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# Effects of Arbuscular Mycorrhizal Fungi on Growth and Physiological Performance of *Catalpa bungei* C.A.Mey. under Drought Stress

Wei Chen <sup>†</sup>, Panpan Meng <sup>†</sup>, Huan Feng and Chunyan Wang <sup>\*ID</sup>

College of Forestry, Northwest A&F University, Yangling 712100, China; chenwei18298345663@163.com (W.C.); 18391861476@163.com (P.M.); fenghuan19930327@163.com (H.F.)

\* Correspondence: chunyan@nwsuaf.edu.cn; Tel.: +86-187-2954-9495

<sup>†</sup> These authors contributed equally to this work.

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**Abstract:** *Catalpa bungei* C.A.Mey. is a common ornamental timber species. Its survival and growth are greatly affected by water scarcity in arid and semi-arid areas of Northwest China. Evidence suggests arbuscular mycorrhizal fungus (AMF) may improve plant drought resistance. However, there is limited information on the systematic effects of AMF on drought resistance in *C. bungei* seedlings. Here, a pot experiment was used to explore the effects of inoculation with the AMF *Rhizophagus intraradices* on the growth and physiological performance of *C. bungei* under different water treatment conditions. Three water levels and two mycorrhizal inoculation treatments were used with factorial design. The results showed that drought stress noticeably affected the growth and physiological performance of *C. bungei* seedlings. However, inoculation with *R. intraradices* significantly ameliorated the growth, and alleviated the effects of drought stress. The growth parameters of AMF-inoculated seedlings significantly increased regardless of water status. AMF changed the biomass allocation in seedlings by reducing the root mass ratio (RMR) and root/shoot ratio. AMF-inoculated seedlings displayed higher gas exchange parameters, photosynthetic pigment concentrations, specific leaf area (SLA), but lower specific leaf weight (SLW), regardless of water status. AMF alleviated drought-induced oxidative stress by attenuating the excess generation of reactive oxygen species (ROS), especially H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup>, in leaves. Inoculation with AMF under drought stress also dramatically augmented indole-3-acetic acid (IAA) and gibberellins (GA<sub>3</sub>) levels and the IAA/abscisic acid (ABA) and GA<sub>3</sub>/ABA ratios, but reduced ABA and zeatin (ZT) levels in leaves. AMF symbiosis improved root morphology and promoted the absorption of nitrogen (N) and phosphorus (P) in seedlings. We conclude that inoculation with *R. intraradices* is potentially useful for afforestation and cultivation of *C. bungei* in Northwest China. Furthermore, AMF improved soil structure by increasing the glomalin-related soil protein (GRSP) contents and the proportion of macro-aggregates (0.25–0.5 mm) in the rhizosphere soil.

**Keywords:** *Rhizophagus intraradices*; *Catalpa bungei*; growth parameters; physiological performance; root morphology; soil aggregates; drought stress

## 1. Introduction

Drought is one of the most important factors affecting plant growth in terrestrial ecosystems [1]. Climate change increases the odds of severe drought conditions around the world [2,3]. Drought stress can change plant water status, and has obvious effects on seed germination, plant morphological structure, biomass distribution, soil nutrient availability, photosynthesis, and metabolism, thereby seriously restricting the normal growth, development, survival, and productivity of plants [3–6]. Plants adapt to drought by making morphological and physiological adjustments through the synergistic

action of various physiological processes [7]. Currently, attempts to enhance plant drought resistance are being made to improve forestry and agricultural productivity and reduce water consumption. One of the strategies used to enhance plant drought resistance is inoculation with AMF [2,7,8].

AMF are one of the most widely distributed endophytic mycorrhizal fungi, able to form arbuscular mycorrhizal symbiosis with more than 80% of terrestrial plant species [2,9]. The symbiosis between AMF and plants is beneficial for plant growth, stress resistance, nutrient cycling, and soil quality improvement [1,10,11]. The symbiosis between AMF and plants is important for drought adaptation and increased drought resistance in the latter [2,12,13]. This is partly explained by the large extraradical hyphal network formed in the soil upon AMF colonization, which can expand the root uptake area and promote the absorption and utilization of water and nutrients (mainly phosphorus (P) and nitrogen (N)) by the plant [2,10,11]. In exchange, to maintain a symbiotic relationship, plants provide 4–20% of their total photosynthate (in the form of lipids and sugars) for the AMF growth and reproduction [14,15]. The regulation of plant drought resistance by AMF is a very complex process, involving a variety of metabolites and metabolic pathways [3,16]. Under drought conditions, AMF can enhance seedling survival [7,17], promote absorption and transportation of water by the host plant [8,18], change the root morphology [7,19], improve the gas exchange ability and water use efficiency (WUE) [3,8], regulate the plant endogenous hormone levels [6,20], and accelerate reactive oxygen species (ROS) removal [1,7], all of which are aimed at reducing the negative impact of drought on plants. In addition, AMF can produce glomalin—defined as glomalin-related soil protein (GRSP), which serves as a super glue—to promote the formation of water-stable soil aggregates through the physical entanglement of extraradical hyphae, thus improving and stabilizing soil structure and its water-holding capacity [11,15,21]. Therefore, AMF can enhance the adaptability of plants to drought by the up- and down-regulation of various physiological and biochemical processes [3,22].

*Catalpa bungei*, native to China, is a common, high-quality, ornamental timber tree species. Owing to its strong root tillering ability, fast growth rate, excellent material quality, corrosion resistance, and wide application, it is broadly planted in the warm temperate and subtropical regions of China [23,24]. However, its self-sterility, low seed setting, low germination rate, and low seedling emergence rate make seedling propagation difficult [25,26]. The shortage of water resources in the arid and semi-arid areas of Northwest China is a key factor affecting the survival and rapid growth of *C. bungei* seedlings. Therefore, new approaches to improve the survival and growth performance of *C. bungei* seedlings and enhance their adaptability to drought are necessary for afforestation in northern China. The coupling effects of water and fertilizers [27], light conditions [28], drought stress [29,30], and exponential nitrogen fertilizations [31] can affect the growth of *C. bungei* seedlings. AMF have been shown to improve drought resistance in many woody plants [2,8,13,16]. However, in-depth information on the systematic effects of AMF on drought resistance, and its corresponding mechanisms of action in *C. bungei* seedlings, is limited. Therefore, in this study, we used a pot experiment to simulate drought stress and explore the effects of AMF inoculation on the growth characteristics of *C. bungei* under different water conditions. Specifically, we aimed to elucidate the relevant mechanisms of action of AMF with regard to root morphology, nutrient absorption, photosynthesis, ROS removal capacity, endogenous hormone regulation, and the effect of AMF on soil structure. We intend to provide a theoretical basis for the application of AMF in the afforestation and cultivation of *C. bungei* in Northwest China.

## 2. Materials and Methods

### 2.1. Arbuscular Mycorrhizal Inoculum, Plant Material, and Cultivation Substrate

The mycorrhizal inoculum (AMF, *Rhizophagus intraradices*) used in this study contained spores, mycelium, and infested maize root fragments that were obtained from the Institute of Plant Nutrition and Resources, Beijing Academy of Agriculture and Forestry Sciences, China.

*C. bungei* seeds were collected from the Northwest A&F University campus. Following surface sterilization (with  $\text{KMnO}_4$ ) and germination, they were seeded in trays (disinfected with 0.5% sodium hypochlorite) containing sterilized substrate ( $121\text{ }^\circ\text{C}$ , 2 h) filled with nutrient soil, vermiculite, and perlite (V:V:V = 1:1:1). After they reached the 3–6 leaf stage, the seedlings were transplanted into pots (20 cm in diameter, 20 cm in depth, and disinfected with 0.5% sodium hypochlorite) for planting.

The study area is located in Yangling District, Shaanxi Province, China ( $34^\circ 16' \text{ N}$ ,  $108^\circ 4' \text{ E}$ ) (Figure 1). The soil type is loam, with an average annual precipitation of 650 mm and an average annual temperature of  $13.7\text{ }^\circ\text{C}$ . It belongs to the semi-humid and semi-arid climate zone in the warm temperate zone of East Asia. Topsoil was collected from the nursery of the College of Forestry, Northwest A&F University, Yangling District, and sifted through a 2 mm sieve. Sifted river sand was washed with clean water (8–9 times) and dried under natural light. Both were sterilized ( $121\text{ }^\circ\text{C}$ , 2 h) separately, and then mixed evenly at a 1:1 volume ratio. The amounts of total N, available P, and potassium (K) in the soil were  $830$ ,  $25.43$ , and  $183.88\text{ mg}\cdot\text{kg}^{-1}$ , respectively.

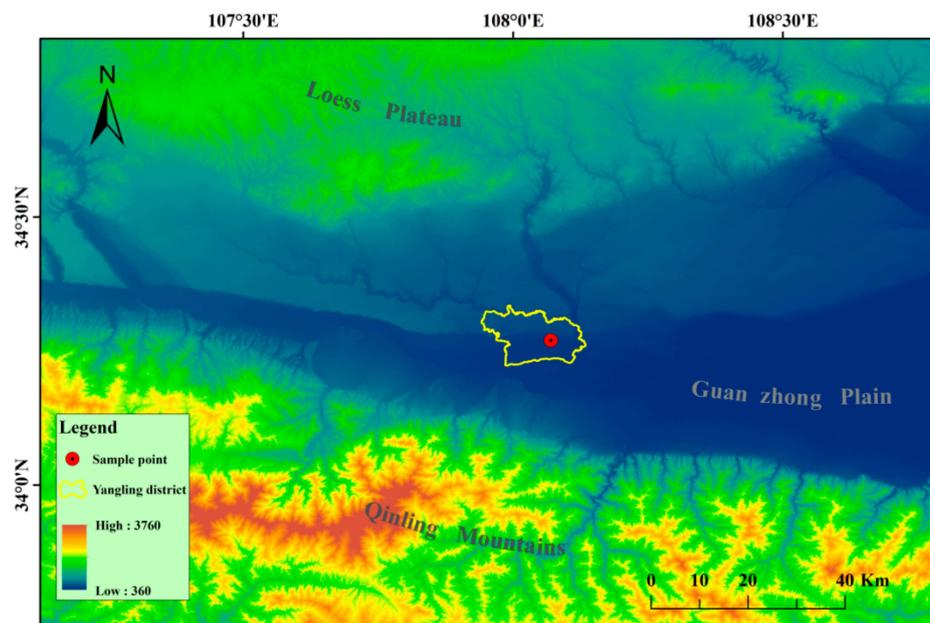


Figure 1. Map of study area.

## 2.2. Experimental Design

The pot experiment was conducted from April to September 2019 in the greenhouse of the Northwest A & F University, using factorial design, including water and inoculation treatments. Six treatments were performed, with 10 replicates each. Water treatments were divided into three categories: well-watered (WW), moderate drought (MD), and severe drought (SD), which corresponded to a soil water content of 70%, 50%, and 30% of the maximum field capacity (Maximum field capacity was determined according to the method reported by Yan et al. [32]), respectively. The inoculation treatments were performed with *R. intraradices* and non-inoculated seedlings were used as controls.

*C. bungei* seedlings with uniform growth trends were selected and transplanted into plastic flowerpots containing about 4.2 kg of cultivation substrate on 15 May 2019. At the time of transplanting, 3 g of *R. intraradices* inoculum was spread evenly on the roots until full contact (inoculated treatment group). The control group was treated with the same amount of inactivated inoculum ( $121\text{ }^\circ\text{C}$ , 2 h). After transplanting, normal water supply was maintained until the seedlings grew stably (approximately 45 d). Root samples of three seedlings were randomly selected for the detection of AMF colonization. After successful colonization, water treatments were conducted, and the soil water content was controlled using the weighing method for 2.5 months (from 10 July to 25 September 2019).

### 2.3. Measurement of AMF Colonization

Seedling roots were first washed with running tap water and then several times with distilled water. Samples were randomly cut into small root segments (approximately 1 cm) and immersed in 10% KOH solution, after which they were placed in a water bath at 90 °C for 30 min. After the root segments became transparent, any residual KOH was washed off with distilled water, and the root segments were dyed with 0.05% Trypan blue staining solution at 90 °C for 30 min. Samples were then decolorized with a mixture of lactic acid and glycerin ( $v/v = 1:1$ ) three times [33]. Next, samples were observed under a 400× optical microscope (Olympus Bx43, Tokyo, Japan), and the AMF colonization rate was calculated according to the magnifying cross method [34].

### 2.4. Measurement of Gas Exchange Parameters and Photosynthetic Pigments

Gas exchange parameters, including net photosynthetic rate ( $P_n$ ,  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ), stomatal conductance ( $G_s$ ,  $\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ), transpiration rate ( $T_r$ ,  $\mu\text{mol}\cdot\text{mol}^{-1}$ ), and intercellular  $\text{CO}_2$  concentration ( $C_i$ ,  $\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ), were determined before seedling harvest using a Li-6400 portable photosynthetic apparatus (LI-COR, Lincoln, NE, USA). Seedling leaves (3rd, 4th, and 5th from the top) were measured on a sunny day from 9:00 to 12:00 a.m. (stomatal opening was the largest) with Li-6400-02B (red and blue light sources). Light intensity was  $1000 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . For each leaf, three randomly selected measuring points were used. WUE is expressed as the ratio of  $P_n$  to  $T_r$  [8] (Yang et al., 2014). Chlorophyll (Chl a and Chl b) and carotenoid contents were determined by direct extraction with acetone [35].

### 2.5. Determination of Plant Growth Parameters and Biomass Allocation

Seedling height and basal diameter were measured before harvest using a tape measure and vernier caliper, respectively. Root, stem, and leaf fresh weights were measured immediately after the harvest, whereas dry weights were determined after drying the biomass (at 65 °C, until constant weight). Next, the biomass allocation of each part was calculated. Leaf mass ratio (LMR), stem mass ratio (SMR), and root mass ratio (RMR) were determined as the ratios of leaf, stem and root biomass to the total biomass, respectively. Root/shoot ratio was calculated as the ratio of underground biomass to the aboveground biomass [36].

### 2.6. Analysis of Nutrient Absorption and Distribution

Powdered root, stem, and leaf samples were sieved through a 0.45 mm sieve, and N, P, and K concentrations were determined using the Kjeldahl, molybdenum-stibium colorimetric, and flame photometric method [37], respectively.

### 2.7. Determination of Specific Leaf Area and Specific Leaf Weight

Leaf area was calculated from a single leaf at the same position, using the transparent grid method [34]. Leaves were dried at 65 °C (to constant weight), and their dry weights were determined. Specific leaf area (SLA) was calculated as the ratio of a single leaf area to its dry weight, whereas the specific leaf weight (SLW) was calculated as the ratio of a single leaf dry weight to its area, which is essentially the reciprocal of the SLA.

### 2.8. Estimation of the Root System Architecture

Cleaned roots of the fresh seedlings were scanned and analyzed using the root scanner system (WinRHIZO 2013e). Morphological parameters, such as root length, root surface area, projected area, average root diameter, and root branching number were calculated [38].

### 2.9. Determination of Phytohormone Levels

Cytokinin (CTKs), auxin (indole-3-acetic acid, IAA), gibberellin (GA<sub>3</sub>), and abscisic acid (ABA) levels in leaves were determined using high performance liquid chromatography [39,40].

### 2.10. Determination of ROS

Fresh leaf and root samples were ground in pre-cooled mortars with liquid nitrogen, and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and superoxide anion radical (O<sub>2</sub><sup>-</sup>) contents were determined according to the method reported by Gao [35].

### 2.11. Determination of GRSP Content

The rhizosphere soil was shaken off, collected, air-dried and filtered through a 1 mm sieve. Half gram of soil was put into a plastic centrifuge tube with 4 mL of extraction solution. The mixture was well suspended, and placed in an autoclave (121 °C, 30–60 min), to extract the easily extractable glomalin-related soil protein (EE-GRSP; 20 mmol·L<sup>-1</sup> sodium citrate solution, pH 7.0) and the total glomalin-related soil protein (T-GRSP; 50 mmol·L<sup>-1</sup> sodium citrate solution, pH 8.0). Tube contents were centrifuged at 10,000 rpm for 15 min, and the supernatants were collected. Absorbance of the supernatants was determined at 595 nm, using the Coomassie Brilliant Blue method. EE-GRSP and T-GRSP contents were calculated according to a standard bovine serum albumin curve [41].

### 2.12. Analysis of Soil Aggregate Distribution

The particle size distribution of the aggregates (including micro- and macro-aggregates) was determined using a Malvern laser particle size analyzer (Mastersizer 2000, Worcestershire, UK) [42]. Based on their size, soil particles were classified into five different categories: <0.002, 0.002–0.01, 0.01–0.25, 0.25–0.5, and 0.5–1 mm.

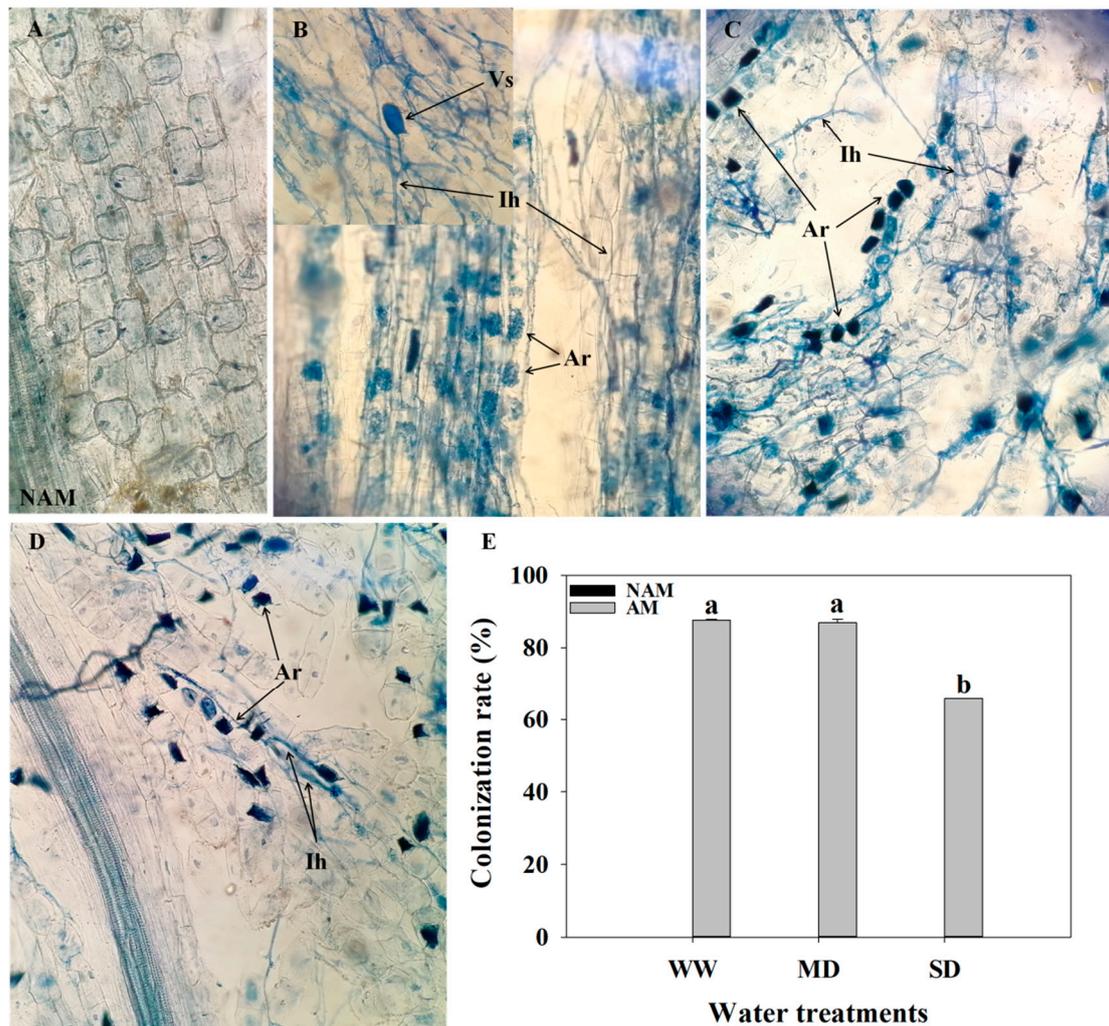
### 2.13. Statistical Analysis

Data were analyzed using the SPSS 21.0 software, and the differences among treatments were compared using one-way analysis of variance (ANOVA) and independent-sample T test. Differences among the treatments were examined using the Duncan test at  $p < 0.05$ . A two-way ANOVA was used to determine the significance of the water and inoculation treatments, and their interaction with the experimental parameters. Pearson's correlation coefficients among parameters were analyzed using SPSS [8]. All data are reported as mean ± standard error (SE). Mapping was carried out using SigmaPlot 12.0.

## 3. Results

### 3.1. AMF Root Colonization

No AMF colonization was found in the roots of non-inoculated *C. bungei* seedlings (Figure 2A), whereas clear colonization by *R. intraradices* was observed in the mycorrhizal inoculated seedlings. Mycorrhizal hyphae ultimately formed distinct morphological structures, such as intraradical hyphae, arbuscules, and vesicles, under WW (Figure 2B), MD (Figure 2C), and SD (Figure 2D) conditions. Root colonization rate in the *R. intraradices*-inoculated seedlings was above 60% under the three water treatment conditions (Figure 2E), indicating that *R. intraradices* could form a good symbiotic relationship with the seedling roots. Colonization rate of the mycorrhizal fungi was 86.89% under the MD treatment, which was only slightly lower than that for WW (87.55%), whereas it decreased sharply under SD stress (65.74%,  $p < 0.05$ ) (Figure 2E).

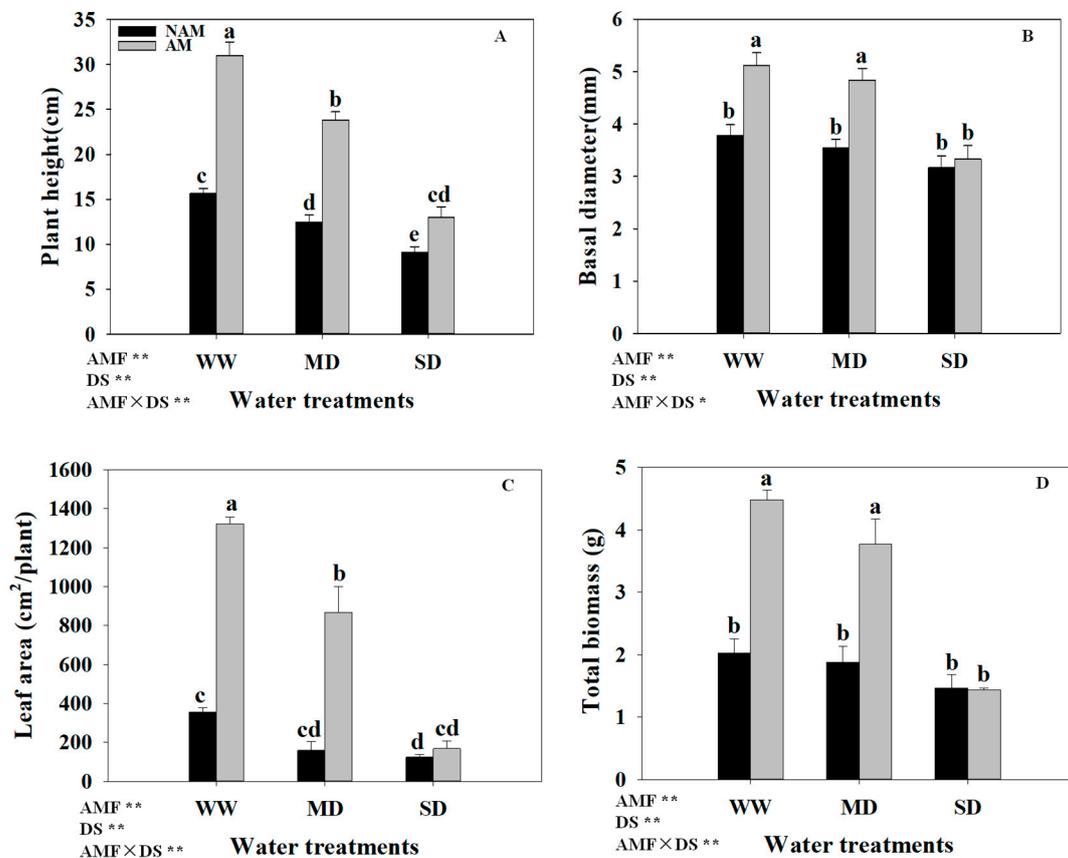


**Figure 2.** Development of arbuscular mycorrhizal fungus (AMF) *Rhizophagus intraradices* in *Catalpa bungei* seedling roots by Trypan blue staining. (A) The root of a non-inoculated plant. (B) Inoculated roots under well-watered (WW), (C) moderate drought (MD), and (D) severe drought (SD) conditions. (E) Colonization rate of mycorrhizal *C. bungei* seedlings. Ih, intraradical hyphae; Ar, arbuscule; Vs, vesicles; (A, B, C and D, G:  $\times 400$ ). NAM, non-AMF-inoculated; AM, AMF-inoculated. Different lowercase letters above the bars indicate significant differences ( $p < 0.05$ ) among treatments. Values are means  $\pm$  SE ( $n = 3$ ).

### 3.2. Growth Parameters

Drought stress noticeably inhibited the growth of *C. bungei* seedlings (Figure 3). Both under inoculated and non-inoculated conditions, the plant height and leaf area of the *C. bungei* seedlings showed significant decreases with an increase in drought stress (Figure 3A,C); the basal diameter and total biomass also decreased, but only under SD conditions (in the inoculation treatment) was this difference significant ( $p < 0.05$ ) (Figure 3B,D). Plant height, basal diameter, leaf area, and the total biomass of mycorrhizal inoculated *C. bungei* seedlings increased significantly under both WW and MD conditions, compared with those for the control treatment (Figure 3). Plant height and total biomass increased more than 1.9-fold, basal diameter approximately 1.35-fold, and leaf area 3.7- (WW condition) and 5.4- (MD stress) fold, compared with those for the control treatment. Under SD stress, the growth parameters of mycorrhizal inoculated seedlings (basal diameter, leaf area, and total biomass) increased, but these differences were not significant ( $p < 0.05$ ). Thus, AMF inoculation could significantly promote

the growth of *C. bungei* seedlings and alleviate the inhibition of growth by drought stress, but this effect was not obvious under SD stress.



**Figure 3.** Effect of arbuscular mycorrhizal fungus (AMF) *Rhizophagus intraradices* on (A) plant height, (B) basal diameter, (C) leaf area and (D) total biomass of *Catalpa bungei* seedlings under DS. NAM, non-AMF-inoculated; AM, AMF-inoculated; WW, well-watered; MD, moderate drought; SD, severe drought; DS, drought stress. Different lowercase letters above the bars indicate significant differences ( $p < 0.05$ ) among treatments. Values are means  $\pm$  SE. Plant heights and basal diameters are based on 9 biological replicates, while leaf area and total biomass represents the average of three biological replicates. Two-way ANOVA output: ns, not significant; \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

### 3.3. Biomass Allocation

Drought stress and AMF inoculation significantly changed the biomass allocation pattern of *C. bungei* (Table 1). Increasing drought stress caused a decrease in LMR and leaf and stem biomass of the *C. bungei* seedlings (both inoculated and non-inoculated). There was a slight increase in SMR under the same condition. The root biomass of both inoculated and non-inoculated seedlings, together with RMR and the root/shoot ratio of non-inoculated seedlings, increased under MD stress and decreased under SD stress, but there were no significant differences between the water treatments ( $p > 0.05$ ). RMR and the root/shoot ratio of inoculated seedlings, on the other hand, showed a notable increase (2.43 and 3.41-fold higher than that under WW, respectively; Table 1). Under SD stress, the leaf biomass of the non-mycorrhizal treated seedlings, as well as the leaf and stem biomass and LMR of the mycorrhizal inoculated seedlings, decreased significantly (52.13%, 20.91%, 34.34%, and 65.63% compared to that of the WW conditions, respectively). Under WW conditions, most of the non-inoculated seedling biomass was distributed in the leaves, followed by the roots, and with the least proportion allocated to stems (leaf > root > stem), whereas under drought stress it showed a root > leaf > stem distribution. The biomass of the mycorrhizal inoculated seedlings, however, tended to be distributed in leaves,

followed by the stems, while the least allocated proportion was to the roots (leaf > stem > root). Under drought stress, the biomass allocation trend was as follows: leaf > root > stem.

AMF inoculation promoted the biomass accumulation in leaves and stems of *C. bungei* seedlings, increased LMR and SMR, but inhibited the accumulation of root biomass, and decreased RMR and root/shoot ratio under both WW and drought stress, compared with those in the non-inoculated conditions (Table 1). Leaf and stem biomass of the mycorrhizal inoculated seedlings were significantly higher, while RMR was significantly lower both under WW (3-fold, 3.5-fold and 40%, respectively) and MD (2.8-fold, 2.7-fold and 44.23%, respectively), than in the non-mycorrhizal treated seedlings. However, there were no significant differences in all the indices between the inoculated and control treatments under SD stress ( $p > 0.05$ ).

#### 3.4. Photosynthetic Gas Exchange Capacity

Drought stress significantly reduced the gas exchange parameters of *C. bungei* seedlings (Table 2). The Pn, Gs, Ci, Tr, and WUE of the *C. bungei* seedlings decreased gradually with the intensification of stress, with or without AMF inoculation. Among those, Pn, Gs, and Tr were significantly lower under MD and SD stress than under WW, and WUE was significantly lower under SD stress than under WW. Under SD stress, Pn, Gs, Ci, Tr and WUE of both the control (or inoculated) treatments were only 30.12% (37.88%), 27.27% (33.33%), 65.14% (83.82%), 38.12% (48.48%) and 77.65% (76.22%) of those under WW conditions. Under the same water conditions, Pn, Gs, Tr, and WUE of seedlings inoculated with AMF were all higher than those of non-inoculated seedlings, while Ci was lower than that of the non-inoculated seedlings (Table 2). Under MD stress, Gs and WUE of the mycorrhizal inoculated seedlings were significantly higher, whereas Ci was significantly lower than those of the non-inoculated seedlings (1.5-fold, 1.15-fold and 81.03%, respectively). However, there was no significant difference between the inoculated and control treatments under SD stress ( $p > 0.05$ ). These results showed that AMF could improve the photosynthetic capacity and WUE of *C. bungei* seedlings to some extent, but this positive effect was inhibited under SD stress.

#### 3.5. Photosynthetic Pigment Concentrations

Regardless of the AMF status, the concentrations of photosynthetic pigments (chlorophyll a, b, and carotenoids) decreased in the *C. bungei* leaves with increasing drought stress, whereas the chlorophyll a/b values increased gradually (Table 3). Under SD stress, the concentrations of pigments decreased significantly both in the non-inoculated (or inoculated) seedlings, being only 31.32% (45.65%), 27.45% (41.38%), and 35.29% (55.88%) of those under WW treatments, respectively. AMF inoculation increased leaf photosynthetic pigment concentrations to some extent, especially under drought stress (Table 3). Although there was no significant difference in the concentration of photosynthetic pigments between the inoculated and control treatments ( $p > 0.05$ ), the pigment concentrations of the mycorrhizal inoculated seedlings was higher than those of the non-mycorrhizal treated seedlings—except for the chlorophyll a/b value, which was lower—under both WW and drought stress.

#### 3.6. SLA and SLW

With increasing drought stress, the SLA of both inoculated and non-inoculated *C. bungei* seedlings decreased gradually, while SLW increased significantly (Figure 4). SLA and SLW were significantly different between WW and drought stress treatments ( $p < 0.05$ ). Under SD, the SLA and SLW of AMF-inoculated (or non-inoculated) seedlings were 66.53% (61.19%) and 1.5-fold (1.6-fold) of those under WW treatments, respectively. The SLA of mycorrhizal inoculated seedlings was significantly higher under WW and MD stress, while the SLW was significantly lower than that of non-mycorrhizal treated seedlings under the three water treatments (Figure 4).

**Table 1.** Effect of the arbuscular mycorrhizal fungus (AMF) *Rhizophagus intraradices* on the biomass allocation in different parts of the *Catalpa bungei* seedlings under DS.

Water Status	AMF Status	Leaf Biomass(g)	Stem Biomass(g)	Root Biomass(g)	Leaf Mass Ratio	Stem Mass Ratio	Root Mass Ratio	Root/Shoot Ratio
WW	NAM	0.94 ± 0.07 c	0.36 ± 0.02 b	0.72 ± 0.15 a	0.47 ± 0.03 abc	0.18 ± 0.01 a	0.35 ± 0.04 ab	0.55 ± 0.10 bc
	AM	2.87 ± 0.06 a	0.99 ± 0.25 a	0.62 ± 0.15 a	0.64 ± 0.01 a	0.22 ± 0.05 a	0.14 ± 0.04 c	0.17 ± 0.05 c
MD	NAM	0.60 ± 0.17 c	0.32 ± 0.02 b	0.97 ± 0.18 a	0.31 ± 0.06 c	0.17 ± 0.02 a	0.52 ± 0.06 a	1.14 ± 0.27 a
	AM	2.10 ± 0.34 b	0.85 ± 0.12 a	0.83 ± 0.06 a	0.55 ± 0.04 ab	0.22 ± 0.01 a	0.23 ± 0.04 bc	0.31 ± 0.08 bc
SD	NAM	0.49 ± 0.06 c	0.32 ± 0.03 b	0.66 ± 0.18 a	0.34 ± 0.01 c	0.23 ± 0.05 a	0.43 ± 0.06 a	0.79 ± 0.20 ab
	AM	0.60 ± 0.15 c	0.34 ± 0.04 b	0.50 ± 0.12 a	0.42 ± 0.10 bc	0.24 ± 0.02 a	0.34 ± 0.08 ab	0.58 ± 0.21 bc
Significance	AMF	**	**	ns	**	ns	**	**
	DS	**	*	ns	*	ns	ns	ns
	AMF × DS	**	*	ns	ns	ns	ns	ns

NAM, non-AMF-inoculated; AM, AMF-inoculated; WW, well-watered; MD, moderate drought; SD, severe drought; DS, drought stress. Different lowercase letters within each column indicate significant differences ( $p < 0.05$ ) among treatments. Values are means ± SE ( $n = 3$ ). Two-way ANOVA output: ns, not significant; \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

**Table 2.** Effect of the arbuscular mycorrhizal fungus (AMF) *Rhizophagus intraradices* on the photosynthetic gas exchange parameters of *Catalpa bungei* seedlings under DS.

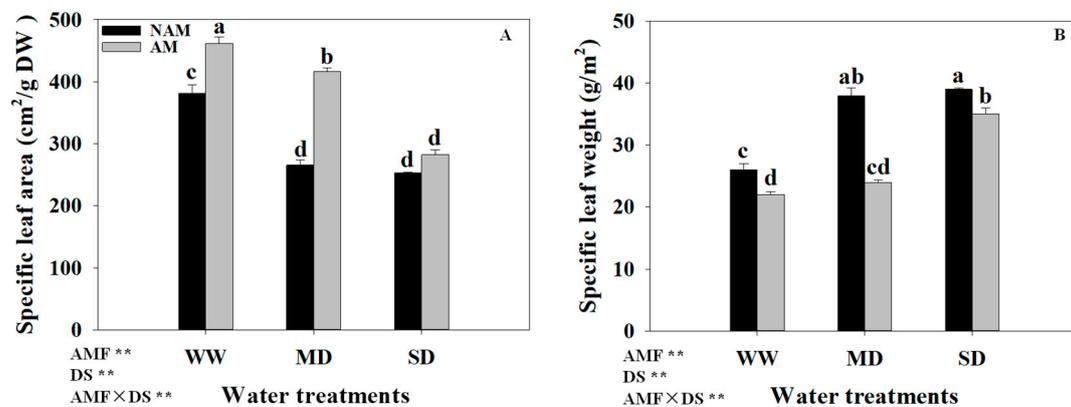
Water Status	AMF Status	$P_n$ ( $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ )	$G_s$ ( $\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ )	$C_i$ ( $\mu\text{mol}\cdot\text{mol}^{-1}$ )	$T_r$ ( $\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ )	WUE ( $\mu\text{mol}/\text{mmol}$ )
WW	NAM	6.01 ± 0.25 a	0.11 ± 0.01 ab	312.15 ± 5.21 a	3.41 ± 0.24 a	1.79 ± 0.06 a
	AM	6.52 ± 0.26 a	0.12 ± 0.01 a	241.89 ± 16.82 bc	3.63 ± 0.29 a	1.85 ± 0.10 a
MD	NAM	3.78 ± 0.21 b	0.06 ± 0.01 c	277.79 ± 24.51 ab	2.54 ± 0.10 b	1.50 ± 0.09 b
	AM	4.40 ± 0.45 b	0.09 ± 0.01 b	225.08 ± 15.72 c	2.53 ± 0.23 b	1.72 ± 0.05 a
SD	NAM	1.81 ± 0.19 c	0.03 ± 0.00 d	203.35 ± 12.91 c	1.30 ± 0.13 c	1.39 ± 0.05 b
	AM	2.47 ± 0.13 c	0.04 ± 0.00 cd	202.75 ± 9.82 c	1.76 ± 0.11 c	1.41 ± 0.03 b
Significance	AMF	**	**	**	ns	ns
	DS	**	**	**	**	**
	AMF × DS	ns	ns	ns	ns	ns

NAM, non-AMF-inoculated; AM, AMF-inoculated; WW, well-watered; MD, moderate drought; SD, severe drought; DS, drought stress.  $P_n$ , net photosynthetic rate;  $G_s$ , stomatal conductance;  $T_r$ , transpiration rate;  $C_i$ , intercellular  $\text{CO}_2$  concentration; WUE, Water use efficiency; Different lowercase letters within each column indicate significant differences ( $p < 0.05$ ) among treatments. Values are means ± SE ( $n = 9$ ). Two-way ANOVA output: ns, not significant; \*\*  $p < 0.01$ .

**Table 3.** Effect of the arbuscular mycorrhizal fungus (AMF) *Rhizophagus intraradices* on photosynthetic pigment concentrations in *Catalpa bungei* seedlings under DS.

Water Status	AMF Status	Chlorophyll a(mg/g)	Chlorophyll b(mg/g)	Chlorophyll a/b	Carotenoid(mg/g)
WW	NAM	1.66 ± 0.08 ab	0.51 ± 0.03 ab	3.28 ± 0.07 a	0.34 ± 0.01 a
	AM	1.84 ± 0.07 a	0.58 ± 0.04 a	3.18 ± 0.10 a	0.34 ± 0.01 a
MD	NAM	1.00 ± 0.46 bcd	0.31 ± 0.17 abc	3.51 ± 0.32 a	0.21 ± 0.08 ab
	AM	1.41 ± 0.23 abc	0.42 ± 0.09 ab	3.44 ± 0.18 a	0.30 ± 0.02 a
SD	NAM	0.52 ± 0.16 d	0.14 ± 0.04 c	3.75 ± 0.13 a	0.12 ± 0.04 b
	AM	0.84 ± 0.00 cd	0.24 ± 0.00 bc	3.55 ± 0.03 a	0.19 ± 0.06 ab
Significance	AMF	ns	ns	ns	ns
	DS	**	**	ns	**
	AMF × DS	ns	ns	ns	ns

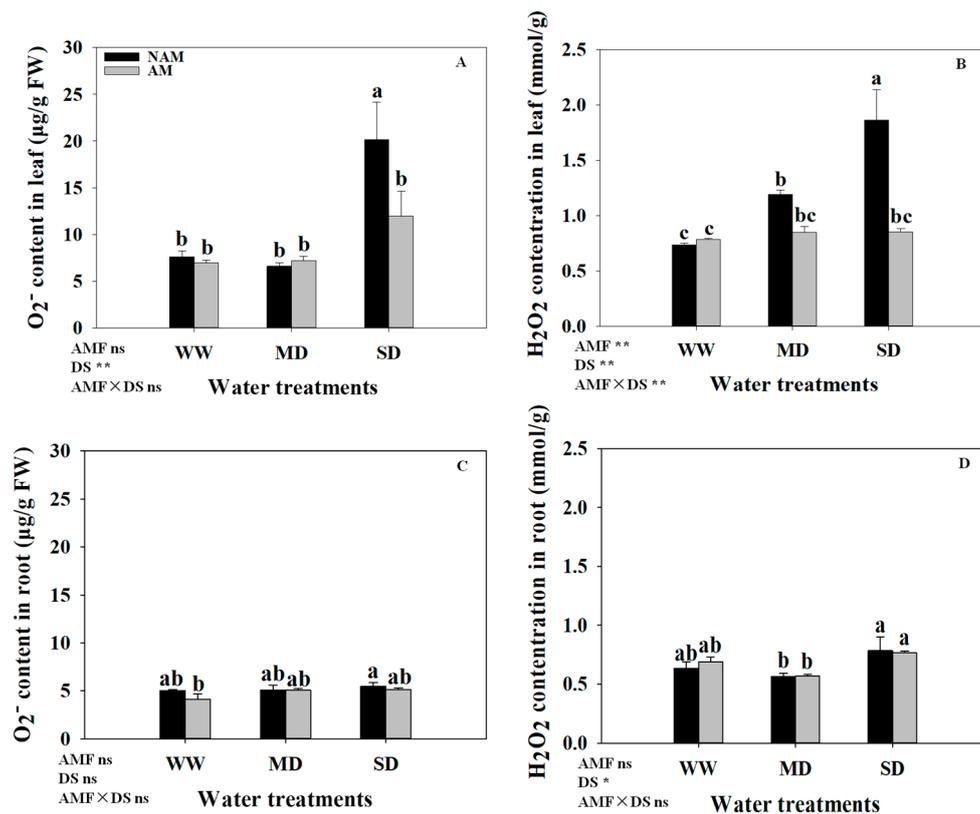
NAM, non-AMF-inoculated; AM, AMF-inoculated; WW, well-watered; MD, moderate drought; SD, severe drought; DS, drought stress. Different lowercase letters within each column indicate significant differences ( $p < 0.05$ ) among treatments. Values are means ± SE ( $n = 3$ ). Two-way ANOVA output: ns, not significant; \*\*  $p < 0.01$ .



**Figure 4.** Effect of the arbuscular mycorrhizal fungus (AMF) *Rhizophagus intraradices* on (A) specific leaf area (SLA) and (B) specific leaf weight (SLW) of *Catalpa bungei* seedlings under DS. NAM, non-AMF-inoculated; AM, AMF-inoculated; WW, well-watered; MD, moderate drought; SD, severe drought; DS, drought stress. Different lowercase letters above the bars indicate significant differences ( $p < 0.05$ ) among treatments. Values are means ± SE ( $n = 3$ ). Two-way ANOVA output: ns, not significant; \*\*  $p < 0.01$ .

### 3.7. ROS ( $H_2O_2$ and $O_2^-$ ) Contents

Regardless of the inoculation status, the  $O_2^-$  and  $H_2O_2$  content of *C. bungei* leaves and roots increased gradually with the intensification of drought (with the exception of  $H_2O_2$  in roots under MD stress) (Figure 5). Moreover, the  $O_2^-$  and  $H_2O_2$  content of the inoculated (or non-inoculated) roots showed no significant difference between WW and drought stress ( $p > 0.05$ ) (Figure 5C,D). The above parameters were significantly higher (2.64- and 2.55-fold, respectively) in the leaves of non-inoculated seedlings under SD stress, than those under WW treatments (Figure 5A,B). However, there was no significant difference in the ROS content of inoculated seedlings among different water treatments ( $p > 0.05$ ). Under both WW and drought stress conditions, neither  $H_2O_2$  nor  $O_2^-$  were significantly different between the roots of mycorrhizal and non-mycorrhizal treated seedlings, while their contents were significantly reduced (to 45.70% and 59.25% of those in the control treatments, respectively) in the leaves under SD stress.

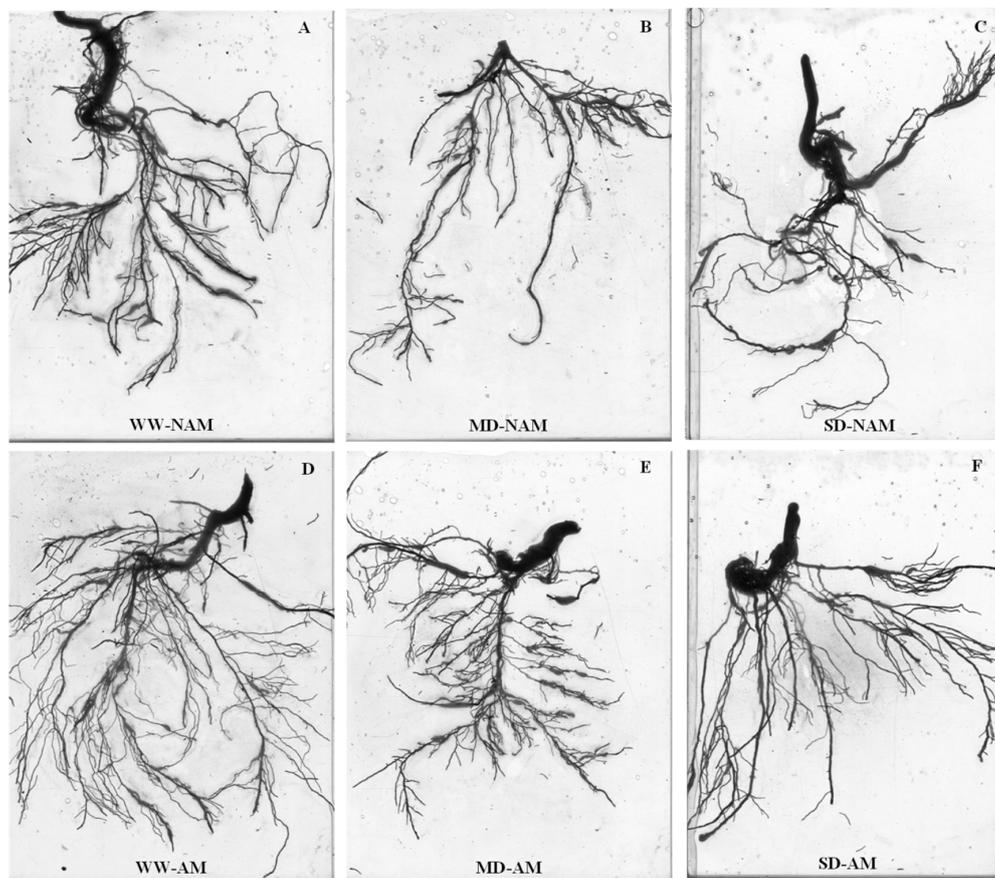


**Figure 5.** Effect of the arbuscular mycorrhizal fungus (AMF) *Rhizophagus intraradices* on the  $O_2^-$  and  $H_2O_2$  contents in (A,B) leaves and (C,D) roots of *Catalpa bungei* seedlings under DS. NAM, non-AMF-inoculated; AM, AMF-inoculated; WW, well-watered; MD, moderate drought; SD, severe drought; DS, drought stress. Different lowercase letters above the bars indicate significant differences ( $p < 0.05$ ) among treatments. Values are means  $\pm$  SE ( $n = 3$ ). Two-way ANOVA output: ns, not significant; \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

### 3.8. Root System Architecture

Drought stress significantly inhibited root growth, but AMF inoculation significantly improved root development (Figure 6). Regardless of the inoculation status, all root morphological parameters—except the average root diameter—decreased with an increase in drought stress (Table 4). None of the total root length, surface area, projected area, root volume, or branching number were significantly different under MD in non-inoculated seedlings from those under WW, but they all significantly decreased under SD conditions. In addition, the total root tips of non-inoculated seedlings were reduced significantly, while the length and surface area of fine roots ( $0 < d \leq 0.5$  mm) were not significantly different among the three watering conditions ( $p > 0.05$ ). Most of the root morphological parameters, such as total root length, surface area, volume, root tips, length, and surface area of fine roots ( $0 < d \leq 0.5$  mm) decreased significantly under drought stress, while total root projected area and branching number were reduced notably only under SD stress. The average root diameter of either inoculated or non-inoculated seedlings did not change significantly among the three water treatments. Compared with those of the non-inoculated seedlings under WW conditions, all root morphological parameters of mycorrhizal inoculated seedlings were significantly higher than those of non-mycorrhizal treated seedlings, except the average root diameter, which decreased significantly (by 29.52%) (Table 4). Under MD stress, the total root volume, the number of root tips, branching number, and the surface area of fine roots ( $0 < d \leq 0.5$  mm) were significantly higher in the inoculated vs. the non-inoculated seedlings (1.41-, 1.56-, 1.45-, and 1.89-fold, respectively) (Table 4). However, under SD stress, only the root branching number of the mycorrhizal inoculated seedlings was significantly higher (1.84-fold), while the other

parameters showed no significant difference between inoculated and control treatments ( $p > 0.05$ ) (Table 4).



**Figure 6.** Effect of the arbuscular mycorrhizal fungus (AMF) *Rhizophagus intraradices* on the root morphology of *Catalpa bungei* seedlings under drought stress. (A), WW-NAM; (B), MD-NAM; (C), SD-NAM; (D), WW-AM; (E), MD-AM; (F), SD-AM; NAM, non-AMF-inoculated; AM, AMF-inoculated; WW, well-watered; MD, moderate drought; SD, severe drought.

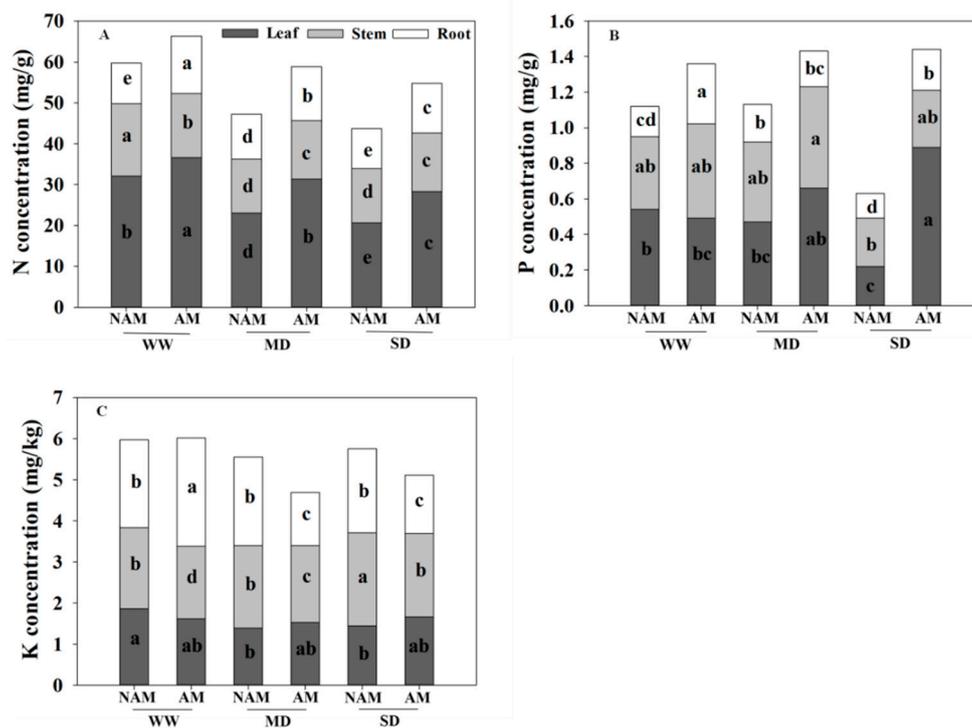
### 3.9. Nutrient Absorption and Distribution

N concentration was highest in the roots, stems, and leaves of *C. bungei*, while K concentration was the lowest. The concentrations of N and P in the aboveground parts, especially leaves, were significantly higher than those in the underground part under the three water treatments (Figure 7). Compared with the WW conditions, drought stress significantly reduced the N, P, and K concentrations in leaves, N in the stems of non-mycorrhizal treated seedlings, the N, P, and K concentrations in the roots, N in the leaves and stems of the mycorrhizal inoculated seedlings, and increased the concentration of K in the stems of both mycorrhizal and non-mycorrhizal treated seedlings, but had no significant effect on the P concentration in the stem ( $p > 0.05$ ). Moderate drought stress significantly increased the N and P concentrations in the roots of non-mycorrhizal treated seedlings but decreased them under SD stress. Compared with those in the control treatments, root, stem, and leaf N and P concentrations and leaf K were all higher in inoculated seedlings under both WW and drought stress conditions (except for N concentration in stems, and P and K in leaves, under WW conditions) (Figure 7). N concentration reached a significant difference under the three water conditions, while P concentration in leaves and roots was significantly different under SD stress ( $p < 0.05$ ). In addition, AMF inoculation significantly reduced K concentration in the stems and roots of *C. bungei* seedlings under drought stress ( $p < 0.05$ ).

**Table 4.** Effect of the arbuscular mycorrhizal fungus (AMF) *Rhizophagus intraradices* on the root morphological parameters of *Catalpa bungei* seedlings under DS.

Water Status	AMF Status	Total Root Length/(cm)	Total Root Surface Area/(cm <sup>2</sup> )	Root Projected Area/(cm <sup>2</sup> )	Total Root Volume/(cm <sup>3</sup> )	Mean Root Diameter/(mm)	Root Tips	Branching Number	LF (cm)	SAF (cm <sup>2</sup> )
WW	NAM	602.81 ± 13.10 b	179.67 ± 14.81 b	57.98 ± 3.97 b	603.66 ± 12.31 bc	1.05 ± 0.09 a	2861.67 ± 73.26 b	3142.00 ± 177.69 b	189.71 ± 4.43 bc	14.65 ± 0.11 c
	AM	1022.21 ± 136.34 a	234.84 ± 23.94 a	74.74 ± 7.62 a	1022.54 ± 136.31 a	0.74 ± 0.04 b	3645.33 ± 183.39 a	4288.00 ± 397.20 a	473.60 ± 85.65 a	45.84 ± 8.30 a
MD	NAM	548.39 ± 39.65 b	165.64 ± 7.74 b	52.72 ± 2.46 b	548.39 ± 39.65 c	0.99 ± 0.07 a	1844.67 ± 104.97 c	2635.33 ± 153.78 b	200.70 ± 25.40 bc	17.98 ± 3.08 c
	AM	692.37 ± 43.32 b	191.82 ± 3.97 b	64.08 ± 2.36 ab	774.15 ± 71.81 b	0.89 ± 0.06 ab	2882.67 ± 71.39 b	3823.67 ± 61.43 a	315.47 ± 49.39 b	33.92 ± 2.77 b
SD	NAM	302.77 ± 44.66 c	94.07 ± 11.30 c	29.66 ± 3.53 c	316.10 ± 47.65 d	1.00 ± 0.03 a	1296.33 ± 138.36 d	970.50 ± 43.60 d	127.02 ± 16.68 c	12.06 ± 0.26 c
	AM	315.67 ± 6.44 c	87.62 ± 7.35 c	27.89 ± 2.34 c	315.67 ± 6.44 d	0.88 ± 0.06 ab	1144.67 ± 28.98 d	1787.00 ± 17.90 c	137.35 ± 17.98 c	11.79 ± 1.04 c
Significance	AMF	**	*	*	**	**	**	**	**	**
	DS	**	**	**	**	ns	**	**	**	**
	AMF×DS	*	ns	ns	*	ns	**	ns	*	**

NAM, non-AMF-inoculated; AM, AMF-inoculated; WW, well-watered; MD, moderate drought; SD, severe drought; DS, drought stress; LF, length of fine roots (0 < d ≤ 0.5 mm); SAF, surface area of fine roots (0 < d ≤ 0.5 mm). Different lowercase letters within each column indicate significant differences ( $p < 0.05$ ) among treatments. Values are means ± SE ( $n = 3$ ). Two-way ANOVA output: ns, not significant; \*  $p < 0.05$ ; \*\*  $p < 0.01$ .



**Figure 7.** Effect of the arbuscular mycorrhizal fungus (AMF) *Rhizophagus intraradices* on the (A) nitrogen (N), (B) phosphorus (P) and (C) potassium (K) concentrations in *Catalpa bungei* seedlings under drought stress. NAM, non-AMF-inoculated; AM, AMF-inoculated; WW, well-watered; MD, moderate drought; SD, severe drought. Different lowercase letters above the bars indicate significant differences ( $p < 0.05$ ) among treatments. Values are means  $\pm$  SE ( $n = 3$ ).

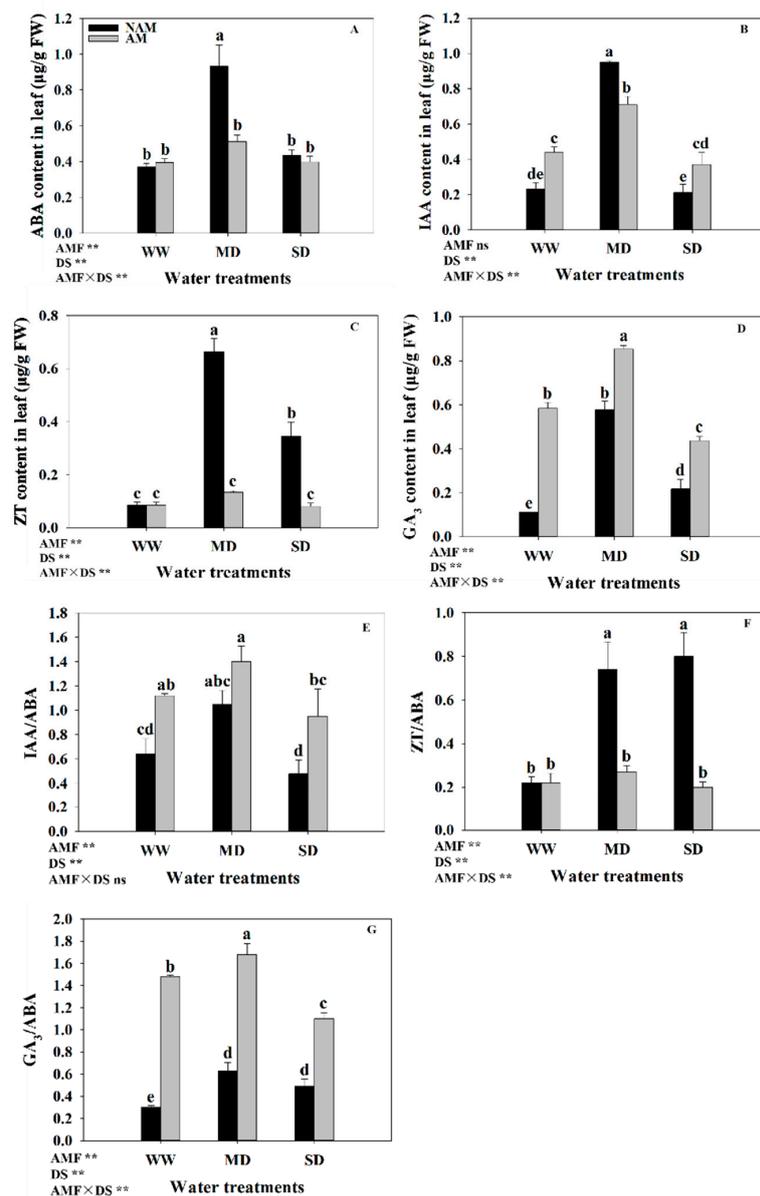
### 3.10. Phytohormones Levels

Phytohormone contents (ABA, IAA, ZT, GA<sub>3</sub>) and proportions (IAA/ABA, ZT/ABA, GA<sub>3</sub>/ABA) increased initially in the *C. bungei* leaves, after which they decreased with the aggravation of drought stress, reaching the maximum under MD stress (except ZT/ABA without inoculation), regardless of the AMF inoculation status (Figure 8). Under MD stress, the ABA, IAA, GA<sub>3</sub>, and ZT contents in non-mycorrhizal treated seedlings, and the IAA and GA<sub>3</sub> contents in mycorrhizal inoculated seedlings were significantly ( $p < 0.05$ ) higher than those under WW or SD conditions. In addition, under drought stress, the ZT/ABA and GA<sub>3</sub>/ABA ratios were significantly higher in the leaves of non-mycorrhizal treated seedlings than under WW conditions. These results showed that drought stress could stimulate the accumulation of hormones to a certain extent, could change their proportion, and had an overall greater impact on these growth regulators in the leaves of non-mycorrhizal rather than mycorrhizal inoculated seedlings. Under both WW and drought stress, the ABA and ZT contents, as well as the ZT/ABA ratio, were lower in the leaves of inoculated compared to the non-inoculated seedlings (Figure 8A,C,F). IAA and GA<sub>3</sub> contents, and the IAA/ABA and GA<sub>3</sub>/ABA ratios, on the other hand, were higher than those under control treatments (except the IAA content under MD stress) (Figure 8B,D,E,G). Thus, hormone contents and proportions showed significant differences between inoculated and control treatments ( $p < 0.05$ ) under drought stress.

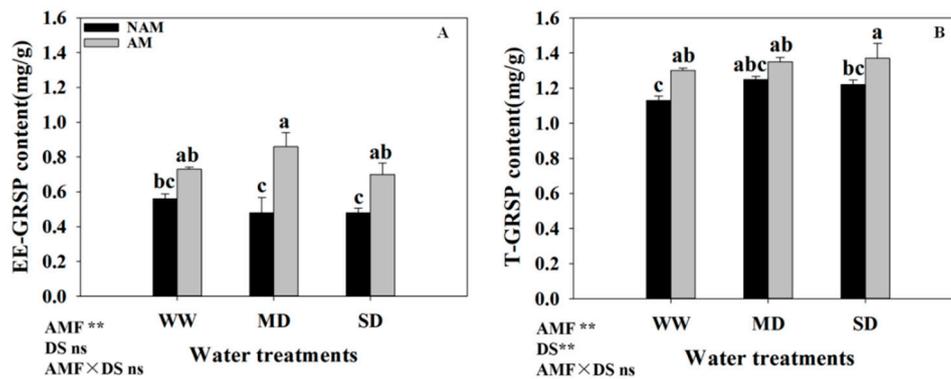
### 3.11. GRSP Content

Under drought stress, there was a slight decrease in the EE-GRSP content in the non-inoculated rhizosphere soil, while T-GRSP (both in inoculated and control treatments) and EE-GRSP (in the inoculated treatment) were higher than those under WW conditions (Figure 9). However, there was no significant difference in the EE-GRSP and T-GRSP contents among the three water treatments,

regardless of the inoculation status ( $p > 0.5$ ), which indicated that water stress had little effect on the GRSP content in the rhizosphere soil of *C. bungei*. Compared with the non-inoculated control, the EE-GRSP and T-GRSP contents were higher in the AMF-inoculated rhizosphere soils vs. the non-inoculated soils, under both WW and drought stress conditions (Figure 9). EE-GRSP content increased significantly—by 1.79-fold (in MD) and 1.46-fold (in SD), compared to the non-inoculated control—under drought stress, while the T-GRSP content increased significantly under WW and SD (1.15- and 1.12-fold that of control), respectively.



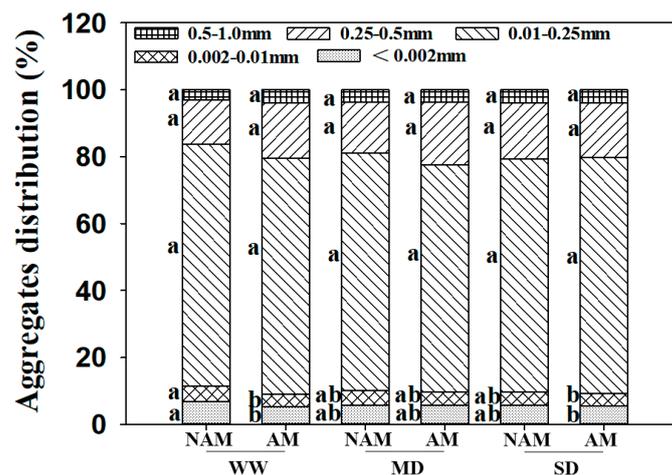
**Figure 8.** Effect of the arbuscular mycorrhizal fungus (AMF) *Rhizophagus intraradices* on the phytohormone content and ratio in *Catalpa bungei* seedlings under DS. (A), abscisic acid (ABA); (B), indole-3-acetic acid (IAA); (C), zeatin (ZT); (D), gibberellin (GA<sub>3</sub>); (E), IAA/ABA; (F), ZT/ABA; (G), GA<sub>3</sub>/ABA. NAM, non-AMF-inoculated; AM, AMF-inoculated; WW, well-watered; MD, moderate drought; SD, severe drought; DS, drought stress. Different lowercase letters above the bars indicate significant differences ( $p < 0.05$ ) among treatments. Values are means  $\pm$  SE ( $n = 3$ ). Two-way ANOVA output: ns, not significant; \*\*  $p < 0.01$ .



**Figure 9.** Effect of the arbuscular mycorrhizal fungus (AMF) *Rhizophagus intraradices* on the easily extractable glomalin-related soil protein (EE-GRSP) (A), and total glomalin-related soil protein (T-GRSP) (B) content in the rhizosphere soil under DS. NAM, non-AMF-inoculated; AM, AMF-inoculated; WW, well-watered; MD, moderate drought; SD, severe drought; DS, drought stress. Different lowercase letters above the bars indicate significant differences ( $p < 0.05$ ) among treatments. Values are means  $\pm$  SE ( $n = 3$ ). Two-way ANOVA output: ns, not significant; \*\*  $p < 0.01$ .

### 3.12. Soil Aggregates

Regardless of the AMF status, the soil particle size in the *C. bungei* rhizosphere was mainly distributed in the 0.01–0.25 mm range (micro-aggregates), accounting for 68%–72%, followed by 0.25–0.5 mm (macro-aggregates), 13%–18%, and 0.5–1.0 mm (macro-aggregates), which was the smallest proportion (Figure 10). With an increase in drought stress, the percentage of soil aggregates with different particle sizes was not significant in the rhizosphere of both inoculated and control treatments. Compared with the control treatment, AMF inoculation reduced the proportion of soil micro-aggregates (0–0.25 mm), but increased the proportion of macro-aggregates (0.25–0.50 mm) under both WW and MD stress (Figure 10), indicating that AMF can promote the formation of soil micro-aggregates into macro-aggregates to a certain extent.



**Figure 10.** Effect of the arbuscular mycorrhizal fungus (AMF) *Rhizophagus intraradices* on the distribution of soil aggregates under drought stress. NAM, non-AMF-inoculated; AM, AMF-inoculated; WW, well-watered; MD, moderate drought; SD, severe drought. Different lowercase letters above the bars indicate significant differences ( $p < 0.05$ ) among treatments. Values are means  $\pm$  SE ( $n = 5$ ).

### 3.13. Correlation Between AMF Colonization and Other Parameters

We carried out Pearson correlation analysis between AMF colonization and the other parameters to clarify the potential mechanisms surrounding mycorrhizal colonization and drought stress response. Results are presented in Table 5. Significant positive correlations were found between the AMF colonization and seedling growth parameters (except root biomass and SLW), root morphological parameters (except average root diameter), gas exchange parameters (Pn, Gs, WUE), photosynthetic pigment concentration, nutrient concentration (N in leaves and roots), and hormone levels (GA<sub>3</sub>) (*r* ranged from 0.695 to 0.962, *p* < 0.01, or 0.05). Positive correlations were also found with EE-GRSP, root biomass, N and P in stems, P and K in roots, IAA, ZT, and ABA in leaves, albeit not to a significant level. In contrast, significant negative correlations were found between AMF colonization and SLW, K concentration in stems, P and O<sub>2</sub><sup>-</sup> content in leaves, and H<sub>2</sub>O<sub>2</sub> content in roots (*r* in the range of -0.971 to -0.702, *p* < 0.01, or 0.05). Moreover, correlations with root/shoot ratio, average root diameter, leaf K and H<sub>2</sub>O<sub>2</sub> content, and root O<sub>2</sub><sup>-</sup> concentration were also negative, but these differences were not significant.

**Table 5.** Pearson correlation coefficients between AMF colonization and the other parameters.

Variable	AMF Colonization	Variable	AMF Colonization		
Growth parameters	Plant height	0.906 **	Total root length	0.828 **	
	Basal diameter	0.728 *	Surface area	0.912 **	
	Leaf area	0.917 **	Projected area	0.922 **	
	SLA	0.954 **	Root volume	0.851 **	
	SLW	-0.971 **	Root average diameter	-0.279	
	Total biomass	0.962 **	Branching number	0.946 **	
	Root biomass	0.463	Root tips	0.946 **	
	Stem biomass	0.783 *	LF	0.749 *	
Leaf biomass	0.922 **	SAF	0.848 **		
Gas exchange parameters	Pn	0.695 *	Biomass allocation	Root/shoot ratio	-0.658
	Gs	0.762 *	Nutrient absorption	Root N	0.910 **
	Ci	0.141		Stem N	0.495
	Tr	0.566		Leaf N	0.751 *
WUE	0.809 **	Root P		0.336	
Phytohormone	IAA	0.578	Stem P	0.626	
	GA <sub>3</sub>	0.747 *	Leaf P	-0.702 *	
	ZT	0.432	Root K	0.423	
	ABA	0.386	Stem K	-0.830 **	
GRSP	EE-GRSP	0.418	Reactive oxygen species	Root H <sub>2</sub> O <sub>2</sub>	-0.716 *
	T-GRSP	-0.013		Root O <sub>2</sub> <sup>-</sup>	-0.417
Soil aggregates	<0.002 mm	0.046	Photosynthetic pigments	Leaf H <sub>2</sub> O <sub>2</sub>	-0.270
	0.002–0.01 mm	0.099		Leaf O <sub>2</sub> <sup>-</sup>	-0.714*
	0.01–0.25 mm	0.132		Chlorophyll a	0.800 **
	0.25–0.5 mm	-0.122		Chlorophyll b	0.757 *
	0.5–1 mm	0.170	Carotenoid	0.910 **	

SLA, specific leaf area; SLW, specific leaf weight; Pn, net photosynthetic rate; Gs, stomatal conductance; Tr, transpiration rate; Ci, intercellular CO<sub>2</sub> concentration; WUE, Water use efficiency; IAA, indole-3-acetic acid; GA<sub>3</sub>, gibberellins; ZT, zeatin; ABA, abscisic acid; EE-GRSP, easily extractable glomalin-related soil protein; T-GRSP, total glomalin-related soil protein; LF, length of fine roots (0 < d ≤ 0.5 mm); SAF, surface area of fine roots (0 < d ≤ 0.5 mm); \* *p* < 0.05; \*\* *p* < 0.01.

#### 4. Discussion

AMF promotes the absorption of water and nutrients by plant roots and facilitates water and nutrient transport from the roots to the leaves, leading to enhanced photosynthetic rate and other gas exchange-related traits. Moreover, it improves plant nutrition by increasing the availability and affecting the distribution of nutrients, and promotes the growth of woody tree seedlings (height and basal diameter), biomass accumulation, and stress resistance [8,13,16,43].

The colonization rate of mycorrhizal fungi reflects the degree of infection and affinity between AMF and the host plant [6,44]. Under all water conditions, the mycorrhizal colonization rate was over 60%, indicating a relatively high affinity between the selected AMF and *C. bungei*. The degree of arbuscule staining was deeper under drought stress than the WW treatments, which could be due to the stimulation of branching or thickening of the arbuscular by the drought stress, resulting in morphological changes. Under SD stress, however, mycorrhizal colonization rate decreased significantly, which likely had an impact on the microbial activities, and affected the function of mycorrhizal symbionts to a certain extent. These findings are in line with those reported in *Cyclobalanopsis glauca* seedlings [45], black locust (*Robinia pseudoacacia* L.) [46], and trifoliolate orange (*Poncirus trifoliata*) [47]. This decrease may be due to the low carbon availability in the host plants under drought stress, or because drought stress could have inhibited spore germination and hyphal growth in the rhizosphere soil [1,45]. However, the response of different AMF to drought stress may be significantly different because of their diverse stress resistance. Chen et al. showed that the mycorrhizal colonization rate of *Amorpha fruticosa* by *Funneliformis mosseae* and *F. constrictum* was significantly higher under drought stress than in WW conditions [48]. In addition, previous studies on the hybrid poplar [49], *Astragalus adsurgens* Pall [50], and *Malus hupehensis* [3] reported that the AMF colonization rate was not affected by water deficit conditions. These phenomena indicate that the mycorrhizal colonization rate depends not only on the external environment factors, but also on the fungi and the colonized plant species [51].

Plant adaptability to the external environment and the influence of the environment on plant growth are to some extent reflected by biomass. Drought stress inhibits plant root extension along with stem and leaf growth, which ultimately results in a reduced biomass. Thus, biomass is the most intuitive index to assess plant performance under drought [52]. In this study, drought stress reduced the growth parameters (plant height, basal diameter, leaf area and biomass) of the *C. bungei* seedlings. However, colonization by AMF had positive effects on the growth parameters of *C. bungei* seedlings under both WW and drought stress. Similar effects were previously reported in other species; *Sophora davidii* [53], black locust [8], *Cyclobalanopsis glauca* [45] and Arizona cypress (*Cupressus arizonica* G.) [16]. We recorded significant positive correlations ( $p < 0.01$ ) between AMF colonization and morphological parameters, which corroborate the above effects. This growth benefit is likely due to the large extracellular hyphae AMF network, which transports water and nutrients to roots, increasing their absorption range and helping plants cope with the drought stress [8,52]. However, the relationship between AMF and plants under drought stress is very complex. Other studies found no detectable effect of AMF colonization [54,55], or even a negative effect on plant growth under drought stress [56], possibly for a variety of reasons, such as high root density, nutrient depletion, or insufficient light [57,58]. These indicate a myriad of interspecific differences between different AMF species and plants, and that their symbiosis may also be affected and regulated by environmental conditions [59].

Pronounced changes in the biomass allocation pattern could be interpreted as an adaptive strategy to overcome water and/or nutrient depletion more quickly [60,61]. In this study, water status significantly affected biomass allocation, regardless of the inoculation status. Under MD stress, *C. bungei* seedlings allocated more biomass to their roots, increasing their root/shoot ratio. This adaptive response indicates a preferential distribution of carbohydrates to the roots, probably to increase water absorption and to accelerate root growth under water scarcity. This survival strategy is complemented by a decrease in leaf area and the induction of leaf senescence and abscission, forming a drought resistance strategy that is consistent with results of previous studies [59,60]. However, inoculation with *R. intraradices* promoted biomass accumulation in the stems and leaves of *C. bungei* seedlings but inhibited it in the

root. Under the same drought stress level, both the biomass ratio of inoculated seedlings to roots and the root/shoot ratio were significantly lower than those in the non-inoculated seedlings. A negative correlation between AMF colonization and root/shoot ratio ( $r = -0.658$ ,  $p > 0.05$ ) in mycorrhizal inoculated seedlings was also observed. This indicated that AMF changed the biomass allocation in the seedlings. Under drought stress, roots of the symbiotic seedlings could absorb relatively large amounts of water or could allocate a relatively high proportion of carbohydrates for the aboveground growth, thus alleviating the restriction imposed by water scarcity on the growth of *C. bungei* seedlings. Similar results were also obtained for mycorrhizal inoculated *Broussonetia papyrifera* [60], *Bauhinia faberi* var. *microphylla* [4], *Retama monosperma*, and *Acacia gummifera* [58] under water deficit conditions. In this study, the biomass allocation pattern of non-mycorrhizal treated seedlings under WW conditions was consistent with that of inoculated seedlings under drought stress (leaf > root > stem), which was also the most direct evidence that AMF could promote the absorption of water in *C. bungei* seedlings and alleviate drought stress.

Stomata are channels for gas exchange and transpiration control between plants and the external environment. They effectively control water loss and CO<sub>2</sub> absorption [62]. Stomatal behavior is closely related to the soil and climate conditions of plants, especially water and nutrient status. Stomata function is the key to modulate transpiration and photosynthesis in leaves [63]. Both inoculated and non-inoculated *C. bungei* seedlings subjected to drought stress had strongly reduced Gs, and consequently reduced photosynthesis. Reducing Gs to decrease the transpiration-induced water loss is a key strategy for maintaining adequate levels of tissue hydration and cell expansion to sustain growth and biomass production, even under water deficit conditions [64,65]. Studies have shown the Gs, Tr, and Pn of mycorrhizal inoculated plants to be higher than those of non-mycorrhizal treated plants under water deficit [8,47]. In the current study, mycorrhizal inoculated seedlings displayed higher Pn, Gs, and Tr, but lower Ci concentrations under both WW and drought stress conditions than their non-mycorrhizal treated counterparts, which was similar in woody species, such as black locust [8], *P. trifoliata* [66], carob (*Ceratonia siliqua* L.) [65], and *M. hupehensis* [3]. Studies have also shown that by increasing leaf area and P content, to regulate the physiological status, AMF optimizes the photosynthetic rate in the host plants [12]. Our results of a higher leaf area and P content in the inoculated seedlings also confirmed this. On the other hand, the higher Pn of the inoculated plants under drought stress was also caused by the increase in Gs, indicating that the stomatal opening of inoculated plants was prolonged, to the benefit of both gas exchange and photosynthesis [15]. This was also indicated by the correlations found between Pn and Gs ( $r = 0.967$ ,  $p < 0.01$ ) in the present study (data not shown in table).

Leaf water status is an important feature for assessing the effects of AMF on drought stress, as it can provide information on how to evaluate the physiological and biochemical changes in plants [67]. A higher WUE is conducive to moving water to the evaporating surface and keeping leaf stomata open [8]. Therefore, a higher WUE in the mycorrhizal inoculated plants under drought stress is an indicator of their higher photosynthetic performance [65]. This was also confirmed by our results. Indeed, AMF improved WUE of the *C. bungei* seedlings, irrespective of water status. A positive and highly significant correlation ( $r = 0.809$ ,  $p < 0.01$ ) was also evident between AMF colonization and WUE. Similarly, inoculation with AMF enhanced WUE in *S. davidii* [53], black locust [8] and hybrid poplar [49] under drought stress conditions. The positive effects of AMF on WUE can be attributed to the absorption of water and nutrients from the soil by the external hyphae, the regulation of Gs through hormonal signals, optimization of osmotic regulation, or the enhancement of root hydraulic conductivity [8,68,69]. However, contrary to the results of most studies, Doubková et al. [70] documented that inoculation with AMF in *Knautia arvensis* had no positive effect on WUE. In fact, the WUE of mycorrhizal inoculated plants was lower than that of the non-mycorrhizal treated plants under WW conditions. This possible discrepancy was explained by larger root systems connected to the extraradical hyphal network, that made the use of soil water more efficient.

Chlorophyll is one of the most important pigments in higher plants. It is crucial for photosynthesis, through which plants obtain energy from light [3,71]. Therefore, chlorophyll content is an important index for the evaluation of photosynthetic intensity, efficiency, and environmental stress [8]. Several studies have shown that inoculation with AMF can notably increase the chlorophyll content of plants under drought stress [22,46,47,72]. Similarly, our study also shows that inoculation with AMF increased the chlorophyll a, chlorophyll b, and carotenoid concentrations, and enhanced the utilization of light energy in the leaves of *C. bungei*. We found significant positive correlations between AMF colonization and chlorophyll a ( $r = 0.800, p < 0.01$ ), chlorophyll b ( $r = 0.757, p < 0.05$ ), and carotenoids ( $r = 0.757, p < 0.05$ ), which further confirmed the beneficial role of mycorrhizal fungi on the photosynthetic pigments. The increase in chlorophyll content may be related to the increase in P and Mg uptake by AMF [71]. Indeed, AMF promoted the absorption of P in leaves also in our study. Moreover, increased chlorophyll and gas exchange parameters indicate that AMF increased photosynthetic carbon (C) fixation, which would not only stimulate AMF growth, but would also increase the photosynthetic rate because of the reciprocal C–P relationship between AMF and their host plants [71].

SLA is the main leaf functional trait connected with the nutrient retention capacity, which reflects the plant's ability to exploit and utilize the environmental resources [64,73]. SLA directly affects the assimilation of CO<sub>2</sub> and the light, water, and nutrient utilization efficiency [64]. Our results showed that seedlings inoculated with *R. intraradices* had markedly increased SLA and decreased SLW under both WW and drought stress conditions. Moreover, the correlation analysis revealed a significant positive relationship between AMF colonization and SLA ( $r = 0.954, p < 0.01$ ) and Pn ( $r = 0.695, p < 0.05$ ). Increased SLA enhances the photosynthetic area and thus the leaf photosynthetic rate, which is conducive to the accumulation of photosynthetic products [61]. Similar results were also found in *Cynophalla flexuosa* [64] and wheat (*Triticum* spp.) [74], confirming that mycorrhizal inoculated plants are beneficial for the adaptation to arid environments. In addition, SLA is also related to photosynthetic N allocation, meaning that a higher SLA would enable a higher N allocation ratio to photosynthesis [75]. A significantly positive correlation ( $r = 0.882, p < 0.01$ ) between SLA and leaf N concentration was also confirmed by our results (data not shown in table). Therefore, a high SLA may help plants increase their ability to acquire available resources for better growth and reproduction [75]. In contrast, Hernández-Ortega et al. [76] reported that *Melilotus albus* inoculated with the *Glomus* Zac-19 AMF-inoculum had lower SLA on diesel-contaminated substrate. Busquets et al. [77] found that the SLW of *Anthyllis cytisoides* inoculated with *G. mosseae* was significantly higher than that of the non-inoculated plants following drought. This may be due to an improved cell wall resistance and leaf thickening (typical xerophytic characteristics), as a result of the symbiotic relationships [73,77].

In plants, ROS production and removal (e.g., O<sub>2</sub><sup>•−</sup> and H<sub>2</sub>O<sub>2</sub>) are in a dynamic equilibrium state to maintain normal physiological and metabolic functions [78]. When plants are subjected to drought stress, this balance is disrupted, leading to an overproduction of ROS that will result in oxidative stress and consequently membrane lipid peroxidation, protein denaturation, nucleic acid chain fracture, cell membrane damage, and even cell death in severe cases [6,15,79]. Avoidance of oxidative stress by preventing ROS accumulation is the most effective way for mycorrhizal inoculated plants to cope with drought stress [80]. The extraradical hyphae of mycorrhizal fungi may be involved in H<sub>2</sub>O<sub>2</sub> efflux because mycorrhizal hyphae have functional aquaporins that can transport H<sub>2</sub>O and H<sub>2</sub>O<sub>2</sub> [7]. Here, we demonstrated that drought stress induced the accumulation of H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>•−</sup> in *C. bungei* seedlings, irrespective of their AMF status. However, AMF inoculation decreased ROS formation, especially in leaves, under different watering regimes. In addition, AMF colonization showed a significant negative correlation with leaf O<sub>2</sub><sup>•−</sup> ( $r = -0.714, p < 0.05$ ) and root H<sub>2</sub>O<sub>2</sub> content ( $r = -0.716, p < 0.05$ ) in mycorrhizal inoculated seedlings. These results are similar to those previously reported in black locust [46], *C. arizonica* [16] and *M. hupehensis* [3], indicating that mycorrhizal fungi could mitigate drought-induced oxidative stress in plants by reducing the production of ROS, and thus can maintain the integrity of cell membranes and stabilize proteins under drought stress [15,80]. Low ROS

accumulation in the mycorrhizal inoculated seedlings may be related to an intensive hyphal and arbuscular growth within the roots [46].

Plant roots not only provide physical support for plants but are also important in providing the material basis for photosynthesis (water and nutrient absorption). Root development directly affects plant growth, performance, and survival [4]. It has been confirmed that AMF inoculation can significantly affect the host root architecture and promote plant growth under drought stress [7,19]. Our results showed significantly higher root length, total surface area, projected area, volume, tips, branching number, length, and surface area of fine roots in the AMF-inoculated *C. bungei* seedlings under WW conditions. These morphological parameters were also improved under drought stress when compared with the non-AMF seedlings. This is consistent with previous reports from *Prunus cerasifera* L. [81], *P. trifoliata* [82], *Amygdalus pedunculata* [83], and *Acacia seyal* Del. seedlings [84]. AMF colonization was positively correlated ( $p < 0.01$ ) with all root morphological parameters (except average root diameter), indicating that likely due to a better root morphology, mycorrhizal inoculated plants have a stronger capacity to adapt to drier soils than their non-mycorrhizal treated counterparts.

Although AMF can affect root plasticity in a number of ways, the most common one is to increase root branching and to generate a larger proportion of roots with small diameters [81,85]. An increased root branching caused by mycorrhizal symbiosis resulted in the enhancement of root hairy zones, which are the main sites for P acquisition [85]. This was also supported by our findings since mycorrhizal symbiosis significantly increased P uptake and root branching in the *C. bungei* seedlings. The average root diameter is a parameter that reflects the absorption efficiency of the root system, and to a certain extent reflects the soil volume that can be occupied by the root system per unit biomass [60]. In this study, under the same water conditions, the average root diameter of the inoculated seedlings was lower than that of the non-inoculated ones. This is similar to the results of Zhang et al. [86] in *Cyclobalanopsis glauca* seedlings inoculated with AMF, indicating that AMF inoculation can induce inoculated plant roots to become smaller, thus making it easier for the roots to absorb water and nutrients. The above results indicate that AMF can effectively regulate the root morphological parameters of the host plants to improve the absorption of water and nutrients, thereby promoting growth, root development and stress resistance. However, some studies have suggested that inoculation had no detectable effect on the root morphology of *Bidens frondosa* L., such as root length, volume, or surface area, under three levels of water availability [55]. This may be possible, as the regulation of AMF on plant root morphology is dependent on nutrient and water availability, the AMF species, and the host species itself [81,86,87].

As basic macronutrients, N, P and K play important roles in plant growth and metabolism [88]. Nutrient distribution and accumulation are results of the interaction between plant and environmental factors and reflect not only the adaptability of plants to environmental factors, but also the impact of the environment on plants [89]. Previous studies suggested that the promotion of nutrient uptake by AMF is an important physiological mechanism of the mycorrhiza-induced drought resistance of the host plant [2,7]. Yang et al. [8] discovered that black locust seedlings inoculated with *F. mosseae* and *R. intraradices* had enhanced leaf C, N, and P concentrations under both WW and drought stress conditions. Zhang et al. [90] reported that inoculation with *A. laevis*, *G. mosseae*, and *G. caledonium* increased N, P, K, calcium (Ca), Mg, zinc (Zn), and copper (Cu) uptake in loquat (*Eriobotrya japonica* Lindl.) seedlings. Zarik et al. [91] conducted similar research on *Cupressus atlantica*, and the results suggested that inoculation with AMF significantly improved the uptake of minerals independently of water regimes. A recent study by Al-Arjani et al. [22] also found that under drought stress, mycorrhizal inoculated *Ephedra foliata* seedlings had higher uptake of essential nutrients, such as K, Mg, and Ca, than the non-inoculated seedlings. In the present study, AMF inoculation increased the N and P concentrations in roots, stems, and leaves, and K concentration in the leaves of *C. bungei*. These data suggest that the higher drought resistance of *C. bungei* seedlings following AMF inoculation could partly be attributed to the improved nutrient uptake by AMF, as nutrient deficiency would greatly

contribute to stress. Moreover, the enhanced drought resistance is likely connected to the improved photosynthetic performance of *C. bungei* by the AMF inoculation.

Phytohormones, including ABA, GA<sub>3</sub>, CTKs, and IAA, are known to act as signaling molecules and to be involved in various developmental processes of the mycorrhizal symbiosis [10,92–94]. AMF could improve drought resistance by regulating endogenous hormones after symbiosis with host plants [6,7]. Typically, ABA is the most involved hormone in the drought responses, mainly because stomatal behavior is closely related to changes in the ABA levels [66,94,95]. According to Ouledali et al. [94], ABA content increased in the leaves of AMF-inoculated olive tree (*Olea europaea* L.) seedlings under MD stress, but decreased significantly under SD stress, compared with that in the non-inoculated seedlings. Some studies suggested ABA content to be significantly higher in drought-stressed mycorrhizal inoculated plants than in non-mycorrhizal treated plants [19,66,96]. Their view was that plants respond to drought by synthesizing ABA to promote stomatal closure, thereby reducing water loss. However, contrary to their findings, our study showed that AMF-inoculated *C. bungei* seedlings exhibited considerably lower ABA levels in leaves under drought stress, which supports other findings [18,97]. This apparent discrepancy might be because of the delayed stomatal closure in mycorrhizal inoculated seedlings by the reduction of ABA levels, which improved the gas exchange capacity and enhanced the photosynthetic effect. Our previous results also supported the hypothesis that seedlings inoculated with AMF had higher Gs and Pn than non-inoculated seedlings, regardless of the water status.

In addition, CTKs may also affect leaf senescence and nutrient mobilization [98]. These hormones, alone or in combination, regulate plant growth and development [22,66]. Our results indicated that inoculation with AMF considerably augmented IAA (with the exception of MD stress) and GA<sub>3</sub> levels, but reduced zeatin (ZT, a cytokinin) in the leaves of *C. bungei* seedlings, exposed either to WW or drought stress conditions. This is in accordance with previous findings [22,66,99]. It implies that AMF could postpone leaf senescence and cause a quick adaptation to drought stress by influencing the auxin signal transduction. Regardless of the water balance, AMF inoculation significantly increased the IAA/IBA and GA<sub>3</sub>/ABA values in the leaves of *C. bungei*, indicating that the positive effects of *C. bungei* on growth were strengthened under drought stress. As proposed by Al-Arjani et al. [22], the decrease in GA<sub>3</sub> levels and the increase in ABA levels are supposed to be the key determinants of stress tolerance. However, in our study, the opposite results were obtained. This, however, should not be alarming, as plants inoculated with AMF had a disposition to maintain both growth promotion and stress adaptation, and the final result of the hormone interaction seemed to have a minimal impact on cell metabolism and the increased stress resistance [100]. Such changes in phytohormones by AMF may provide important clues to improve the drought resistance of the host plants.

AMF hyphae and spores release GRSP, a type of iron-containing, heat-stable glycoprotein [7,11] while growing with the root system. GRSP is an important binding agent for the formation of soil aggregates [50,101]. It is abundant in soils, usually in the range of several to 10 mg·g<sup>-1</sup> soil, which helps combine tiny particles into small aggregates of different sizes [11]. In addition, to ensure the transportation of water into the mycelium, it can seal the AMF extraradical hyphae in the rhizosphere soil [7], improve soil environment (enter the soil along with the degradation of fungal biomass), which can enhance soil stability, porous structure and moisture penetration, and provide necessary space and improved gas exchange channels for plant root growth [41,50]. Most studies suggest that T-GRSP is a stable glycoprotein accumulated by AMF over a long period of time, while EE-GRSP is a newly produced glycoprotein [102]. In this study, we found no detectable changes in the EE-GRSP and T-GRSP levels in the rhizosphere soil without AMF inoculation upon the aggravation of drought stress. This may be due to the fact that the sterilized substrate was mixed evenly, and AMF colonization did not occur, therefore, no new GRSP could be produced. After inoculation, EE-GRSP and T-GRSP increased under MD stress, indicating that a certain degree of drought stress could promote the production of GRSP, and this increase in the EE-GRSP further led to an increase in T-GRSP.

Wu [103] investigated GRSP in the rhizosphere of pot-cultured *P. trifoliata* seedlings (without AMF inoculation) and concluded that there was no GRSP without AMF colonization. However, our results

showed the presence of GRSP (both EE-GRSP and T-GRSP) in sterilized, non-inoculated soil. This may be because the GRSP having been present in the rhizosphere soil before sterilization, which was also confirmed by Bedini et al. [41]. Indeed, GRSP is very stable in nature and highly persistent in the soil [7,11]. In the current study, under the same water regime, the EE-GRSP and T-GRSP contents increased significantly in the rhizosphere soils inoculated with AMF, compared to those without inoculation, which was in accordance with the results of Ji et al. [50], who reported a significant increase in the GRSP content in the rhizosphere of *A. adsurgens* Pall., inoculated with AMF, under both WW and drought stress conditions. AMF also elevated GRSP content in the rhizosphere soil of black locust [104]. GRSP concentration in potted soil was significantly higher after inoculation than the initial value without inoculation, indicating a causal relationship between mycorrhizal symbiosis and GRSP content. Our results provide strong evidence for the differences in GRSP production between mycorrhizal and non-mycorrhizal treated plants.

Soil structure refers to the grouping of soil particles into porous compounds, also known as aggregates. Particle size distribution and the stability of soil aggregates impacts not only soil pore distribution but is also related to the movement and storage of water [104,105]. In addition to improving soil structure by producing GRSP, the extraradical mycelia of AMF can directly create the skeletal structure of soil aggregates by physically entangling soil particles, and then form micro-aggregates. Finally, hyphae and the roots can combine these smaller micro-aggregates (<0.25 mm) to form larger and more stable macro-aggregates (>0.25 mm) [101,104–106]. Due to their better air and water permeability and stability towards wind and water erosion, well-aggregated soils are conducive to plant and microbial growth [11]. Zhang et al. [104] reported that soil aggregate stability parameters, such as water stable aggregates, geometric mean diameter, and mean weight diameter were elevated by AMF inoculation in the rhizosphere of black locust. Wu et al. [105] showed that mycorrhizas-enhanced > 2, 1–2, and > 0.25 mm water-stable aggregate fractions in the rhizosphere soil of *P. trifoliata* seedlings. In the current study, AMF inoculation reduced the proportion of soil micro-aggregates (<0.002, 0.002–0.01, and 0.01–0.25 mm, respectively) and increased the proportion of macro-aggregates (0.25–0.5 mm) under the same water conditions, compared with the control treatments. These indicate that AMF inoculation improved soil structure and maintained soil permeability and water-holding capacity by consolidating small micro-aggregates into large aggregates. We therefore conclude that beyond a direct mineral nutritional effect, AMF colonization—through the effects of GRSP production and hyphal entanglement on soil aggregates—promoted the growth of *C. bungei* seedlings also indirectly, under drought stress. Although the results obtained in pot experiments cannot represent field conditions, microscopic approaches are helpful to better understand the causal relationship between AMF symbiosis and soil quality. Our findings underscore the link between GRSP and AMF and add new proof to their ability to enhance soil aggregate stability.

## 5. Conclusions

This study demonstrated that inoculation of *R. intraradices* significantly ameliorated the growth of *C. bungei* seedlings under drought conditions. AMF could enhance photosynthesis and WUE (by increasing leaf area), SLA and photosynthetic pigment concentration, change the biomass allocation in seedlings (by reducing RMR and root/shoot ratio) and promote the growth of aboveground parts (leaves and stems). Moreover, mycorrhizal treatment alleviated drought-induced oxidative stress in plants by reducing the excess generation of ROS (especially  $H_2O_2$  and  $O_2^-$  in leaves). Inoculation with AMF under drought stress dramatically augmented IAA and  $GA_3$  levels, but reduced ABA, and ZT levels in the leaves. Under drought stress, *R. intraradices* could form a good symbiotic relationship with *C. bungei* seedlings, and AMF symbiosis indeed improved root morphology and structure, and promoted the absorption of N and P in seedlings, which was conducive to plant adaptation to a dry environment. Furthermore, by increasing the GRSP content and proportion of macro-aggregates (0.25–0.5 mm) in the rhizosphere soil, AMF improved soil structure. Thus, it is feasible to use mycorrhizal biotechnology for vegetation restoration and reconstruction in arid and semi-arid areas.

This article mainly involved physiological and biochemical processes; however, we cannot ignore that the genetics of the AMF samples chosen to treat plants with could have a significant impact on the outcome of the interaction, which should be regarded as another area of uncertainty and future focus. The molecular mechanism of AMF improving drought resistance of *C. bungei* also needs to be further explored.

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