6.76 mg/kg and 19.9 mg/kg, respectively. The soil was mixed with sand (2:1) and autoclaved (121 °C, 1.2 kg·cm^{-2}) for 1 h to kill naturally occurring AMF propagules. Six hundred and fifty grams of the sterilized mixture were dispensed into 25 plastic pots (Φ, 7.50 cm; high, 16 cm).

Experiments were conducted in the phytotron of the Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences (Beijing, China). All experiments were carried out in an environmentally controlled growth room with a 24 h cycle of 14 h at 30 °C in light and 10 h at 20 °C in darkness, with a photon flux density of 350 photon μmol·m^{-2}·s^{-1} (photosynthetic active radiation) at the plant-canopy level, as well as 70% relative air humidity. Seeds of *S. miltiorrhiza* were provided by the *S. miltiorrhiza* GAP (Good Agricultural Practice) planting base of Tianjin TianShiLi pharmaceutical Co., Ltd. in Shangluo, Shan Xi Province, China. First, seeds were sterilized in 10% H_2O_2 for 1 h; then they were rinsed several times with deionized water; finally, they were sowed 0.5 cm below the soil surface. One seedling was reserved in each pot at the 2-leaf stage. The original inocula of AM fungi *Glomus mosseae* (*G. mosseae*, BGC-XZ01), *Glomus aggregatum* (*G. aggregatum*, BGC-BJ107), *Glomus versiforme* (*G. versiforme*, BGC-NM04B), and *Glomus intraradices* (*G. intraradices*, BGC-USA04) were kindly supplied by the AMF Culture Center in the Institution of Plant Nutrition and Resource Sciences, Beijing Academy of Agriculture and Forestry Sciences. Inoculum was collected from pot cultures of sorghum plants that were a mixture of spores, mycelium, sand, vermiculite and root fragments that contained approximately 1000 spores per 100 g.

There were five treatments in the present study. The mycorrhizal treatments consisted of fresh inocula (25 g) of one of the following AMFs: 1. CK (inactivated inocula, non-AMF); 2. *G. mosseae*; 3. *G. aggregatum*; 4. *G. versiforme*; 5. *G. intraradices*. AMFs inocula were sown homogeneously below the soil surface (1–2 cm) of each pot. The non-AMF pot was added with the same amount of inactivated inoculum. Each treatment was replicated by five pots, and plant samples of each pot were gathered as one composite sample for analysis.

2.2. Plant Growth and Determination

Plants were harvested after 90 days, and the following was recorded: plant height, leaf number, length and width of leaves, leather leaf length, top leaflet size and base leaflet size, leaf dry weight, root fresh and dry weight, taproot length and the diameter, lateral root number.

2.3. Determination of Infection Rate and Intensity

Fifty fresh fibrous roots were selected randomly and washed cleanly; then, they were cut into 1 cm root pieces and fixed by Formalin-Aceto-Alcohol (FAA). Root samples were washed with 10% KOH solution and stained with 0.05% trypan blue in lactophenol [17]. An Olympus BH-2 (Japan) with an anymicro DSS YT-5M digital shoot system was used at 1000 times for inoculation observation. Infection rate, infection intensity and mycorrhizal dependency were calculated as described by Janos [18] and Trouvelot [19]. The AMFs infection rate was counted by the following formula: AMF infection ratio (%) = 100 × root length infected/root length observed. Infection intensity (%) was as follows: $=(95n_5 + 70n_4 + 30n_3 + 5n_2 + n_1)$ /total root segments, where n_5 means the number of roots with the infection level of 5 (infection ratio 90%–100%); n_4 is the root number at level 4 (infection ratio 50%–90%); n_3 is the root number at level 3 (infection ratio 10%–50%); n_2 is the root number at level 2 (infection ratio 1%–10%); n_1 is the root number at level 1 (infection ratio 0%–1%). AMF dependency based on dry matter was calculated according to the following formula: AMF dependency (%) = mean total dry matter inoculated with AMF/mean dry matter of untreated plants × 100%.

2.4. Determination of Elements

Oven-dried samples (0.2 g each) were ground for elements' analysis. A reagent blank was prepared by following the whole extraction procedure without a sample. For determination of nitrogen, 10 mL of sulfuric acid was added to samples at room temperature for 30 min; next, 1.5 g $Na_2S_2O_3$, 0.4 g of zinc powder and 10 mL of water was added and incubated for 10 min; then it was

digested until the liquid became greenish gray. The digestion liquid was diluted with deionized water to 100 mL. N concentration was determined by the Kjeldahl Method (K9840 Hanon, Jinan, China). For determination of K and P, samples were digested with a tri-acid mixture (HNO_3 (68%)-H_2SO_4 (98%)-$HClO_4$ (70%), $v/v/v$ = 8:1:1); then a digestion liquid was diluted to 50 mL with deionized water after cooling. K concentration was determined by flame spectrophotometry (FP6400 Yuefeng, Shanghai, China). P concentration was determined by the vanadium molybdate yellow colorimetric method with a UV-vis spectrophotometer (UV-2600 SHIMADZU, Kyoto, Japan) at 700 nm. Accumulation was calculated for N, P, K by N, P, K concentration × mean biomass of each plant. For determination of Ca, Mg, Fe, Mn, Cu and Zn, 0.5 g samples were carbonized in a quartz crucible with a hot plate; then they were transferred into a muffle furnace at 500 °C for 3 h. Ashes were dissolved with 5 mL 1:1 nitric acid solution; then they were subsequently diluted to 50 mL with deionized water. Then, the filtrate was directly measured by an atomic absorption spectrophotometer (AA-130 SHIMADZU, Kyoto, Japan).

2.5. Determination of Phyto-Active Compounds Concentration

2.5.1. Tanshinones

Leaf and root sample powder (~0.15 g) was incubated with 25 mL methanol in an ultrasonic at 30 °C for 1 h; then it was replenished with methanol. Next, the extraction was filtered with a 0.45 μm membrane, and the filtrate was detected by HPLC.

High Performance Liquid Chromatography (HPLC, Waters Alliance e2696, Milford, MA, USA) consisting of a model 2695 HPLC pump, a model 2998 photodiode array detector (PAD) and a Empower-3 work-station (Milford, MA, USA), as well as a Zorbax XDB-C18 column (4.6 mm × 250 mm, i.d. 5 μm) was used for tanshinones concentration determination. A binary gradient elution system consisting of water (A), acetonitrile (B) and separation was achieved using the following gradient program: 0–10 min 90%–80% B; 10–12 min 80% B; 12–13 min 80%–75% B; 13–15 min 75%–40% B; 15–20 min 40%–0% B; 20–25 min 0%–90% B. Flow-rate was 1.0 mL/min; sample injection volume was 20 μL; column temperature was 30 °C; monitor wavelength was 270 nm. Phyto-active compounds' concentrations were calculated from standard curves using peak areas, which were attained from HPLC. Standard samples of dihydrogen tanshinone I (E06-110116), implicit tanshinone (Y53-110616), tanshinone (1428-070321) and tanshinone II_A (1067-080314) were supplied by Jiangxi Herbfine High-technological Co., Ltd. in Nanchang, Jiangxi Province, China.

2.5.2. Salvianolic Acid B

Samples (~0.1 g) were extracted with 10 mL of 75% methanol in boiling water for 1 h; then, their loss was replenished with 75% methanol after being cooled. The filtrate through the 0.45 μm membrane was prepared for salvianolic acid B analysis.

Salvianolic acid B concentration was determined as described in the Chinese Pharmacopoeia (2010 edn) [20] with a Waters Alliance e2696 HPLC system as shown above. A Zorbax XDB-C18 column (4.6 mm × 250 mm, i.d. 5 μm) was used at 30 °C with an isocratic elution of methanol-acetonitrile-formic acid-water (30:10:1:59, $v/v/v/v$). The flow-rate was 0.8 mL/min and the sample injection volume was 20 μL; and it was monitored at 286 nm. Peak areas were used to calculate salvianolic acid B concentration by standard curves, then salvianolic acid B accumulation was calculated by concentration determined by HPLC × mean dry weight per plant. Standard sample of salvianolic acid B was supplied by Jiangxi Herbfine High-technological Co., Ltd. in Nanchang, Jiangxi Province, China.

2.6. Statistical Analysis

Effects of the treatments were determined by one-way analysis of variance (ANOVA), and the differences between treatments were determined using Tukey's pairwise comparison test at a significance level of 95%; this excludes where it was otherwise indicated in the text. Different letters

indicated significant differences between treatments. All statistical data were performed with software SPSS 12.0.

3. Results

3.1. AMFs Colonization of S. miltiorrhiza Seedlings Roots

Plants were successfully colonized by *G.a*, *G.m*, *G.v* and *G.i*. *S. miltorrhiza* roots had intra-radical hyphae and spores, while the roots of CK treatment remained uncolonized (Figure 1). Infection rates among *G.i*, *G.v* and *G.a* inoculated were not statistically different, but those treatments were significantly higher than with *G.m*. Infection intensities among the four inoculated treatments were significantly different; they were in the order of *G.i* > *G.v* > *G.a* > *G.m* (Table 1). Results above suggested that *S. miltiorrhiza* was a mycotrophic plant, which could form a well-developed symbiotic system with various AMFs.

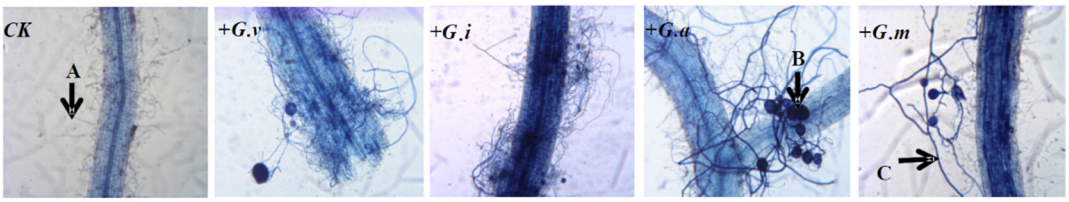

Figure 1. Effect of arbuscular mycorrhizae fungi (AMFs) inoculation on the colonization of *S. miltiorrhiza* root. Roots were stained by trypan blue, then photographed under a 1000 times microscope. (**A**): root hair; (**B**): spore; (**C**): hypha. CK means with non-AMF inoculation; *G.v.* means inoculation with arbuscular mycorrhizae (AM) fungus *Glomus versiforme*; *G.i.* means inoculation with AM fungus *Glomus intraradices*; *G.a.* means inoculation with AM fungus *Glomus aggregatum*; *G.m.* means inoculation with AM fungus *Glomus mosseae*.

Table 1. Infection rate and intensity with different AMFs inoculation.

Treatment	Infection Rate (%)	Infection Intensity (%)
CK	0.00 ^{c}	0.00 ^{e}
G.m	84.61 ± 10.42 ^{b}	23.86 ± 12.08 ^{d}
G.a	93.37 ± 5.03 ^{a}	59.93 ± 6.05 ^{c}
G.i	100.00 ± 0.00 ^{a}	83.58 ± 3.19 ^{a}
G.v	99.04 ± 1.93 ^{a}	70.94 ± 7.96 ^{b}

CK means with non-AMF inoculation; *G.v.* means inoculation with AM fungus *Glomus versiforme*; *G.i.* means inoculation with AM fungus *Glomus intraradices*; *G.a.* means inoculation with AM fungus *Glomus aggregatum*; *G.m.* means inoculation with AM fungus *Glomus mosseae.* Different letters in the same column indicate significant differences at 0.05 level. Data are means ± SD (n = 5).

3.2. Growth and Biomass of S. miltiorrhiza Seedlings

S. miltiorrhiza seedlings that were colonized with AMFs had greater biomass than those with non-AMF plants at the two growth stages (30 days and 90 days after seeding), as shown in Figure 2. Seedlings with non-AMF were short and had small yellow leaves, while seedlings with inoculation of AMFs were tall and had large green leaves. Table 2 also indicated that plant height, leather-leaf length, top leaflet size and base leaflet length of inoculation with AMFs treatments were significantly higher than those with non-AMF inoculation. However, no significant difference of leaf number was observed between inoculated treatments and non-AMF inoculated treatment. Among the four AMFs treatments, there were no significant differences for leaf number, leather leaf length and top leaflet size. Results above indicate that AMFs inoculation could notably accelerate the growth of *S. miltiorrhiza* seedlings.

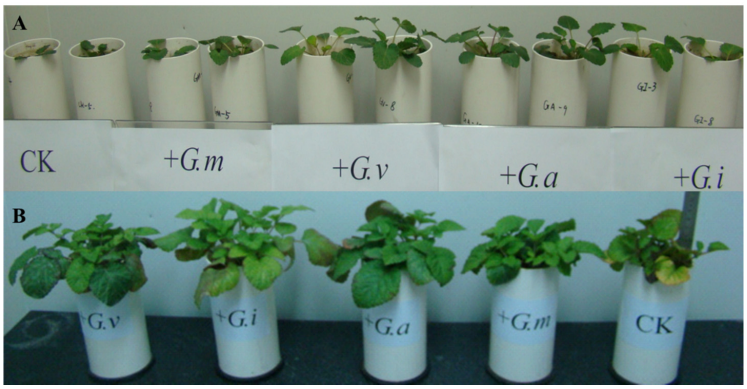

Figure 2. Effect of AMFs inoculation on growth of *S. miltiorrhiza* seedlings. (**A**): 30 days after sowing; (**B**): 90 days after sowing. CK means with non-AMF colonized; CK means with non-AMF inoculation; *G.v.* means inoculation with AM fungus *Glomus versiforme*; *G.i.* means inoculation with AM fungus *Glomus intraradices*; *G.a.* means inoculation with AM fungus *Glomus aggregatum*; *G.m.* means inoculation with AM fungus *Glomus mosseae.*

Table 2. Effect of AMFs inoculation on shoot growth traits of *S. miltiorrhiza* seedlings.

Treatment	Plant Height (cm)	Leaf Number	Leather Leaf Length (cm)	Top Leaflet Size (cm)		Base Leaflet Size (cm)	
				Length	Width	Length	Width
CK	10.58 ± 0.66^{c}	21.00 ± 6.00^{a}	9.41 ± 0.22^{b}	4.01 ± 0.56^{b}	3.72 ± 0.32^{b}	2.22 ± 0.28^{c}	1.78 ± 0.27^{c}
G.m	12.50 ± 1.20^{ab}	22.50 ± 4.12^{a}	11.91 ± 0.43^{a}	4.89 ± 0.42^{a}	4.66 ± 0.21^{a}	2.78 ± 0.10^{b}	2.30 ± 0.08^{ab}
G.v	13.60 ± 0.43^{a}	23.50 ± 4.12^{a}	11.27 ± 0.73^{a}	5.24 ± 0.40^{a}	4.39 ± 0.24^{a}	2.98 ± 0.26^{ab}	2.20 ± 0.20^{bc}
G.a	12.38 ± 0.81^{ab}	22.00 ± 9.38^{a}	11.85 ± 0.72^{a}	5.32 ± 0.54^{a}	4.62 ± 0.44^{a}	3.42 ± 0.24^{a}	2.67 ± 0.33^{a}
G.i	12.28 ± 0.82^{b}	20.00 ± 1.63^{a}	11.67 ± 0.98^{a}	4.97 ± 0.57 a	4.62 ± 0.53^{a}	3.28 ± 0.42^{a}	2.40 ± 0.30^{ab}

CK means with non-AMF inoculation; *G.v.* means inoculation with AM fungus *Glomus versiforme*; *G.i.* means inoculation with AM fungus *Glomus intraradices*; *G.a.* means inoculation with AM fungus *Glomus aggregatum*; *G.m.* means inoculation with AM fungus *Glomus mosseae.* Different letters in the same column indicate significant differences at 0.05 level. Data are means ± SD (n = 5).

AMFs colonization significantly promoted the growth and development of *S. miltiorrhiza* roots (Figure 3; Table 3). Seedlings with non-AMF inoculation had a short root with a few fibrous roots and protruding taproots, while seedlings with AMFs inoculation had significant longer roots and more lateral roots. Taproot length and the lateral root number of seedlings with AMFs inoculation were 1.21–1.32 and 1.42–1.71 times longer and greater than those with non-AMF inoculation, respectively. There was no significant difference of taproot length and lateral root number among the four AMF treatments. Nevertheless, seedlings with *G.v* and *G.i* inoculation had the longest taproot length. In addition, seedlings inoculation with *G.v* also had the biggest taproot diameter. In conclusion, AMFs colonization could promote the roots' growth, especially for lateral roots' formation. *G.v* fungi had a better effect on the roots' growth than the others used in the experiment.

Figure 3. Effect of AMFs inoculation on roots growth of *S. miltiorrhiza* seedlings after 90 days sowing. CK means with non-AMF inoculation; *G.v.* means inoculation with AM fungus *Glomus versiforme*; *G.i.* means inoculation with AM fungus *Glomus intraradices*; *G.a.* means inoculation with AM fungus *Glomus aggregatum*; *G.m.* means inoculation with AM fungus *Glomus mosseae.*

Table 3. Effect of AMFs inoculation on root traits of *S. miltiorrhiza* seedlings.

Treatment	Taproot Length (cm)	Taproot Diameter (cm)	Lateral Root Number
CK	10.05 ± 5.53^{c}	0.92 ± 0.10^{ab}	7 ± 4^{b}
G.m	12.13 ± 1.32^{ab}	0.92 ± 0.09^{ab}	17 ± 10^{a}
G.v	13.15 ± 2.45^{a}	0.95 ± 0.06^{a}	17 ± 7^{a}
G.a	12.64 ± 1.34^{ab}	0.69 ± 0.21^{c}	17 ± 4^{a}
G.i	13.33 ± 0.97^{a}	0.89 ± 0.18^{bc}	19 ± 4^{a}

CK means with non-AMF inoculation; *G.v.* means inoculation with AM fungus *Glomus versiforme*; *G.i.* means inoculation with AM fungus *Glomus intraradices*; *G.a.* means inoculation with AM fungus *Glomus aggregatum*; *G.m.* means inoculation with AM fungus *Glomus mosseae.* Different letters in the same column indicate significant differences at 0.05 level. Data are means ± SD (n = 5).

AMFs colonization had a positive influence on the seedlings' biomass of *S. miltiorrhiza* (Table 4). Compared to CK, leaf and root biomass with AMFs inoculation increased by 34%–65% and 58%–128%, respectively. The root of *S. miltorrhiza* is a raw material for Chinese patent medicine. Therefore, a big root/shoot ratio and root drying ratio is a promotion for production. Results indicated that AMFs inoculation could increase the root/shoot ratio by 2%–30% when compared to CK. *G.v* inoculation treatment which had a significantly bigger root/shoot ratio. AMFs treatments significantly increased the root drying rate by 1.18%–3.00% when compared to non-AMF treatment. In addition, inoculation with *G.v* had the highest AMF dependency among the four AM fungi. In total, *G.v* fungus was better for the root/shoot ratio and root drying rate than the others used in the experiment.

Table 4. Effect of AMFs inoculation on biomass of *S. miltiorrhiza* seedlings.

Treatment	Leaf Weight (g/Plant, *DW*)	Root		Total Weight (g/Plant, *DW*)	Root/Shoot Ratio	AMF Dependency (%)
		Weight (g/Plant, *DW*)	Drying Rate (%)			
CK	1.83 ± 0.57^{c}	1.33 ± 0.17^{c}	18.62^{c}	3.16 ± 0.59^{d}	0.77 ± 0.20^{bc}	-
G.m	2.47 ± 0.13^{b}	2.11 ± 0.44^{b}	19.80^{b}	4.57 ± 0.33^{c}	0.86 ± 0.22^{b}	144.62
G.v	2.85 ± 0.10^{ab}	3.04 ± 0.29^{a}	21.62^{a}	5.89 ± 0.39^{a}	1.07 ± 0.07^{a}	186.39
G.a	2.79 ± 0.14^{ab}	2.22 ± 0.22^{b}	20.48^{ab}	5.01 ± 0.32^{bc}	0.79 ± 0.07^{c}	158.54
G.i	3.03 ± 0.28^{a}	2.62 ± 0.72^{ab}	21.13^{a}	5.65 ± 0.87^{ab}	0.86 ± 0.21^{b}	178.80

CK means with non-AMF inoculation; *G.v.* means inoculation with AM fungus *Glomus versiforme*; *G.i.* means inoculation with AM fungus *Glomus intraradices*; *G.a.* means inoculation with AM fungus *Glomus aggregatum*; *G.m.* means inoculation with AM fungus *Glomus mosseae.* DW means dry weight. Different letters in the same column indicate significant differences at 0.05 level. Data are means ± SD (n = 5).

3.3. Mineral Elements Concentration and Accumulation of S. miltiorrhiza Seedlings

Compared to CK, inoculation with AMFs significantly decreased N concentration in leaves and roots of *S. miltiorrhiza* seedlings. There was no significant difference of N concentration in roots among the four AMFs inoculation treatments; however, *G.a* treatment had a significantly higher N concentration in leaves than the *G.i* treatment. Furthermore, in both leaves and roots of seedlings with non-AMF, the N accumulation was significantly lower than those with AMFs inoculation. Moreover, in both leaves and roots, N accumulation among the four AMFs treatments was statistically different. The *G.a* treatment had the highest N accumulation in leaves, which was significantly higher than *G.m* and *G.v* treatments. N accumulation in roots with *G.a* inoculation was significantly higher than other treatments (Table 5). Inoculation with AMFs increased the P concentration in leaves significantly, but inoculation with *G.v* and *G.i* decreased the P concentration in roots. Even so, inoculation with the four AMFs remarkably enhanced the P accumulation, both in leaves and roots. The P accumulation was in order of *G.v* > *G.i* > *G.m* > *G.a* in leaves; and *G.v* > *G.a* > *G.m* > *G.i* in roots (Table 5). In spite of K concentrations in leaves and roots with AMFs, inoculation was not significantly higher than CK, even in roots' K concentration of inoculation with *G.v.* Furthermore, *G.a* was lower than CK, but inoculation with AMFs could notably enhance K accumulation in leaves and roots. Effects of the four AMFs for

increasing K accumulation was in the order of *G.i* > *G.v* > *G.a* > *G.m* in leaves, and *G.v* > *G.i* > *G.m* > *G.a* in roots (Table 5).

Table 5. Effect of AMFs on N, P and K concentration and accumulation of *S. miltiorrhiza* seedlings.

Treatment	N Concentration (%, *DW*)		N Accumulation (mg/Plant, *DW*)		P Concentration (%, *DW*)		P Accumulation (mg/Plant, *DW*)		K Concentration (%, *DW*)		K Accumulation (mg/Plant, *DW*)	
	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
CK	1.14 ^{a}	0.87 ^{a}	20.86 ^{d}	11.57 ^{d}	0.25 ^{c}	0.20 ^{a}	4.58 ^{d}	2.66 ^{e}	1.57 ^{a}	1.12 ^{a}	28.73 ^{d}	14.90 ^{d}
G.m	0.96 ^{bc}	0.73 ^{b}	23.71 ^{c}	15.40 ^{c}	0.34 ^{a}	0.21 ^{a}	8.40 ^{bc}	4.43 ^{bc}	1.69 ^{a}	1.05 ^{ab}	41.74 ^{bc}	22.16 ^{bc}
G.v	0.92 ^{bc}	0.67 ^{b}	26.22 ^{b}	20.37 ^{a}	0.32 ^{ab}	0.16 ^{b}	9.12 ^{a}	4.86 ^{a}	1.59 ^{a}	0.98 ^{b}	45.32 ^{b}	29.79 ^{a}
G.a	1.02 ^{b}	0.71 ^{b}	28.46 ^{a}	15.76 ^{c}	0.30 ^{b}	0.21 ^{a}	8.37 ^{bc}	4.66 ^{ab}	1.54 ^{a}	1.01 ^{ab}	42.97 ^{bc}	22.42 ^{bc}
G.i	0.89 ^{c}	0.68 ^{b}	26.97 ^{ab}	17.82 ^{b}	0.30 ^{b}	0.16 ^{b}	9.09 ^{ab}	4.19 ^{cd}	1.62 ^{a}	0.94 ^{b}	49.09 ^{a}	24.63 ^{b}

CK means with non-AMF inoculation; *G.v.* means inoculation with AM fungus *Glomus versiforme*; *G.i.* means inoculation with AM fungus *Glomus intraradices*; *G.a.* means inoculation with AM fungus *Glomus aggregatum*; *G.m.* means inoculation with AM fungus *Glomus mosseae.* DW means dry weight. Different letters in the same column indicate significant differences at 0.05 level. Data are means ± SD (n = 5).

Inoculation with AMFs did not influence Ca and Mg concentration (except Mg in leaves). However, it significantly affected Cu, Zn, Fe and Mn concentration (Table 6). Both in leaves and roots, Cu and Zn concentration (except *G.m* inoculation treatment leaves) with AMFs inoculation were significantly higher than non-AMF inoculation. In addition, both in leaves and roots, inoculation with *G.i* had the highest Cu and Zn concentration among the four AMFs treatments. In seedling leaves, only *G.v* treatment notably increased Fe concentration; while in seedling roots, *G.m* treatment only demonstrated a lack of a significant increase in Fe concentration. Moreover, both in leaves and roots, inoculation with AMFs (except leaves with *G.i* treatment) demonstrated a significant decrease in Zn concentration (Table 6).

Table 6. Effect of AMFs on Ca, Mg, Cu, Zn and Mn concentration of *S. miltiorrhiza* seedlings.

Treatment	Ca (g/kg, *DW*)		Mg (g/kg, *DW*)		Cu (mg/kg, *DW*)		Zn (mg/kg, *DW*)		Fe (mg/kg, *DW*)		Mn (mg/kg, *DW*)	
	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
CK	12.47 ^{ab}	2.34 ^{a}	8.76 ^{c}	4.43 ^{a}	9.38 ^{d}	7.33 ^{d}	28.65 ^{cd}	12.03 ^{e}	820.03 ^{b}	302.49 ^{c}	127.32 ^{a}	39.56 ^{a}
G.m	13.50 ^{a}	2.52 ^{a}	9.22 ^{bc}	4.12 ^{a}	15.32 ^{c}	12.90 ^{c}	30.46 ^{c}	16.71 ^{cd}	864.16 ^{b}	329.84 ^{bc}	111.42 ^{bc}	28.53 ^{d}
G.v	12.83 ^{ab}	2.54 ^{a}	10.08 ^{a}	4.04 ^{a}	25.44 ^{ab}	24.08 ^{b}	35.18 ^{b}	17.71 ^{c}	1077.59 ^{a}	365.99 ^{ab}	115.18 ^{b}	27.53 ^{de}
G.a	11.77 ^{b}	2.52 ^{a}	8.21 ^{cd}	4.20 ^{a}	24.26 ^{ab}	28.46 ^{a}	36.63 ^{b}	29.25 ^{ab}	886.15 ^{a}	446.79 ^{a}	111.05 ^{bc}	37.27 ^{bc}
G.i	12.36 ^{ab}	2.67 ^{a}	9.94 ^{ab}	3.93 ^{a}	26.36 ^{a}	28.01 ^{a}	42.07 ^{a}	30.13 ^{a}	856.83 ^{a}	372.69 ^{ab}	128.02 ^{a}	37.12 ^{bc}

CK means with non-AMF inoculation; *G.v.* means inoculation with AM fungus *Glomus versiforme*; *G.i.* means inoculation with AM fungus *Glomus intraradices*; *G.a.* means inoculation with AM fungus *Glomus aggregatum*; *G.m.* means inoculation with AM fungus *Glomus mosseae.* DW means dry weight. Different letters in the same column indicate significant differences at 0.05 level. Data are means ± SD (n = 5).

3.4. Phyto-Active Compounds' Concentration of S. miltiorrhiza Seedlings

There was a detection of different phyto-active compounds' concentrations in roots of *S. miltiorrhiza* seedlings with different treatments (Table 7). Results indicated that seedlings' inoculation with *G.m* significantly increased the concentration of dihydrogen tanshinone I, implicit tanshinone, tanshinone I and tanshinone II_A, which were 1.97, 1.69, 1.91 and 2.11 times that of CK treatment, respectively. Then, the total concentration of four compounds was 1.18–1.94 times that of CK. *G.i* treatment significantly enhanced implicit tanshinone, tanshinone and tanshinone IIA concentration; and the total concentration was 1.77 times that of CK, but had no significant impact on the dihydrogen tanshinone concentration. Concentrations of implicit tanshinone and tanshinone were remarkably improved by inoculation with *G.a*, while total concentration was increased by 0.2622%. In addition, inoculation with *G.v* could not promote phyto-active compounds concentration when compared to CK. In conclusion, enhancement of phyto-active compounds with four AMFs inoculation was in the order of *G.m* > *G.i* > *G.a* > *G.v*.

Table 7. Effect of AMFs on tanshinones concentration of *S. miltiorrhiza* seedling roots.

Treatment	Dihydrogen Tanshinone I (%, *DW*)	Implicit Tanshinone (%, *DW*)	Tanshinone (%, *DW*)	Tanshinone II_A (%, *DW*)	Total (%, *DW*)
CK	0.0524 ± 0.0220^{b}	0.1484 ± 0.0286^{c}	0.0525 ± 0.0134^{c}	0.2162 ± 0.0677^{c}	0.4695
G.m	0.1033 ± 0.0325^{a}	0.2502 ± 0.0393^{ab}	0.1002 ± 0.0194^{ab}	0.4560 ± 0.0526^{a}	0.9097
G.v	0.0781 ± 0.0065^{ab}	0.1339 ± 0.0356^{c}	0.0382 ± 0.0043^{c}	0.3052 ± 0.0986^{bc}	0.5554
G.a	0.0613 ± 0.0024^{b}	0.2914 ± 0.1079^{a}	0.0812 ± 0.0043^{b}	0.2978 ± 0.0300^{bc}	0.7317
G.i	0.0817 ± 0.0220^{ab}	0.2618 ± 0.0716^{a}	0.1133 ± 0.0256^{a}	0.3743 ± 0.0319^{ab}	0.8311

CK means with non-AMF inoculation; *G.v.* means inoculation with AM fungus *Glomus versiforme*; *G.i.* means inoculation with AM fungus *Glomus intraradices*; *G.a.* means inoculation with AM fungus *Glomus aggregatum*; *G.m.* means inoculation with AM fungus *Glomus mosseae.* DW means dry weight. Different letters in the same column indicate significant differences at 0.05 level. Data are means ± SD (n = 5).

Table 8 showed that salvianolic acid B accumulation of the *S. miltiorrhiza* seedling was greatly increased by 115%, 81%, 50% and 181% in leaves; 180%, 142%, 125% and 179% in roots, respectively, with *G.m*, *G.v*, *G.a* and *G.i* inoculation; meanwhile, the distribution rate in roots was amplified, except with *G.i* inoculation. The improvement effect for roots was stronger than for leaves with AMFs inoculation, and followed with the order of *G.i* > *G.m* > *G.v* > *G.a* for leaves and *G.m* > *G.i* > *G.v* > *G.a* for roots.

Table 8. Effects of AMFs on salvianolic acid B accumulation of *S. miltiorrhiza* seedling roots.

Treatment	Accumulation (mg/Plant, *DW*)		Distribution (%)	
	Leaf	Root	Leaf	Root
CK	23.64 ± 1.27^{e}	69.04 ± 7.73^{e}	25.51	74.49
G.m	50.92 ± 6.06^{b}	193.54 ± 28.65^{a}	20.83	79.17
G.v	42.77 ± 4.34^{c}	167.05 ± 23.31^{c}	20.38	79.62
G.a	35.50 ± 2.89^{d}	155.32 ± 8.05^{d}	18.60	81.40
G.i	66.42 ± 11.44^{a}	192.59 ± 9.95^{ab}	25.64	74.36

CK means with non-AMF inoculation; *G.v.* means inoculation with AM fungus *Glomus versiforme*; *G.i.* means inoculation with AM fungus *Glomus intraradices*; *G.a.* means inoculation with AM fungus *Glomus aggregatum*; *G.m.* means inoculation with AM fungus *Glomus mosseae.* DW means dry weight. Different letters in the same column indicate significant differences at 0.05 level. Data are means ± SD (n = 5).

4. Discussion

Roots and rhizomes of *S. miltiorrhiza* are the main raw materials of the Compound Danshen Dripping Pill, an oral herbal medicine that has been widely used in China, Korea and Russia for the treatment of cardio-cerebrovascular diseases, such as occlusive vasculitis, cerebral infarction, atherosclerosis and coronary artery diseases. AMF colonization can promote plant absorption of mineral elements [21], regulate the synthesis and distribution of plant hormones [22,23], ameliorate the microbial environment of the plant rhizosphere, and enhance plant resistance to disease [24,25]. Then, it can strengthen plant tolerance to environmental stress [26,27] and improve overall growth conditions of host plants [28]. Plant secondary metabolites are commonly used in Chinese medicine, and play a particular role in the symbiotic relationship formed between plant and mycorrhizal fungi [29].

4.1. Growth of S. miltiorrhiza with AMFs Inoculation

A previous study indicates that *S. miltiorrhiza* is a mycotrophic plant, which could be easily colonized by several AMF species (*Glomus, Acaulospora, Scutellospora* and *Entrophospora*) [30]. This study also showed that roots of *S. miltiorrhiza* seedlings were successfully colonized by *G.a*, *G.m*, *G.v* and *G.i*. (Figure 1, Tables 1 and 3). Thus, it was reasonable for the present experimental design.

The growth of plants is generally regulated by light, water and nutrients. Plants inoculated with AMFs can improve photosynthesis by increasing chlorophyll content [10], as well as by stimulating water and minerals uptake by the upregulating of transporter genes [5,6,16] and nutrients

utilization [31], as well as by enhancing productivity by strengthening disease resistance [8,31]. In the present study, large and green leaves of inoculated seedlings could have a highly effective photosynthesis rate, which is beneficial for the accumulation of carbohydrates. In addition, AMFs inoculation enhances the root number by phytohormone regulating [32] and then influences root architecture by endogenous polyamines metabolism [33]. Moreover, AMFs inoculation equips extensive extra-radical mycelium, which would increase surface areas of roots in order to acquire more nutrients and water [34]. *S. miltiorrhiza* inoculated with AMFs had longer roots and more lateral roots (Figure 3, Table 3). Therefore, those seedlings possess higher biomass than those with non-inoculation of AMFs (Table 4). These results were consistent with the studies of Liu et al. on *Glycyrrhiza uralensis* Fisch [35] and Berta et al. on *Prunus cerasifera* [36]. However, Berta et al. suggested that inoculation with AMFs decreased the root/shoot fresh weight ratio [36]; the results of this study increased the root/shoot ratio; it was increased with AMFs inoculation (Table 4). The main reason for this is that the water content of leaves is higher than that of roots, so the drying rate of leaves is lower than roots. The root/shoot ratio in our study was calculated by dry weight; it was calculated by fresh weight in the study of Berta et al. As roots of *S. miltiorrhiza* are a main commodity, inoculation with AMFs can increase economic benefits by increasing the root drying rate and root/shoot ratio. Results also suggested that it could be a better way to improve the growth and biomass of *S. miltiorrhiza* by inoculation of *G.v* and *G.i.*

A prior study indicates that improving accumulation of N, P and K resulted in the increase in the biomass of Anthurium [37]. Since the biomass of *S. miltiorrhiza* seedlings was improved by inoculation with AMFs, N, P and K content, both in leaves and roots, was significantly higher than those with non-AMF inoculation (Table 5). In addition, inoculation with AMFs increased P concentration in leaves, and the result was consistent with Berta et al. [36]. However, it was necessary to note that N and K concentrations were barely affected by AMFs inoculation (Table 5). Dilution effects of plant growth resulted in this phenomenon. AMFs' colonization facilitated increasing biomass by enhancing the assimilation of N and K, but rapid growth resulted in the relative deficiency of N and K. Therefore, inoculation with AMFs and balance fertilization were suggested to be effective ways for *S. miltiorrhiza* cultivation.

The increase of Fe, Cu, Zn and Mg concentrations results in a net increase of photosynthetic activity that has been reported in many plants [38]. In the present study, AMFs inoculation resulted in the higher concentration of Cu and Zn; both in leaves and roots (Table 6). This was consistent with the reports of Guo and Gong [39], as well as Baslam et al. [40]. Several studies showed that AMFs would change composition and activity of the rhizosphere microbes by decreasing Mn reducers, and then negatively influence Mn uptake [41,42]. Thus, Mn concentration was decreased by AMFs inoculation (Table 6).

4.2. Phyto-Active Compounds of S. miltiorrhiza with AMFs Inoculation

Secondary metabolites with different functions are produced during the growth and development of plants. Medicinal plants inoculated with AMFs would promote the accumulation of several secondary metabolites [31]. For example, Liu et al. found that licorice plants that were inoculated with AM enhanced glycyrrhizic acid content by 0.38–1.07-fold after 4 months; also, after 30 months, it enhanced by 1.34–1.43-fold [35].

There are two categories of phyto-active compounds in *S. miltiorrhiza*: fat-soluble tanshinones (e.g., dihydrogen tanshinone I, implicit tanshinone, tanshinone and tanshinone II_A) and phenolic acid water-soluble compounds (salvianolic acid B). It is clear that tanshinones are synthesized in roots of *S. miltiorrhiza* plants. Seedlings inoculated with AMFs could increase content of tanshinones significantly, especially with *G.m* and *G.i* (Table 7). Salvianolic acid B accumulation in leaves and roots were remarkably increased with AMFs inoculation, because the biomass with AMFs inoculation significantly increased (Tables 4 and 8). The results were in accordance with previous studies [30,31,35]. In addition, there was an interesting finding in this study. *S. miltiorrhiza* seedlings' inoculation with *G.m*

had the lowest biomass (Table 4) but it had the highest concentration of tanshinones and accumulation of salvianolic acid B among the four AMFs (Tables 7 and 8). There are two reasons to explain the relationship between biomass and secondary metabolites. First, secondary metabolites are positively correlated with mineral elements [43,44]. However, there were few relations between tanshinones concentration and mineral concentration in the present study (Tables 4–6). This is because mineral nutrition was supplied for photosynthesis, and then photosynthate was transferred to maintain the AM symbiosis [45]. Consequently, carbon was not enough the increase the biomass. Second, it is a result of the plant reaction to AMFs inoculation. The AM symbiosis formation is mediated by gene expression and the production of chemical molecular signals both in plants and fungi [46]. Chemical defense is induced by gene expressions that manipulate their metabolism [1,45]. Therefore, tanshinones were produced as defense chemicals to resist AMFs inoculation. That was the reason for the *G.m* infection rate; also, intensity was lower than the other three fungi (Table 2), while the concentration of tanshinones' concentration was higher than the others (Table 7), which is consistent with Zubek et al. [47].

5. Conclusions

S. miltiorrhiza was easily inoculated with AMFs. Also, the inoculation with AMFs increased plant growth, biomass, mineral elements accumulation and tanshinones concentration, as well as salvianolic acid B accumulation. Inoculation with *G.i* and *G.v* was beneficial for plant growth, while inoculation with *G.v* and *G.m* was beneficial for the secondary metabolism. Thus, *G.v* was the best fungi for the growth and secondary metabolism of *S. miltiorrhiza*.

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