

Effects of five mycorrhizal fungi on biomass and leaf physiological activities of walnut

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Abstract

Arbuscular mycorrhizal fungi (AMF) can benefit many plants, but their effects on walnuts are not yet known. The present study aimed to analyze the effect of five AMF species, namely, *Acaulospora scrobiculata*, *Diversispora spurca*, *Glomus etunicatum*, *G. mosseae* and *G. versiforme* on biomass production, chlorophyll contents, sugar fraction contents, and mineral element contents of walnut (*Juglans regia* L.) seedlings. The five AMF species colonized roots of walnut, established mycorrhizas in roots and hyphae in soil, and released easily extractable glomalin-related soil protein into soil, whilst *D. spurca* exhibited the best effect. All the AMF inoculations, except *A. scrobiculata*, stimulated shoot and root biomass production. Mycorrhizal fungal inoculations collectively increased leaf chlorophyll *a*, chlorophyll *b*, and total chlorophyll *a+b* concentrations, and thus promoted leaf sucrose accumulation, which provides an important mycorrhiza-carbon source to roots. AMF inoculations conferred a positive effect on leaf N, P, K, Mg, Fe, B, Zn and Cu contents, while they reduced leaf Mn contents. These results concluded that AMF were beneficial to the growth and physiological activities of walnut, which gives the support for the AMF application in walnut.

Keywords: arbuscular mycorrhiza; mineral nutrition; photosynthate; walnut

Introduction

Walnut (*Juglans regia* L.) is an important economy forest in many countries of the world. Walnut is often grown in mountainous areas where the soil is very poor, limiting the yield and physiological activities of walnut (Pati and Mukhopadhyay, 2009; Qin *et al.*, 2011; Wang *et al.*, 2016; Bu *et al.*, 2019; Zou *et al.*, 2019; Kong *et al.*, 2020). To adapt to the soil environment, plants coexist with soil microorganisms such as arbuscular mycorrhizal fungi (AMF) to improve nutrient acquisition (López-Ráez *et al.*, 2010; Latef and Chaoxing, 2011; Gill *et al.*, 2016; Wu *et al.*, 2019b, 2020). AMF are a kind of soil inhabiting endophytic fungi, which form arbuscular mycorrhizal symbiosis with roots of 80% of land's plants. Arbuscular mycorrhizas are able to stimulate nutrient absorption, improve plant growth, and enhance stress tolerance of host plants (Wu *et al.*, 2013; Adolfsson *et al.*, 2017; He *et al.*, 2019; Moreira *et al.*, 2019; Rosolino *et al.*, 2019; Zhang *et al.*, 2020; Zou

et al., 2020; Yang *et al.*, 2021). In switchgrass plants, inoculation with *Rhizophagus irregularis* improved K, Mg, and Na contents in shoots (Sun and Yang, 2019). Native AMF isolated from field citrus significantly accelerated P, K, Mg, and Zn contents in leaves of trifoliolate orange seedlings (Wu *et al.*, 2019). Mathur *et al.* (2018) reported that *R. intraradices*, *Funneliformis mosseae*, and *F. geosporum* promoted the synthesis of chlorophyll probably by increasing Mg uptake. However, *Glomus clarum* showed a negative effect on Mn contents in sour orange (Ortas *et al.*, 2002). Arines *et al.* (1990) also reported that inoculating *G. mosseae* or *G. aggregatum* significantly decreased Mn contents in red clover. As a result, more attention should be paid to the effect of AMF on nutrient uptake of host plants.

Earlier studies have shown that the survival of walnut plants in nursery was significantly improved after inoculation with *G. intraradices* or *G. mosseae* (Dolcet-Sanjuan *et al.*, 1996). *G. deserticola* also improved leaf area and root weight in *Juglans nigra* (Dixon, 1988). In addition, inoculation with *Gigaspora margarita*, *G. deserticola*, and *G. etunicatum* markedly stimulated N, P, and K concentrations in leaves of *J. nigra* (Dixon, 1988). Behrooz *et al.* (2019) found that inoculation with *G. mosseae* and *G. etunicatum* alleviated drought stress of walnut. These studies indicated that AMF co-exist well with walnut, to promote its growth and to enhance stress tolerance. However, the effect of AMF on walnut has been little studied and needs to be further explored.

The aims of the present study were to evaluate the effects of five AMF species from three genera on biomass production, chlorophyll concentrations, sugar contents, and mineral element contents of walnut.

Materials and Methods

Experimental design

This experiment was conducted in completely randomized design with six inoculations with *Acaulospora scrobiculata*, *Diversispora spurca*, *Glomus etunicatum*, *G. mosseae*, *G. versiforme* and non-AMF control. Each treatment was replicated six times, resulting in a total of 36 pots.

Plant set-up

Seeds of *Juglans regia* L. Liaohu 1 were sterilized with 75% alcohol for 10 min, sowed in autoclaved (0.11 MPa, 121°C, 2 h) sand, and germinated in an incubator at 28 °C/20 °C (day/night temperature) and 80% relative humidity. A month later, one seedling with uniform size and two-leaf-old was transferred into a 2.1-L plastic pot that was supplied with 2.1 kg autoclaved soil and sand (3:1, v/v). When the seedlings were transplanted, mycorrhizal fungi were inoculated. Five AMF species were *Acaulospora scrobiculata* Trappe, *Diversispora spurca* (C.M. Pfeiff., C. Walker & Bloss) C. Walker & A. Schüßler, *Glomus etunicatum* Becker & Gerd., *Glomus mosseae* (Nicolson & Gerd.) Gerd. & Trappe, and *Glomus versiforme* (P. Karst.) S.M. Berch, which were provided by the Bank of Glomales in China (BGC, Beijing, China). These fungi were propagated with white clover as the host plant for 3 months under potted conditions. Mycorrhizal fungal inoculums consisted of spores, sporocarps, AMF-colonized root segments, soil hyphae, and the growth substrate. For AMF inoculation, 100 g of mycorrhizal inoculum was applied to the rhizosphere of potted walnut seedlings. Non-AMF treatment received 100 g sterilized mycorrhizal inoculum. The seedlings were subsequently placed in a greenhouse with 720 $\mu\text{mol}/\text{m}^2/\text{s}$ average photon flux density, 28/20 °C day/night temperature, and 67% relative humidity from March to June, 2019.

Parameter determinations

At harvest time, the growth of walnut seedlings with different treatments showed a significant difference. The walnut seedlings were divided into the shoot and the root, and their fresh weight was measured. Subsequently, a small amount of root segments with 1-cm-long were stained according to the protocol as described by Phillips and Hayman (1970). The root AMF colonization degree was estimated as the percentage

of AMF-infected root lengths versus total root lengths. Soil mycorrhizal hyphal length was determined using the procedure as outlined by Bethlenfalvai and Ames (1987). Soil easily extractable glomalin-related soil protein (EE-GRSP) was assayed by He *et al.* (2020).

Leaf chlorophyll *a*, chlorophyll *b*, total chlorophyll (chlorophyll *a+b*), and carotenoid concentrations were calculated by Arnon (1949) using the 80% acetone solution.

Leaf samples were dried to a constant weight in air oven at 75 °C for 48 h and ground into 0.5 mm powder, which was used for the analysis of sugars and mineral nutrients. Leaf glucose, fructose, and sucrose contents were determined by Wu *et al.* (2015). The sieved leaf samples were digested by H₂SO₄-H₂O₂ and subjected to chemical analysis by an Electrochemical Analyzer (Smartchem 200) for N contents and by an ICP Specmometer (IRIS Advantage) for other mineral element contents.

Statistical analysis

Data were analyzed using the one-way analysis of variance with the SAS software (SAS Institute, Inc., Cary, NC, USA). The Duncan's Multiple Range Test at the 0.05 level was utilized to compare the significant difference among six treatments.

Results and Discussion

Changes in mycorrhizal status in roots and soils

There was not any mycorrhizal colonization found in the roots of the non-AMF-treated seedlings, while the root colonization of the seedlings inoculated with *A. scrobiculata*, *D. spurca*, *G. etunicatum*, *G. mosseae* and *G. versiforme* ranged from 46.0% to 76.4% (Table 1). Similarly, soil mycorrhizal hyphae were not observed in the non-AMF seedlings, but in the AMF-inoculated seedlings, varied from 1.30 m/g to 1.65 m/g. Moreover, the seedlings colonized by *D. spurca*, *G. etunicatum*, *G. mosseae* and *G. versiforme* exhibited significantly higher soil EE-GRSP concentrations, whereas *A. scrobiculata* did not significantly alter soil EE-GRSP concentrations, compared with non-AMF controls. Among them, mycorrhizal status in soil and root was the highest under the condition of *D. spurca*. As proposed by Davoodian *et al.* (2012), *D. spurca* had better compatibility with walnut seedlings than others AMF species. Previous studies have demonstrated that root AMF colonization was positively correlated with improvement of plant growth and P acquisition (Treseder and Kathleen, 2013). García-González *et al.* (2016) recommended soil EE-GRSP concentration as one indicator of mycorrhizal status. The correlation analysis also showed that there was a significant positive correlation between root mycorrhizal colonization and soil mycorrhizal hyphal length or soil EE-GRSP concentration (Figure 1), which was consistent with previous studies conducted by Curaqueo *et al.* (2010) and Wu *et al.* (2012). Glomalin is released into the soil to increase soil EE-GRSP concentrations for contributing nutrient cycle, when the mycorrhizal hyphae and spores senesced or died (He *et al.*, 2020; Meng *et al.*, 2020).

Table 1. Root AMF colonization, soil hyphal length, and soil easily extractable glomalin-related soil protein (EE-GRSP) of walnut (*Juglans regia* L. Liaohu 1) seedlings after inoculated with *Acaulospora scrobiculata*, *Diversispora spurca*, *Glomus etunicatum*, *G. mosseae*, *G. versiforme*, and non-AMF

AMF treatments	Root AMF colonization (%)	Soil hyphal length (m/g)	Soil EE-GRSP (mg/g)
<i>A. scrobiculata</i>	54.97±1.76c	1.42±0.12b	0.43±0.04cd
<i>D. spurca</i>	76.37±6.70a	1.65±0.10a	0.58±0.04a
<i>G. etunicatum</i>	63.61±5.53b	1.48±0.14ab	0.51±0.04b
<i>G. mosseae</i>	51.57±0.97cd	1.63±0.10a	0.48±0.04bc
<i>G. versiforme</i>	46.99±0.68d	1.30±0.10b	0.52±0.04b
Non-AMF	0±0e	0±0c	0.38±0.03d

Data (means ± SD, *n* = 6) followed by different letters above the bars indicate significant differences (*P* < 0.05) among treatments.

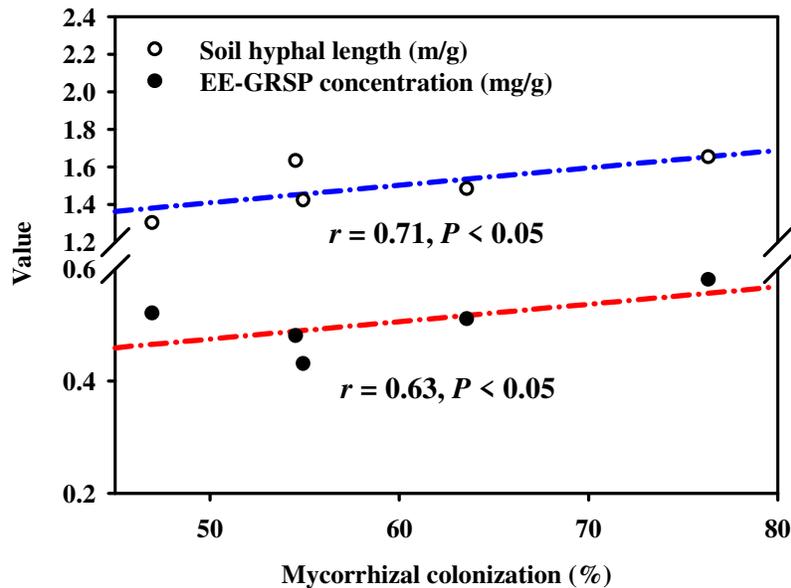


Figure 1. Linear regression between root mycorrhizal colonization and soil hyphal length or EE-GRSP concentration of walnut (*Juglans regia* L. Liaohel) seedlings

Changes in biomass production

The results of this study showed that the shoot and root biomass was positively impacted by AMF colonization (Figure 2). Compared to the non-AMF treatment, shoot and root biomass was significantly increased by 54.6% and 42.9% with *D. spurca*, by 43.1% and 14.3% with *G. etunicatum*, by 22.7% and 6.5% with *G. mosseae*, and by 26.0% and 28.6% with *G. versiforme*. Meanwhile, *A. scrobiculata* did not significantly alter shoot and root biomass, and *D. spurca* exhibited the best promoted effect. Earlier results by Giri *et al.* (2003) showed a significant increase in shoot and root biomass in *Acacia auriculiformis* plants inoculated with *G. fasciculatum* or *G. macrocarpum*. In tea plants, mycorrhiza-improved plant biomass depended on AMF species (*G. etunicatum*, *D. spurca*, *G. versiforme*, and mixed-AMF) (Shao *et al.*, 2018). Also, *G. etunicatum*, *G. mosseae*, and a mix-AMF had notably positive effect on biomass of walnut (Behrooz *et al.*, 2019). Combining these previous results with our study, it can be seen that mycorrhizas could stimulate plant biomass production of walnut, dependent on AMF species, whilst *D. spurca* had the best effect.

Changes in chlorophyll and carotenoid concentrations

The present study showed that compared to non-AMF control, all the mycorrhizal fungal inoculations significantly increased leaf chlorophyll *a*, chlorophyll *b*, carotenoid, and total chlorophyll concentration, except no changes in carotenoid concentrations between *G. versiforme* inoculation and non-AMF treatment (Table 2). Compared with the non-AMF seedlings, chlorophyll *a*, chlorophyll *b*, carotenoid, and total chlorophyll contents were increased by 36.0%, 38.3%, 28.6%, and 36.1% in *A. scrobiculata*-inoculated seedlings, by 65.0%, 68.1%, 57.1%, and 66.0% in *G. etunicatum*-inoculated seedlings, by 68.0%, 63.8%, 51.4%, and 66.0% in *G. mosseae*-inoculated seedlings, by 53.0%, 53.2%, 51.4%, and 53.1% in *D. spurca*-inoculated seedlings, and by 19.0%, 70.2%, 11.4%, and 34.7% with *G. versiforme*-inoculated seedlings. This result was in agreement with Baslam *et al.* (2013) and, Tuo *et al.* (2015), and Ekanayake *et al.* (2015). Chlorophyll and carotenoid have the function of absorbing and transmitting luminous energy (Fester *et al.*, 2005). Better chlorophyll levels in

mycorrhizal plants are involved in higher photosynthates and N, Fe, and Mg contents in mycorrhizal plants (Tuo *et al.*, 2015), as seen in Table 3 and Table 4 in our study.

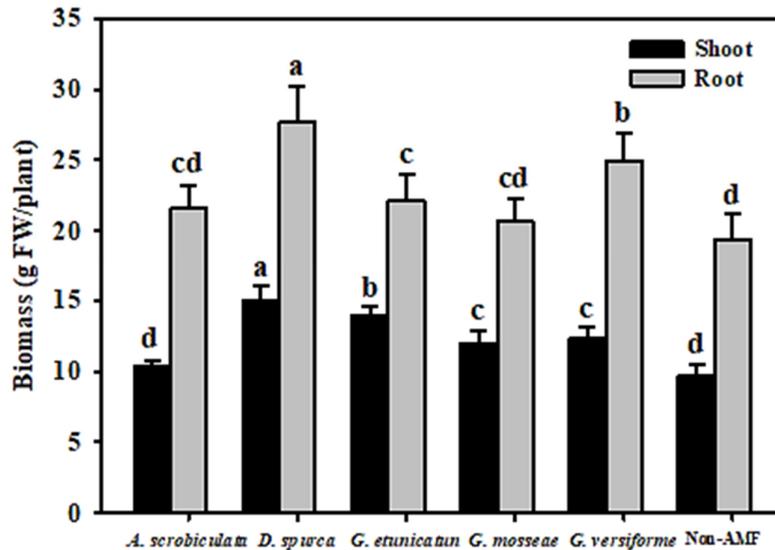


Figure 2. Shoot and root biomass of walnut (*Juglans regia* L. Liaoh 1) seedlings after inoculated with *Acaulospora scrobiculata*, *Diversispora spurca*, *Glomus etunicatum*, *G. mosseae*, *G. versiforme*, and non-AMF

Data (means \pm SD, $n = 6$) followed by different letters above the bars indicate significant differences ($P < 0.05$) among treatments.

Changes in leaf glucose, fructose and sucrose contents

As shown in Table 2, inoculation with AMF had different effects on leaf glucose, sucrose, and fructose contents of walnut. After walnut was inoculated with *A. scrobiculata*, *D. spurca*, *G. etunicatum*, *G. mosseae*, and *G. versiforme*, leaf glucose contents were significantly decreased by 43.9%, 14.7%, 32.2%, 31.4% and 42.7%, and leaf fructose contents were reduced by 46.7%, 30.6%, 15.0%, 53.0% and 31.7%, respectively. Inoculation of *G. mosseae* did not affect the glucose content in leaves, whereas inoculation with *A. scrobiculata*, *D. spurca*, *G. etunicatum*, and *G. versiforme* significantly improved leaf sucrose contents by 65.7%, 117.5%, 69.9%, and 111.4%, respectively. It is known that AMF growth relies on the photosynthate provided by the host plant (Pfeffer *et al.*, 2010). AMF can only absorb and utilize hexoses such as glucose from the cleavage of sucrose (Wu *et al.*, 2015, 2017). Our result indicated that inoculation of AMF significantly increased leaf sucrose content in walnut, and then higher sucrose content in mycorrhizal plants was conducive to the downward transport through the phloem to the root system, for the growth of AMF (Sonia *et al.*, 2010). At the same time, AMF also dramatically reduced leaf glucose and fructose contents of walnut, because the presence of root mycorrhizal carbon pool required a large amount of glucose.

Table 2. Leaf chlorophyll fractions (chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoid) and sugar fractions (glucose, fructose, and sucrose) contents of walnut (*Juglans regia* L. Liaohe 1) seedlings after inoculated with *Acaulospora scrobiculata*, *Diversispora spurca*, *Glomus etunicatum*, *G. mosseae*, *G. versiforme*, and non-AMF

AMF treatments	Chlorophyll contents (mg/g FW)				Sugar contents (mg/g DW)		
	Chlorophyll <i>a</i>	Chlorophyll <i>b</i>	Carotenoid	Total chlorophyll	Glucose	Fructose	Sucrose
<i>A. scrobiculata</i>	1.36±0.11c	0.65±0.04c	0.45±0.03b	2.00±0.11c	37.08±2.25d	83.29±11.11c	177.78±7.39b
<i>D. spurca</i>	1.53±0.10b	0.72±0.06bc	0.53±0.04a	2.25±0.10b	56.41±4.44b	108.53±7.25c	233.42±3.74a
<i>G. etunicatum</i>	1.65±0.14ab	0.79±0.03a	0.55±0.04a	2.44±0.15a	44.88±3.18c	132.85±7.44b	182.39±7.31b
<i>G. mosseae</i>	1.68±0.10a	0.77±0.07ab	0.53±0.05a	2.44±0.06a	45.37±3.71c	73.42±6.81d	113.25±9.05c
<i>G. versiforme</i>	1.19±0.04d	0.80±0.07a	0.39±0.03c	1.98±0.10c	37.90±4.16d	106.77±8.50c	226.84±39.20a
Non-AMF	1.00±0.05e	0.47±0.04d	0.35±0.03c	1.47±0.06d	66.15±3.53a	156.28±5.66a	107.32±13.83c

Data (means ± SD, *n* = 4) followed by different letters above the bars indicate significant differences (*P* < 0.05) among treatments.

Table 3. Leaf mineral element contents of walnut (*Juglans regia* L. Liaohe 1) seedlings after inoculated with *Acaulospora scrobiculata*, *Diversispora spurca*, *Glomus etunicatum*, *G. mosseae*, *G. versiforme*, and non-AMF

AMF treatments	N (mg/plant)	P (mg/plant)	K (mg/plant)	Ca (mg/plant)	Mg (mg/plant)	Fc (µg/plant)	B (µg/plant)	Zn (µg/plant)	Cu (µg/plant)	Mn (µg/plant)
<i>A. scrobiculata</i>	65.76±4.75b	3.41±0.12d	59.10±4.54b	56.91±4.81cd	10.15±0.46ab	1.47±0.15b	259.08±9.07b	178.74±16.49b	782.62±18.89c	583.42±10.13a
<i>D. spurca</i>	80.44±3.12a	6.38±0.51a	65.58±5.94a	78.94±4.65a	10.36±0.99ab	2.71±0.17a	391.90±38.42a	196.20±16.84b	439.19±34.69d	510.50±38.45b
<i>G. etunicatum</i>	75.76±6.13a	4.73±0.44b	69.90±4.79a	67.17±4.14b	11.13±0.79a	1.67±0.08b	209.53±12.32c	188.61±15.05b	1057.17±58.60a	453.58±39.76c
<i>G. mosseae</i>	59.87±1.43bc	4.11±0.19c	52.35±2.97c	66.27±2.58b	9.43±1.43b	1.70±0.14b	217.31±4.16c	292.10±14.30a	978.60±43.83b	504.59±8.59b
<i>G. versiforme</i>	75.67±4.45a	3.92±0.32c	48.39±2.22c	52.57±3.16d	9.82±0.70ab	2.74±0.22a	232.46±17.29bc	197.88±12.14b	440.05±25.18d	467.17±23.90bc
Non-AMF	56.42±5.14c	2.84±0.19e	39.86±3.61d	58.60±0.79c	7.48±0.23c	1.17±0.11c	115.84±10.41d	99.62±9.47c	327.00±17.60e	611.32±31.34a

Data (means ± SD, *n* = 4) followed by different letters above the bars indicate significant differences (*P* < 0.05) among treatments.

Changes in leaf nutrient contents

AM symbiosis plays an important role in improving nutritional acquisition of host plants (Hodge *et al.*, 2010). Compared with non-AMF inoculation, inoculation with different AMF species notably improved leaf K and P content (Table 3), whilst the greatest effect was found in *G. etunicatum* for K improvement and *D. spurca* for P improvement among five AMF treatments. Previous studies have shown that AMF-improved K and P contents were dependent on host plant species, AMF species, and soil environment (Diop *et al.*, 2003; Veresoglou *et al.*, 2011; Kilpeläinen *et al.*, 2020). The reason why AMF promote K and P absorption of host plants may be due to the fact that mycorrhizal extraradical hyphae can extend into the soil inaccessible to the root system. In addition, AMF up-regulated mycorrhiza-specific P transporter genes to promote the P absorption by cells (Amijee *et al.*, 1989). The mycorrhizal extraradical hyphae directly secretes phosphatase and indirectly promotes the root system to secrete organic acid to hydrolyze organophosphorus to inorganic phosphorus, all of these ways promote the uptake of P by plants (Duan *et al.*, 2015; Perumalsamy *et al.*, 2017).

In the present study, except that the *G. mosseae* treatment did not affect leaf N concentrations, the other AMF inoculations notably increased leaf N concentrations, compared to non-AMF control (Table 3). Herein, *D. spurca* inoculation exhibited the highest effect. A similar result is also found in the maize infected with *G. intraradices*, *Acaulospora laevis*, and *Gigaspora margarita* (Frey and Hannes, 1993) and white clover (Xie *et al.*, 2020). It has been suggested that in AM plants, ammonium nitrogen was mainly absorbed by extraradical

hyphae and then synthesized into arginine, which was moved to the intraradical hyphae into urea and ornithine; the ornithine was catalyzed into amino acids and then proteins in root cells (Bago *et al.*, 2001; Govindarajulu *et al.*, 2005; Jin *et al.*, 2005; Johansen and Olsson, 2006; Xie *et al.*, 2020).

All the AMF treatments significantly promoted leaf Ca, Mg, B, Fe, Zn, and Cu contents, except the reduction of Ca after *G. versiforme* treatment. Hereinto, *D. spurca* had the best promotion on Ca and B content, *G. etunicatum* had the strongest positive effect on Mg and Cu contents, *G. versiforme* had the best promotion on Fe, and *G. mosseae* had the best acceleration on Zn content. The important role of the soil AMF mycelium in absorbing mineral elements has been well documented (Labidi *et al.*, 2015; Weisany *et al.*, 2016; Tran *et al.*, 2019; Wu *et al.*, 2019). Earlier studies also showed that AMF had negative effects on Mn content (Arines *et al.*, 1990; Liu *et al.*, 2000). We also found the negative effect on leaf Mn content after AMF inoculation, except *A. scrobiculata*. The reduction of Mn under mycorrhization is mainly because AMs reduce the amount of manganese reductants (Liu *et al.*, 2000). Among five AMF species, *D. spurca* and *G. etunicatum* showed better promotion on Ca, Mg, B, Fe, Zn and Cu acquisition than other AMF species, which is in line with the corresponding root mycorrhizal colonization.

Conclusions

Mycorrhizal fungi could colonize roots of walnut and also positively accelerated shoot and root biomass production and improved physiological activities with regard to leaf chlorophyll production, leaf sucrose accumulation, and leaf nutrient acquisition (e.g., P, K, Mg, B, Fe, Zn, and Cu), dependent on AMF species. Hereinto, *Diversispora spurca* represented the best effect. These results provide critical support for the application of AMF in walnut in the future.

Authors' Contributions

Conceptualization: YJX and QSW; Data curation: GMH and WJC. Formal analysis: WJC, YJX, and QSW; Funding acquisition: YJX; Investigation: GMH and ZYX; Methodology: GMH and WJC; Project administration: YJX; Supervision: YJX and QSW; Writing - original draft: GMH and WJC; Writing - review and editing: MMR and QSW. All authors read and approved the final manuscript.

Acknowledgements

This work was supported by the Local Special Project for Science and Technology Development guided by the central government (2018ZYDD045).

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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