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Growth Performance and Osmolyte Regulation of Drought-Stressed Walnut Plants Are Improved by Mycorrhiza

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Abstract: This study aims to evaluate whether a selected arbuscular mycorrhizal fungus, *Diversispora spurca*, improves growth in drought-stressed walnut (*Juglans regia* L. cv. Qingxiang) plants and whether this improvement is associated with changes in osmolyte (fructose, glucose, sucrose, soluble protein, proline, and betaine) levels. After 60 days of soil drought treatment (50% of maximum field water-holding capacity), root *D. spurca* colonization rate and soil mycelium length decreased by 13.57% and 64.03%, respectively. Soil drought also inhibited the growth performance of aboveground (stem diameter, leaf number, leaf biomass, and stem biomass) and underground (root projected area, surface area, and average diameter) parts, with uninoculated plants showing a stronger inhibition than *D. spurca*-inoculated plants. *D. spurca* significantly increased these growth variables, along with aboveground part variables and root areas being more prominent under drought stress versus non-stress conditions. Although drought treatment suppressed the chlorophyll index and nitrogen balance index in leaves, mycorrhizal inoculation significantly increased these indices. Walnut plants were able to actively increase leaf fructose, glucose, sucrose, betaine, and proline levels under such drought stress. Inoculation of *D. spurca* also significantly increased leaf fructose, glucose, sucrose, betaine, proline, and soluble protein levels under drought stress and non-stress, with the increasing trend in betaine and soluble protein being higher under drought stress versus non-stress. Drought stress dramatically raised leaf hydrogen peroxide (H_2O_2) levels in both inoculated and uninoculated plants, while mycorrhizal plants presented significantly lower H_2O_2 levels, with the decreasing trend higher under drought stress versus non-stress. In conclusion, *D. spurca* symbiosis can increase the growth of drought-stressed walnut plants, associated with increased osmolyte levels and decreased H_2O_2 levels.

Keywords: betaine; mycorrhizal symbiosis; soil water deficit; walnut

Citation: Wen, Y.; Zhou, L.-J.; Xu, Y.-J.; Hashem, A.; Abd_Allah, E.F.; Wu, Q.-S. Growth Performance and Osmolyte Regulation of Drought-Stressed Walnut Plants Are Improved by Mycorrhiza. *Agriculture* **2024**, *14*, 367. https://doi.org/10.3390/agriculture14030367

Academic Editors: Tibor Szili-Kovács and Tünde Takács

Received: 16 January 2024
Revised: 18 February 2024
Accepted: 21 February 2024
Published: 25 February 2024

1. Introduction

Walnut (*Juglans regia* L.) is a globally important commercial tree in the family Juglandaceae, and its nuts are rich in linoleic acid, linolenic acid, tocopherols, and riboflavin [1,2]. Therefore, walnuts have become an important source of human nutrition. Walnuts are grown mostly in Europe, Asia, South America, and North America, with China being the leader in its production [3]. In China, commercial cultivation of walnuts is concentrated in the mountainous areas of the southwest, northwest, and northern regions, where rainfall is minimal and man-made irrigation facilities are limited. This has a negative impact on walnut growth, yield, and nut quality in walnut-producing areas [4]. Therefore, it is particularly urgent to enhance the drought tolerance of walnut plants.

Arbuscular mycorrhizal fungi (AMFs) are a group of soil microorganisms forming symbiotic associations with the roots of many higher plants, with the host providing the fungal partner with carbohydrates and the fungus providing the host partner with water and nutrients in exchange [5,6]. AMFs have been demonstrated to uptake water directly from the soil through well-developed mycorrhizal extraradical hyphae, and hyphae water uptake is critical for mycorrhizal plants' ability to survive drought stress [7]. In addition, the presence of AMFs in roots can alter root architecture, improve osmotic regulation, enhance antioxidant defense systems, optimize endogenous hormone balance, regulate the rhizospheric environment, and accelerate the expression of some stress-responsive genes to improve the host's drought tolerance [8–11]. Therefore, AMFs have been recommended as an important biostimulant for sustainable crop growth in arid regions [12].

AMF populations have been recorded in the rhizosphere of walnuts [13]. AMF inoculation promoted plant growth as well as nutrient uptake in walnuts, which was correlated with its promotion of growth of lateral roots [13]. However, the effect of AMFs on walnuts varied according to the AMF species used, with *Diversispora spurca* (C.M. Pfeiff., C. Walker and Bloss) C. Walker and A. Schüßler having a prominent effect among five AMFs [14]. Moreover, mycorrhizal extraradical hyphae transferred the walnut's juglone outside the rhizosphere, thus affecting the growth of neighboring plants [15]. In addition, root colonization of AMFs promoted mineral nutrient (e.g., N, Zn, and P) acquisition, total phenol levels, and peroxidase activity in drought-stressed walnut plants [16,17]. The expression of some heat shock transcription factors (*Hsfs*) such as *Hsf03*, *Hsf22*, and *Hsf24* was up-regulated by AMFs only under drought stress [18]. These findings suggest that AMFs are an essential pathway for enhancing growth and drought tolerance in walnut plants.

A previous study screened an AMF strain, *D. spurca*, which had an outstanding plant growth-promoting effect on walnuts [14,15], but it is not clear whether this strain affects the osmolytes of walnut plants under drought stress. 'Qingxiang' is a popular walnut cultivar in China, with well-shaped nuts, a strong flavor, and resistance to cold but not drought. This makes it especially important to improve drought tolerance in the walnut Qingxiang cultivar. An earlier study by Zou et al. [16] showed that *D. spurca* could enhance drought tolerance in the walnut Qingxiang cultivar by untargeted metabolomic analysis. A substantial number of differentially expressed metabolites has been identified [16], whereas changes in osmolytes have not been addressed. It is not known whether *D. spurca* affects growth performance and osmolyte levels in drought-stressed walnut plants. The objective of this study was to analyze whether AMFs improve growth in the drought-stressed walnut Qingxiang cultivar and whether this improvement is associated with changes in osmolyte levels.

2. Materials and Methods

2.1. Plant Culture and Experimental Design

Seeds of the walnut Qingxiang cultivar were soaked in tap water until cracked and then buried in autoclaved sand to germinate under natural conditions. After growing four leaves, they were transplanted into a plastic container pre-filled with 1850 g of autoclaved sand–soil mixture in a volume ratio of 1:3. AMF inoculation was performed at the time of transplanting. A trapped proliferation of *D. spurca* was described by Zou et al. [15]. Each inoculated pot received 150 g (approximately 3,900 spores) of *D. spurca* inoculums applied around the roots. In contrast, the uninoculated treatment received the same amount of autoclaved fungal inoculums plus 2 mL of fungal mycorrhizal filtrate through a 30 μm nylon mesh [16].

The details of soil moisture management for potted plants have been described in detail by Zou et al. [16]. Briefly, after *D. spurca* inoculation, the soil moisture of potted plants was maintained at non-stress conditions with 75% of maximum field water-holding capacity from 9 May 2021 to 2 June 2021. Subsequently, soil drought was initiated, where half of the plants were adjusted to 50% of maximum field water-holding capacity, while the other half of the plants were still maintained at non-stress conditions. The soil moisture in

the pots was controlled by weighing at around 6:00 p.m. every day. These plant materials were grown in an environmentally controlled greenhouse with environmental conditions that have been described by Zou et al. [16]. The soil drought treatment was maintained for 60 days and ended on 1 August 2021. When plant materials were harvested, half of the material was frozen in liquid nitrogen and then stored at −75 °C for analysis of biochemical variables; the other half was stored directly at −75 °C for determination of physiological variables.

This study consisted of four treatments including inoculation with *D. spurca* under non-stress conditions (S^-M^+), no inoculation under non-stress conditions (S^-M^-), inoculation with *D. spurca* under drought stress conditions (S^+M^+), and no inoculation under drought stress conditions (S^+M^-). Each treatment was replicated eight times for a total of 32 pots. Each pot contained one walnut plant.

2.2. Determination of Plant Growth, Mycorrhizal Status, and Leaf Physiological Index

Plant height, stem diameter, and leaf number were determined before plant harvest. The soil attached to the root surface was gently shaken off as the rhizosphere. Mycelium length was determined in soil using the method of Bethlenfalvay and Ames [17]. Root segments 1.5 cm long were used to determine mycorrhizal colonization using 0.05% trypan blue staining [18], and the mycorrhizal colonization rate was estimated using the percentage of AMF-colonized length to the observed length.

The nitrogen balance index (Nbi) and chlorophyll index (Chi) of the top second expanded leaf were directly recorded using a Dualex portable plant polyphenol chlorophyll meter, based on the user manual.

Harvested roots were scanned using a scanner, and then a root software (2007b; Regent Instruments Inc., Québec, QC, Canada) was used to analyze root morphological characteristics, including length, diameter, area, and volume.

2.3. Determination of Sugar Concentrations in Leaves

The glucose, fructose, and sucrose concentrations in leaves were determined by a colorimetric method [19]. Fresh leaf samples were oven-dried, ground into a powder, and passed through a 0.5 mm sieve. Then, a 50 mg dried leaf sample was extracted with 4 mL of 80% ethanol in a water bath at 80 °C for 40 min and then centrifuged at 2500× *g* for 6 min. The supernatant was collected, and the residue was continued to repeat the extraction one time according to the above process. The two supernatants were combined, and 10 mg of activated carbon was added, decolorized at 80 °C for 30 min, and filtered. Then, 0.15 mL of extracting solution was incubated with the same volume of 2 mol/L NaOH at 100 °C for 5 min, cooled, reacted with 2.1 mL of 30% hydrochloric acid and 0.6 mL of 0.1% resorcinol at 80 °C for 10 min, and measured by colorimetry at 480 nm for sucrose concentration. A 4 mL solution for fructose determination consisted of extracting solution (0.4 mL), 30% HCl (2.8 mL), and 0.1% resorcinol (0.8 mL), which was then incubated at 80 °C for 10 min and analyzed at 480 nm. Glucose concentration was determined by 0.5 mL of extracting solution and 1 mL of enzyme preparation (0.1 mg/mL *o*-anisidine–HCl, 0.1 mg/mL horseradish peroxidase, and 25 U/mL glucose oxidase) for 5 min at 30 °C and measured by colorimetry at 460 nm after addition of 2 mL of 10 mol/L sulfuric acid.

2.4. Determination of Proline, Soluble Protein, and Hydrogen Peroxide Concentrations in Leaves

Proline concentration in leaves was determined using the sulfosalicylic acid method described by Zheng et al. [20], where 5 mL of reaction solution consisted of 1 mL of 3% sulfosalicylic acid leaf extract, 1 mL of glacial acetic acid, 1 mL of acidic ninhydrin reagent, and 2 mL of toluene. Hydrogen peroxide (H_2O_2) levels in leaves were measured as per the protocol described by Velikova et al. [21], where the reaction solution consisted of 1 mL of 0.1% trichloroacetic acid leaf extract, 1 mL of 10 mmol/L potassium phosphate buffer (pH 7.0), and 2 mL of 1 mol/L potassium iodide solution. Leaf soluble protein levels were analyzed by the Coomassie Brilliant Blue G250 staining method outlined by Bradford [22].

2.5. Determination of Betaine Concentrations in Leaves

Betaine levels in leaves were assayed using the procedure described by Zhou et al. [23]. First, 0.1 g of fresh leaf samples was homogenized with 2 mL of distilled water and extracted at 150× *g* on a shaking table for 24 h. The homogenate was centrifuged at 10,000× *g* at 20 °C for 15 min. The pH value of the supernatant was adjusted to 1.0 with hydrochloric acid. Subsequently, the supernatant (0.5 mL) was incubated in 0.5 mL of 3% saturated Lehman's salt solution for 5 h at 4 °C before centrifugation at 10,000× *g* for 15 min. The supernatant was discarded, and the precipitate was washed three times with ether before being solubilized with 2 mL of 70% acetone. The absorbance was recorded at 525 nm, using 1.5 mg/mL betaine as a standard.

2.6. Statistical Analysis

Each of these selected variables had four biological replicates. The analysis of variance of the data (means ± standard error, n = 4) was performed by SAS 8.1 software, and differences among treatments were tested by Duncan's multiple range test ($p < 0.05$) for significance. Sigmaplot13.0 software was used for graphing.

3. Results

3.1. Mycorrhizae in Roots and Soil

Mycorrhizal colonization was observed in walnut plants inoculated with *D. spurca* under drought stress and non-stress conditions, where root AMF colonization was 81.00 ± 1.84% and 70.01 ± 2.26% under non-stress and drought stress conditions, respectively [16]. Mycorrhizal mycelium was also found in the inoculated plants' rhizosphere, with 38.39 ± 1.87 cm/g and 13.81 ± 0.91 cm/g under non-stress and drought stress conditions, respectively (Table 1). Drought treatment significantly inhibited the rate of mycorrhizal colonization in roots and soil mycelium length by 13.57% and 64.03%, respectively, compared with the non-stress treatment.

Table 1. Effects of AMF inoculation on shoot growth in walnut seedlings.

Treatments	Soil Mycelium Length (cm/g)	Stem Diameter (mm)	Leaf Number	Height (cm)	Leaf Biomass (g/plant)
S^-M^+	38.39 ± 1.87 a	7.35 ± 0.48 a	44.4 ± 2.70 a	30.4 ± 2.30 a	9.61 ± 0.60 a
S^-M^-	0 c	6.68 ± 0.34 b	43.8 ± 3.11 a	26.0 ± 1.87 b	8.82 ± 0.74 b
S^+M^+	13.81 ± 0.91 b	5.54 ± 0.32 c	25.4 ± 1.52 b	20.4 ± 1.14 c	4.80 ± 0.30 c
S^+M^-	0 c	4.72 ± 0.45 d	20.2 ± 0.45 c	19.4 ± 0.96 c	2.75 ± 0.35 d

Different letters following the means ± standard error (n = 4) indicate significant differences at the $p < 0.05$ level. Treatment abbreviations: S^+, drought stress; S^-, non-stress; M^+, inoculation with *Diversispora spurca*; M^-, no inoculation.

3.2. Aboveground Part Growth Response

Soil drought significantly inhibited the aboveground part growth of walnut plants, as evidenced by a significant decrease in stem diameter, leaf number, plant height, leaf biomass, and stem biomass by 24.63%, 42.79%, 32.89%, 50.05%, and 48.21% in inoculated plants and by 29.34%, 53.88%, 25.38%, 68.82%, and 59.90% in uninoculated plants (Table 1). On the other hand, inoculated plants presented significantly higher stem diameter, leaf biomass, and stem biomass by 10.03%, 8.96%, and 9.95% under non-stress conditions and 17.37%, 74.55%, and 42.01% under soil drought, respectively. Mycorrhizal plants also presented significantly higher leaf numbers (25.74%) under drought stress as well as significantly higher plant height (16.92%) under non-stress than non-mycorrhizal plants.

3.3. Underground Part Growth Response

Root total length, projected area, surface area, and average diameter of inoculated plants decreased significantly under soil drought versus non-stress conditions by 21.43%, 11.03%, 20.06%, and 19.23%, respectively; root projected area, surface area, and average

diameter of uninoculated plants also decreased significantly under soil drought versus non-stress conditions by 16.17%, 20.65%, and 16.13%, respectively (Table 2). The root volume was not affected significantly by soil drought and *D. spurca* inoculation. However, inoculation with *D. spurca* significantly increased root total length, projected area, surface area, and average diameter by 35.32%, 7.55%, 10.84%, and 25.81%, respectively, under non-stress conditions and by 15.54%, 14.15%, 11.66%, and 21.15%, respectively, under drought stress.

Table 2. Effects of AMF inoculation on root characteristics in walnut seedlings.

Treatments	Total Length (cm)	Projected Area (cm^2)	Surface Area (cm^2)	Average Diameter (mm)	Volume (cm^3)
S^-M^+	267.8 ± 4.2 a	13.24 ± 0.17 a	20.24 ± 0.57 a	0.78 ± 0.01 a	4.51 ± 1.80 a
S^-M^-	197.9 ± 6.2 bc	12.31 ± 0.43 b	18.26 ± 0.21 b	0.62 ± 0.01 b	2.91 ± 0.64 ab
S^+M^+	210.4 ± 9.8 b	11.78 ± 0.13 b	16.18 ± 0.19 c	0.63 ± 0.03 b	2.43 ± 0.58 ab
S^+M^-	182.1 ± 2.4 c	10.32 ± 0.00 c	14.49 ± 0.62 d	0.52 ± 0.02 c	1.37 ± 0.51 b

Different letters following the means ± standard error (n = 4) indicate significant differences at the $p < 0.05$ level. Treatment abbreviations: S^+, drought stress; S^-, non-stress; M^+, inoculation with *Diversispora spurca*; M^-, no inoculation.

3.4. Leaf Physiological Index Response

Soil drought treatment significantly reduced leaf Nbi and Chi by 33.36% and 11.47% in inoculated plants and 24.33% and 9.67% in uninoculated plants, respectively, as compared with the non-stress treatment (Figure 1). Moreover, inoculation with *D. spurca* significantly increased leaf Nbi and Chi by 38.14% and 34.93% under non-stress conditions and 21.65% and 32.24% under drought conditions, respectively.

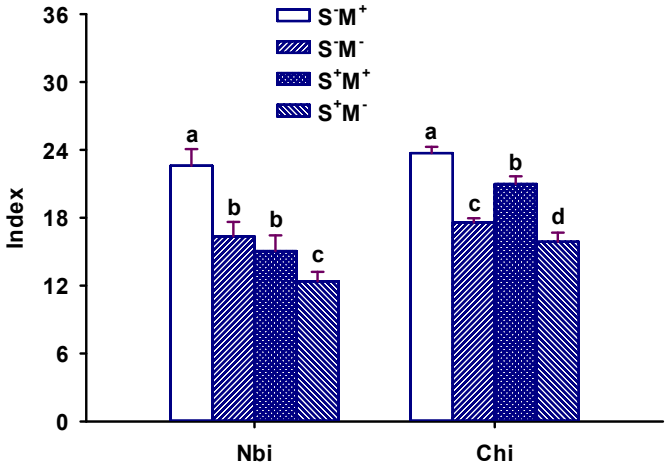

Figure 1. Effects of *Diversispora spurca* inoculation on leaf Nbi and Chi in walnut seedlings. Different letters above the bars (means ± standard error, n = 4) indicate significant differences at the $p < 0.05$ level. Treatment abbreviations: S^+, drought stress; S^-, non-stress; M^+, inoculation with *Diversispora spurca*; M^-, no inoculation.

3.5. Leaf Sugar Concentration Response

Drought treatment significantly boosted fructose and glucose levels in leaves of inoculated plants by 27.46% and 26.19%, respectively, as well as fructose, glucose, and sucrose levels in leaves of uninoculated plants by 68.07%, 53.06%, and 49.40%, respectively, along with no significant change in sucrose levels in the leaves of inoculated plants (Figure 2). When the plants were inoculated with *D. spurca*, leaf fructose, sucrose, and glucose levels were significantly increased by 69.23%, 80.93%, and 120.27% under non-stress conditions and by 28.34%, 34.03%, and 81.60% under drought conditions, respectively.

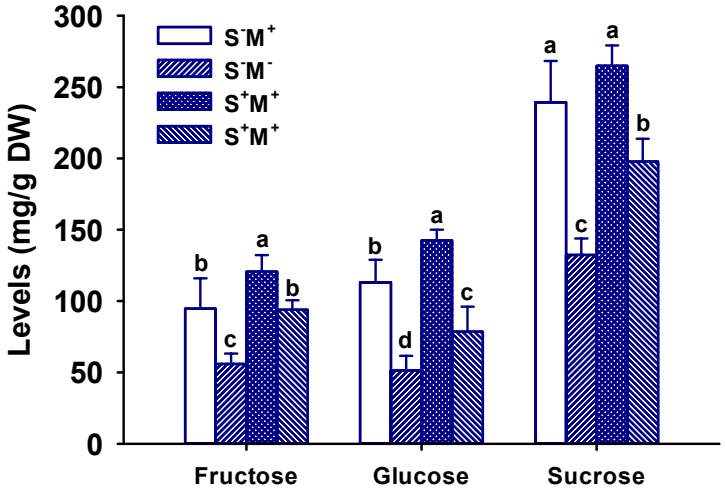

Figure 2. Effects of *Diversispora spurca* inoculation on leaf sugar levels in walnut seedlings. Different letters above the bars (means ± standard error, $n = 4$) indicate significant differences at the $p < 0.05$ level. Treatment abbreviations are the same as in Figure 1.

3.6. Leaf Soluble Protein Level Response

Soil drought treatment significantly suppressed leaf soluble protein levels in inoculated and uninoculated plants by 31.58% and 41.86%, respectively, compared with the non-stress treatment (Figure 3). Nevertheless, inoculation of *D. spurca* significantly increased leaf soluble protein levels under non-stress and drought conditions by 32.56% and 56.00%, respectively, compared with the uninoculated treatment.

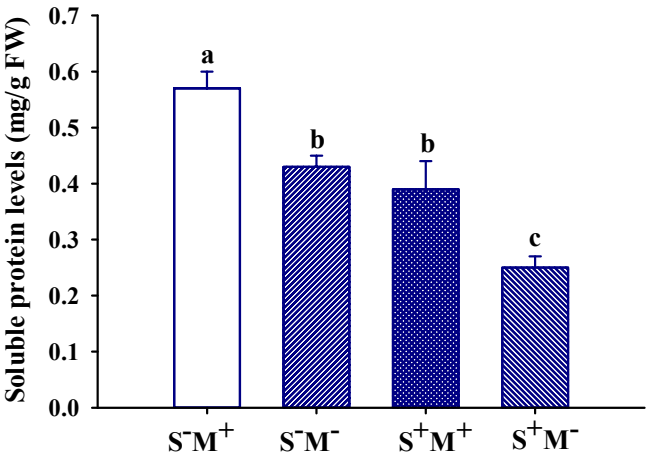

Figure 3. Effects of *Diversispora spurca* inoculation on leaf soluble protein levels in walnut seedlings. Different letters above the bars (means ± standard error, $n = 4$) indicate significant differences at the $p < 0.05$ level. Treatment abbreviations are the same as in Figure 1.

3.7. Leaf Proline and Betaine Concentration Response

Soil drought treatment significantly increased betaine and proline levels in the leaves of inoculated plants by 87.18% and 28.37%, respectively, and in the leaves of uninoculated plants by 52.38% and 31.06%, respectively, compared with the unstressed treatment (Figure 4). On the other hand, inoculation with *D. spurca* significantly increased leaf betaine levels under non-stress and drought conditions by 85.71% and 128.13%, respectively, and also elevated leaf proline levels under non-stress and drought conditions by 20.00% and 17.53%, respectively, compared with the uninoculated treatment.

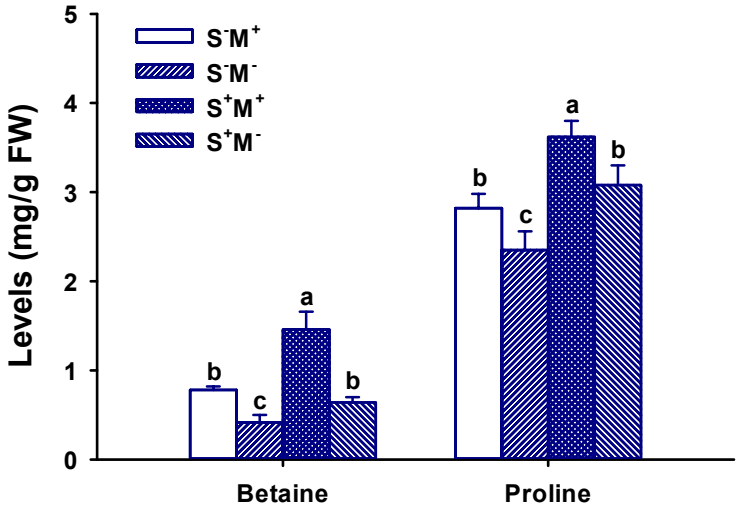

Figure 4. Effects of *Diversispora spurca* inoculation on leaf betaine and proline levels in walnut seedlings. Different letters above the bars (means ± standard error, n = 4) indicate significant differences at the $p < 0.05$ level. Treatment abbreviations are the same as in Figure 1.

3.8. Leaf H_2O_2 Level Response

Soil drought treatment significantly accelerated the accumulation of leaf H_2O_2 levels by 52.06% and 55.43% in inoculated and uninoculated plants, respectively, compared with the non-stress treatment (Figure 5). Inoculation with *D. spurca* significantly decreased leaf H_2O_2 levels by 14.60% and 16.46% under non-stress and drought conditions, respectively, compared with the uninoculated control.

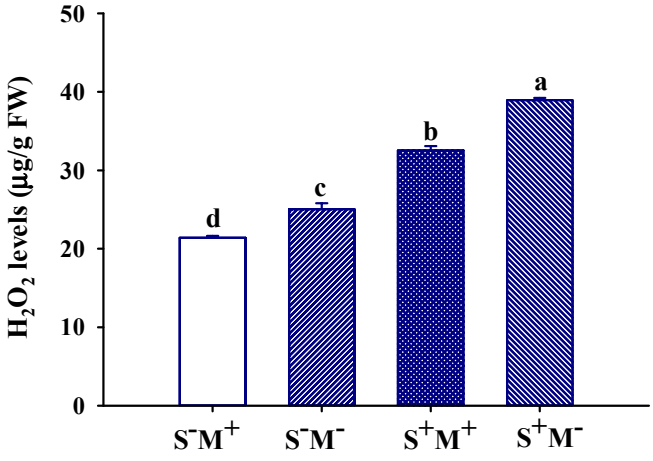

Figure 5. Effects of *Diversispora spurca* inoculation on leaf H_2O_2 levels in walnut seedlings. Different letters above the bars (means ± standard error, n = 4) indicate significant differences at the $p < 0.05$ level. Treatment abbreviations are the same as in Figure 1.

4. Discussion

In this study, soil drought treatment inhibited the colonization rate of walnut variety 'Qingxiang' roots by *D. spurca* and the formation of mycorrhizal mycelium in the soil, as previously described by Zou et al. [16]. This inhibitory effect may originate from the fact that drought reduces soil spore germination, mycelial growth, and the supply of host-made carbohydrates to the fungal partners [11].

Drought treatment also significantly inhibited the aboveground growth performance of walnuts, and the inhibitory effect on leaf and stem biomass production was greater on uninoculated plants than on inoculated plants, demonstrating that inoculated walnut plants were less influenced by soil drought. In addition, inoculation with *D. spurca* significantly

increased stem diameter, leaf biomass, and stem biomass in walnuts, with the increased tendency being more pronounced under soil drought than under non-stress conditions. This is in agreement with the findings of Wang et al. [24] on drought-stressed trifoliate oranges inoculated with *Rhizophagus intraradices* and Mo et al. [25] on drought-stressed watermelons inoculated with *Glomus versiforme*. This demonstrates the role of AMFs in maintaining plant growth vigor in arid zones. Mao et al. [26] also reported that indigenous AMF inoculation had a higher effect on walnut growth improvement than inoculation with commercial AMF species. Mycorrhizal improvement of host aboveground growth is a result of a multifactorial combination, including improved root morphology, increased nutrient uptake, direct water uptake by mycorrhizal hyphae, and regulation by endogenous auxins [7,25,27,28]. The mycorrhizal promotion under drought appears to be important for maintaining plant growth and physiological activities.

The roots are the first part of the plant to sense soil adversity stress [29]. Soil drought usually restricts root growth, leading to a reduction in the soil contact area of roots, a decrease in nutrient and water uptake, and consequent growth inhibition [30,31]. In the present study, drought treatment significantly suppressed root average diameter, projected area, and surface area, which is one of the reasons why drought hindered walnut plant growth. However, *D. spurca* inoculation significantly improved root characteristics under drought stress and non-stress conditions, including length, area, and diameter, but not volume, confirming that inoculated plants may adapt to drought situations by altering root plasticity. Xie et al. [32] also observed increased length, diameter, and fork number in strawberry roots after inoculation of *Glomus mosseae* under mild and moderate drought stress. In a meta-analysis, Chandrasekaran [33] proposed that under drought, root length and root surface area of inoculated plants increased by 37% and 31%, respectively, thus promoting nutrient uptake, particularly P.

Soil drought inhibits the activity of chlorophyll biosynthetic enzymes as well as the uptake of mineral nutrients, thus reducing chlorophyll formation [34]. The present study also showed a significant decrease in leaf Chi in inoculated and uninoculated walnut plants under drought stress versus non-stress conditions. However, inoculation with *D. spurca* significantly increased Chi under both drought stress and non-stress conditions. This demonstrates an important regulatory effect of arbuscular mycorrhizae on chlorophyll synthesis under drought. A similar result was also reported in marigolds under drought stress after inoculation with *Glomus constrictum* [35]. Drought treatment also suppressed leaf Nbi, whereas root colonization of AMFs promoted Nbi under both drought and non-stress conditions, owing to the direct uptake of inorganic N from the soil by mycorrhizal extraradical hyphae and rapid host N assimilation by mycorrhizae [36]. AMF-triggered increase in Nbi is one of the reasons why mycorrhizae increase chlorophyll levels in host plants [37].

Drought can cause damage to plants, but plants also have multiple physiological responses such as osmoregulation to mitigate the damage [38]. Osmolytes in osmoregulation create a more water-absorbent environment in plant cells, allowing them to withstand drought [39]. Moreover, small molecules of osmolytes including soluble carbohydrates, proteins, free amino acids, betaine, and proline have a neutral charge and are required for protective membranes and the denaturation of proteins as well as for osmotic potential and cell expansion under stress [39,40]. In the present study, glucose, fructose, betaine, and proline levels in leaves were increased under drought treatment, indicating that both inoculated and uninoculated walnuts actively accumulate osmolytes to resist drought. Li et al. [41] also demonstrated that leaf soluble sugar, soluble protein, and proline levels in the walnut cultivars 'Liaoning 1' and 'Xiangling' increased with stress duration in an 18-day stress event. All of these indicate that 'Xiangling' has the ability to actively respond to drought stress, but its ability to adapt to drought decreases with the time of drought stress [41]. In addition, this study was conducted under potted conditions, and regular water supplementation may have an effect on Xiangling's stress response. Moreover, inoculation with *D. spurca* also significantly increased glucose, fructose, sucrose, soluble protein,

proline, and betaine levels in walnut leaves under both drought and non-stress conditions, suggesting that mycorrhizal walnuts can actively accumulate more osmolytes in response to drought treatment than non-mycorrhizal controls. Mirshad and Puthur [42] observed that AMF-inoculated *Saccharum arundinaceum* plants had higher soluble protein, proline, and sugar levels under drought than uninoculated plants. Behrooz et al. [43] also reported a considerable rise in proline, total sugar, and starch levels in the mycorrhizal walnut variety 'Chandler' than in non-mycorrhizal walnuts under drought. Of the six osmolytes tested, the increased magnitude in glucose, fructose, sucrose, and proline levels triggered by *D. spurca* was greater under non-stress than under drought stress, while the increased magnitude in soluble protein and betaine was greater under drought stress than under non-stress conditions. This indicated that mycorrhizal walnut plants under non-stress improved osmoregulation predominantly through glucose, fructose, sucrose, proline, and betaine, whereas mycorrhizal walnuts under drought increased osmoregulation preferentially through soluble proteins. This difference needs further study.

H_2O_2 is a reactive oxygen species produced during plant cell metabolism, and excessive levels of H_2O_2 can trigger oxidative bursts in organic molecules, which are regarded to be hazardous to cells [44]. In the present study, drought treatment significantly elevated leaf H_2O_2 levels in walnuts, and the increased magnitude was greater in uninoculated plants than in inoculated plants, suggesting that mycorrhizal walnuts experienced relatively low oxidative bursts. On the other hand, inoculation with *D. spurca* also significantly reduced leaf H_2O_2 levels, with a greater reduction under drought stress than under non-stress. This is strong evidence that mycorrhizal walnut plants have lower oxidative bursts under drought conditions than non-mycorrhizal walnut plants, indicating higher drought tolerance. This is in line with the findings of Liu et al. [45], who inoculated *Funneliformis mosseae* on trifoliate orange under drought conditions. The mycorrhiza-triggered reduction in host H_2O_2 levels under stress is a combined consequence of both the fungus' own H_2O_2-scavenging mechanisms and the activation of plant antioxidant (e.g., polyamine, fatty acids, and flavonoids) defense systems [9].

5. Conclusions

In this study, *D. spurca* inoculation improved the growth performance of aboveground and belowground parts and increased osmolyte levels in the leaves of drought-stressed walnut plants, along with reduced oxidative damage in leaves. *D. spurca* can thus be used as a beneficial fungus to enhance drought tolerance in walnut plants. However, the molecular mechanism underlying the mycorrhizal enhancement of drought tolerance in drought-stressed walnut plants remains unknown and needs to be investigated.

Author Contributions: Conceptualization, Q.-S.W.; data curation, Y.W. and L.-J.Z.; methodology, Y.W. and L.-J.Z.; resources, Q.-S.W. and Y.-J.X.; supervision, Q.-S.W.; writing—original draft, Y.W.; writing—review and editing, A.H., E.F.A. and Q.-S.W. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Hubei Province '14th Five-Year' Major Science and Technology Aid Tibet Project (SCXX-XZCG-22016) and the Key Research and Development Project of Hubei Province (2022BBA153). The authors would like to extend their sincere appreciation to the Researchers Supporting Project Number (RSP2024R134), King Saud University, Riyadh, Saudi Arabia.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: All the data supporting the findings of this study are included in this article.

Acknowledgments: The authors would like to extend their sincere appreciation to the Researchers Supporting Project Number (RSP2024R134), King Saud University, Riyadh, Saudi Arabia.

Conflicts of Interest: The authors declare no conflicts of interest.

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