

Article

# Arbuscular Mycorrhizal Fungus Improves Rhizobium–*Glycyrrhiza* Seedling Symbiosis under Drought Stress

Zhipeng Hao <sup>1,†</sup> , Wei Xie <sup>1,2,†</sup>, Xuelian Jiang <sup>1</sup>, Zhaoxiang Wu <sup>1,3</sup>, Xin Zhang <sup>1</sup> and Baodong Chen <sup>1,2,\*</sup>

<sup>1</sup> State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China; zphao@rcees.ac.cn (Z.H.); xieweibisheng@yeah.net (W.X.); jiangxuelian0518@126.com (X.J.); wuzhaoxiang2004@126.com (Z.W.); xinzhang@rcees.ac.cn (X.Z.)

<sup>2</sup> University of Chinese Academy of Sciences, Beijing 100049, China

<sup>3</sup> Jiangxi Engineering and Technology Research Center for Ecological Remediation of Heavy metal pollution, Institute of Biology and Resources, Jiangxi Academy of Sciences, Nanchang 330096, China

\* Correspondence: bdchen@rcees.ac.cn; Tel.: +86-10-62849068

† These authors contributed equally to this work.

Received: 14 August 2019; Accepted: 20 September 2019; Published: 23 September 2019



**Abstract:** Rhizobia and arbuscular mycorrhizal (AM) fungi can potentially alleviate the abiotic stress on the legume *Glycyrrhiza* (licorice), while the potential benefits these symbiotic microbes offer to their host plant are strongly influenced by environmental factors. A greenhouse pot experiment was conducted to investigate the effects of single and combined inoculation with a rhizobium *Mesorhizobium tianshanense* Chen and an AM fungus *Rhizophagus irregularis* Walker & Schuessler on *Glycyrrhiza uralensis* Fisch. seedling performance under different water regimes. Drought stress inhibited rhizobium nodulation but increased mycorrhizal colonization. Furthermore, co-inoculation of rhizobium and AM fungus favored nodulation under both well-watered and drought stress conditions. *Glycyrrhiza* seedling growth showed a high mycorrhizal dependency. The seedlings showed a negative growth dependency to rhizobium under well-watered conditions but showed a positive response under drought stress. *R. irregularis*-inoculated plants showed a much higher stress tolerance index (STI) value than *M. tianshanense*-inoculated plants. STI value was more pronounced when plants were co-inoculated with *R. irregularis* and *M. tianshanense* compared with single-inoculated plants. Plant nitrogen concentration and contents were significantly influenced by inoculation treatments and water regimes. *R. irregularis* inoculation significantly increased plant shoot and root phosphorus contents. AM fungus inoculation could improve *Glycyrrhiza* plant–rhizobium symbiosis under drought stress, thereby suggesting that tripartite symbiotic relationships were more effective for promoting plant growth and enhancing drought tolerance.

**Keywords:** *Rhizophagus irregularis*; rhizobium; licorice; drought tolerance; nitrogen; phosphorus

## 1. Introduction

The legume *Glycyrrhiza* (licorice) is widely used as a medicinal herb and as an industry material due to the large amount of glycyrrhizin, an important bioactive triterpenoid saponin derived from licorice roots and stolons [1,2]. *Glycyrrhiza* plants are also cultivated to restore degraded ecosystems, particularly in arid and semi-arid regions [3]. However, drought stress and nutrient deficiency are the two main factors limiting the growth and production of *Glycyrrhiza* seedling cultivation [4]. Nevertheless, plants have evolved a series of mechanisms to cope with these stresses. For example,

the mutualism of plant and microbe could affect plant growth, nutrient uptake, and resistance to abiotic and biotic stresses, including drought stress [5].

Rhizobia and arbuscular mycorrhizal (AM) fungi could form symbiosis with legumes and improve plant mineral nutrition, especially nitrogen (N) and phosphorus (P) nutrition [6]. Inoculation with *Mesorhizobium tianshanense* Chen, a rhizobium widely found in dry soil which acts as a nitrogen-fixing symbiotic microbe in at least eight different plant species, significantly increased the plant biomass of *G. uralensis* Fisch. [7]. Inoculation with AM fungus *Glomus mosseae* Gerd. & Trappe or *G. versiforme* S.M. Berch improved the root growth of *G. uralensis* [8]. Moreover, AM inoculation can also increase the glycyrrhizin accumulation of *Glycyrrhiza* plants [3,9,10]. AM fungi and rhizobia are morphologically and physiologically different, so their responses also vary under stress conditions. However, there is no information available on the effect of combined inoculation with rhizobia and AM fungi on *Glycyrrhiza* plants grown under drought stress.

Symbiosis with rhizobia and/or AM fungi improved plant growth and increased the biomass of many legumes even under drought stress [11,12]. The benefits of AM fungi to the legume hosts may complement those of rhizobia, and vice versa. Rhizobia and AM fungi are known to interact either at the colonization stages or at the symbiotic functional procedures [13], and several common plant genes required for early stages of both rhizobial and mycorrhizal symbioses have been identified [14]. Increasing the acquisition of limited phosphorus and micronutrients in mycorrhizal plants may facilitate colonization of rhizobia in the host, because phosphorus has greater priority in nodules than in other plant organs [15–17]. These nitrogen-fixing bacteria may also improve eco-physiological relationships in mycorrhizal symbiosis formation and function [18]. However, the benefit of interspecific symbiosis to host plants could shift to a negative effect depending on nutrient availability and stress level [19]. Both AM fungi and nitrogen-fixing bacteria are heterotrophic and receive carbon from host plants [20,21]. However, the interests of symbiotic interacting partners hardly match because of an imbalance in the traded resources. AM fungi inoculation could suppress the host plant's growth under high soil phosphorus conditions [22], and rhizobia exhibiting low efficiency or even ineffective N<sub>2</sub>-fixation has also been recognized in agricultural soil [23]. The microbial function can be defined as the net benefit of the symbiosis to the associated organisms. Therefore, the negative plant growth outcomes showed that the costs for maintaining symbiosis may counteract the benefits [24,25].

The multiple symbiosis of AM fungus and rhizobium may have positive and negative interactions on plant growth under different water regimes [26]. Plants experiencing microbially induced growth suppression might acquire the majority of necessary nutrients from their symbiotic partners [27]. However, the influence of drought stress on shift of positive–negative microbial dependence is still unclear. An intriguing hypothesis is that the plant growth suppression observed in some microbial symbioses could be compensated by other partner in the multiple symbioses. In the present study, *G. uralensis* seedlings were used to test the interactive effect of AM fungus and rhizobium inoculation on plant growth, mineral nutrition, and drought resistance. The microbial dependency and drought resistance index were calculated to illustrate the microbial functions.

## 2. Materials and Methods

### 2.1. Soil Characteristics

The soil used for the experiment was a calcareous loamy soil collected from Duolun Restoration Ecology Research Station (116°17'29''E, 42°02'20''N). The soil is classified as Haplic Calcisol (FAO (Food and Agriculture Organization of the United Nations) classification) with a pH of 7.02 (1:2.5 soil to water ratio), organic matter content of 30.4 g/kg, total N of 2.4 mg/kg and extractable P (0.5 M NaHCO<sub>3</sub>, pH 8.5) content of 6.73 mg/kg. The soil was air-dried, ground to pass a 2-mm sieve, mixed with quartz sand (<1 mm, 1:1 soil to sand ratio (v/v)) as growth medium and sterilized by  $\gamma$ -irradiation (25 kGy, Institute of Atomic Energy, Chinese Academy of Agricultural Sciences).

## 2.2. Plant Materials

Seeds of *G. uralensis* Fisch. were provided by Chinese Materia Medica Resources Center, China Academy of Chinese Medical Sciences. The seeds were immersed in H<sub>2</sub>SO<sub>4</sub> (50%) for 30 min to break the thick seed coat, washed thoroughly (>3 times) with sterilized distilled water, and then surface sterilized with 10% H<sub>2</sub>O<sub>2</sub> for 10 min. The selected homogeneous seeds were germinated on filter paper soaked with distilled water in Petri dishes at 27 °C in the dark for 2 days in a growth chamber.

## 2.3. Microbial Inoculation

AM fungus *Rhizophagus irregularis* Walker & Schuessler (syn. *Glomus intraradices* Schenck & Smith BGC AH01) was provided by Beijing Academy of Agriculture and Forestry Sciences, China. Mycorrhizal inoculum consisting of spores (78 spores per gram), hyphae, and infected root fragments were used at a 1:10 (v/v) inoculum to growth medium ratio. The inoculum was added to the top 3 cm of the growth medium (i.e., the mycorrhizal inoculum layer) at sowing time just below the seeds for mycorrhizal treatments. Non-inoculated treatment received the same amount of sterilized inoculum together with a 10 mL aliquot of a filtrate (<20 µm) of the inoculum to provide a similar microbial population that was free from AM propagules.

A moderately growing rhizobium *M. tianshanense* Chen (strain CCBAU3306), originally isolated from the *Glycyrrhiza* plant's rhizosphere soil [7], was provided by the Culture Collection of Chinese Agricultural University, Beijing, China. The strain was grown at 28 °C in TY medium (5 g/L tryptone, 3 g/L yeast extract, 9 mmol/L CaCl<sub>2</sub>) and added close to tap roots (10 mL containing 10<sup>7</sup> cells per pot) 3 weeks after sowing to establish the bacterial treatment. Non-inoculated treatment received 10 mL autoclaved (121 °C, 45 min) microbial suspension.

## 2.4. Experimental Design and Growth Conditions

Germinated seeds were sown in 400 mL of growth medium mixed with mycorrhizal inoculum in 0.5 L pots. Before water treatment, all the pots were maintained under well-watered conditions (well-watered, 12% relative water content equating to 55% field water capacity). At 14 weeks after sowing, the water regime was unified for two groups, namely, well-watered and drought stress (drought, 8% relative water content equating to 40% field water capacity). During the first 3 days of the drought period, the drought stress treatments received only 20% of the water consumption to avoid water heterogeneity in soil. The water loss, determined based on container and plant weight, was daily supplemented with deionized water to maintain the desired moisture content. The plants were harvested after 4 weeks of drought stress. All the plants were in vegetative growth during the whole experimental period, and no difference in phenological stages among the treatments was observed.

The experiment was set up in a three-factor randomized complete block design. Experimental treatments included the following: (1) non-AM fungus/rhizobium inoculation as control; (2) inoculated with AM fungus; (3) inoculated with rhizobium; and (4) co-inoculated with AM fungus and rhizobium. All the treatments were cultivated under well-watered and drought stress conditions. There were eight treatments (full combinations of inoculation status and water regimes) with four replicates, giving a total of 32 pots. The experiment was carried out under greenhouse conditions (15–25 °C, 16 h photoperiod with supplementary lighting of 700 µmol m<sup>-2</sup> s<sup>-1</sup>).

## 2.5. Parameter Measurements

Plant roots and shoots were separately harvested. Roots were carefully washed with deionized water, and the nodules were separated. Fresh weight of shoots, roots and total nodules were recorded. The number of nodules were estimated by directly counting. Subsamples of fresh roots (0.5 g) were stored in a 4 °C refrigerator for measuring mycorrhizal colonization. The rest of the roots, shoots, and nodules were dried at 80 °C for 48 h for dry biomass and nutrient content analyses.

The percentage of mycorrhizal colonization was estimated by visual observation after clearing with 10% KOH and staining with 0.05% trypan blue in lactic acid (v/v) [28]. Microscopic quantification was performed using MYCOCALC program [29].

Dry samples of shoots and roots were milled to a fine powder, and N concentration was directly determined by using an element analyzer (Vario EL, Elementar, Hanau, Germany). To determine P concentrations in shoots and roots, plant subsamples (0.2 g) were digested by 10 mL HNO<sub>3</sub> using a microwave accelerated reduction system (Mars 5, CEM Co., Ltd., Matthews, NC, USA) and analyzed by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES, Vista axial, Varian). Plant nutrient contents refer to total P or N contained within the shoot or the root.

The microbial dependency of inoculation treatment was calculated according to van der Heijden [30] as follows. If biomass of  $\sum_1^n a_n > bn$ , then microbial dependency =  $(1 - (bn / (\sum_1^n a_n)) \times 100$ . If biomass of  $\sum_1^n a_n < bn$ , then microbial dependency =  $(-1 + ((\sum_1^n a_n) / bn) \times 100$ , where  $a$  is the plant dry weight of a treatment inoculated with microbe,  $n$  is the number of treatments where plants were inoculated with microbe and “ $b$ ” is the plant dry mass of the non-inoculated treatments. These equations ensured that positive and negative values for microbial dependency were comparable and symmetrical.

Drought stress index as stress tolerance index (STI) was calculated using the following formula:  $STI = B_c \times B_s / M_c^2$ , where  $B_c$  and  $B_s$  were the plant biomass under control and stress conditions, respectively, and  $M_c$  was the mean biomass over all plants under control condition [31].

## 2.6. Statistical Analysis

Data were shown as the mean  $\pm$  standard error (SE) of independent replicates except for microbial dependency and STI, which were calculated from the mean value of the different treatments. The data were checked using the Shapiro–Wilk test for normality and homogeneity of variance before performing the analysis of variance (ANOVA) using SPSS (version 16.0, SPSS Inc. Chicago, IL, USA). For the percentage values of mycorrhizal colonization and arbuscule abundance, the data were transformed into arcsine square before statistical analysis. One-way ANOVA followed by Duncan’s multiple-range test was first performed with treatment as the main factor. Then, a three-way ANOVA was performed to examine the significance of treatment effects and their interactions on the observed parameters using SPSS (version 16.0, SPSS Inc., Chicago, IL, USA).

## 3. Results

### 3.1. Mycorrhizal Colonization and Nodulation

Non-inoculated plants showed no AM fungal colonization or nodulation on their root systems (Table 1). *R. irregularis* inoculated plants showed mycorrhizal colonization, which was significantly increased by 8.7% and 33.2% under drought stress compared with the well-watered conditions in plants inoculated without and with *M. tianshanense*, respectively. *M. tianshanense* inoculation showed a limited effect on root mycorrhizal development because of the reduction of the arbuscular abundance observed in the plants inoculated with *M. tianshanense* compared with that of the non-inoculated control under the well-watered conditions.

A three-way interaction was observed between water regimes, AM fungus, and rhizobium inoculation for nodule numbers and nodule dry weight. The co-inoculation of *M. tianshanense* with mycorrhizal fungus favored the nodulation with a nodule number that is 6.1-fold and nodule dry weight that is 6.4-fold higher compared with single inoculation with *M. tianshanense* under well-watered conditions. Although *M. tianshanense* nodulation was significantly decreased by drought stress, the co-inoculation with *R. irregularis* increased nodule number by 12.7-fold and nodule dry weight by 11.6-fold compared with the *M. tianshanense* single inoculation under drought stress.

**Table 1.** Mycorrhizal colonization and nodulation of plants inoculated with/without arbuscular mycorrhizal (AM) fungus *Rhizophagus irregularis* and rhizobium *Mesorhizobium tianshanense* under well-watered and drought stress conditions (mean ± SE, n = 4). C represents non-inoculation control, F represents inoculation with *R. irregularis*, B represents inoculation with *M. tianshanense* treatments respectively, and FB represents the co-inoculation treatments. Data of columns indexed by the same letter are not significantly different at p < 0.05. ns, not significant; \* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001.

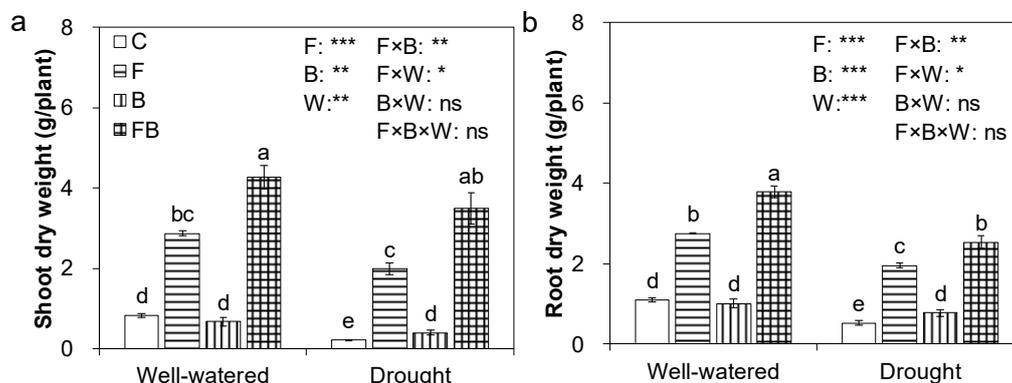
Water Regime (W)	Inoculation	Mycorrhizal Colonization (%)	Arbuscular Abundance (%)	Nodule Number (/plant)	Nodule dry Weight (g/plant)
Well-watered	C	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c	0.000 ± 0.000 c
	F	43.4 ± 2.5 ab	35.2 ± 4.6 a	0.0 ± 0.0 c	0.000 ± 0.000 c
	B	0.0 ± 0.0 c	0.0 ± 0.0 c	5.0 ± 0.6 c	0.028 ± 0.004 c
	FB	37.1 ± 4.9 b	28.5 ± 2.4 b	35.3 ± 2.5 a	0.206 ± 0.016 a
Drought	C	0.0 ± 0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 c	0.000 ± 0.000 c
	F	47.1 ± 3.9 a	35.5 ± 4.0 a	0.0 ± 0.0 c	0.000 ± 0.000 c
	B	0.0 ± 0.0 c	0.0 ± 0.0 c	1.5 ± 0.4 c	0.009 ± 0.002 c
	FB	49.4 ± 5.7 a	38.3 ± 3.3 a	20.5 ± 1.5 b	0.113 ± 0.009 b

Significance of					
F	***	***	***	***	***
B	ns	ns	***	***	***
W	*	ns	***	***	***
F × B	ns	ns	***	***	***
F × W	*	ns	*	*	*
B × W	ns	ns	***	***	***
F × B × W	ns	ns	*	*	*

### 3.2. Plant Biomass

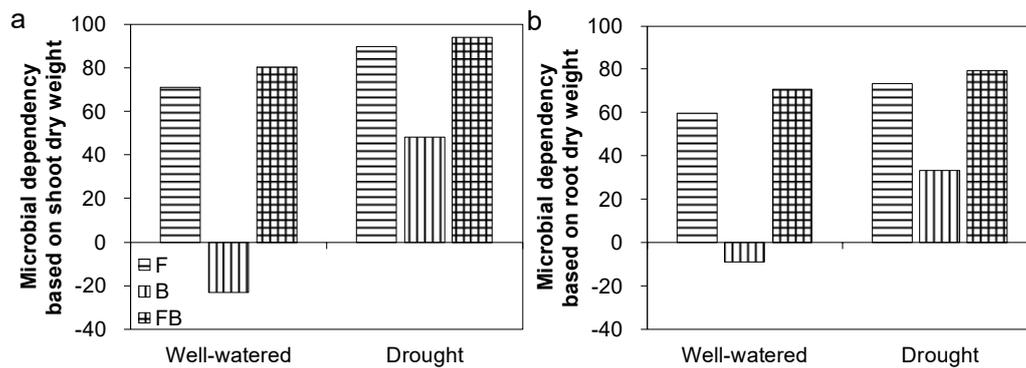
As expected, drought stress decreased plant growth (Figure 1). In general, *R. irregularis* inoculation promoted a plant's shoot and root growth irrespective of water regimes. No difference was observed on dry weight of plants inoculated with *M. tianshanense* compared with non-inoculated control under well-watered conditions, whereas the promotion effects of *M. tianshanense* inoculation on shoot and root growth were significant under drought stress conditions. Significant synergetic effects of *R. irregularis* and *M. tianshanense* inoculation on plant shoot and root growth were recorded under both water regimes.



**Figure 1.** Shoot (a) and root (b) dry weights of plants inoculated with/without AM fungus *Rhizophagus irregularis* and rhizobium *Mesorhizobium tianshanense* under well-watered and drought stress conditions (mean ± SE, n = 4). C represents non-inoculation control, F represents inoculation with *R. irregularis*, B represents inoculation with *M. tianshanense* treatments respectively, and FB represents the co-inoculation treatments. W represents water regime. The same letter above the error bars indicates no significant difference at p < 0.05. ns, not significant; \* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001.

### 3.3. Microbial Dependency

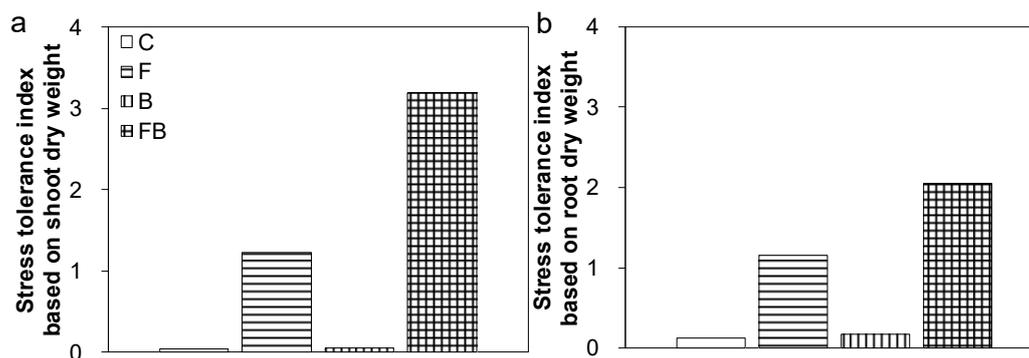
Based on plant shoot and root dry weight, microbial dependency was calculated to evaluate contribution of mycorrhizal fungus and rhizobium inoculations to plant growth. A positive contribution of *R. irregularis* and a negative contribution of *M. tianshanense* inoculation were observed on shoot and root growth under well-watered condition (Figure 2). A significant increase of microbial dependency was observed when plants were co-inoculated with *R. irregularis* and *M. tianshanense* as compared with a single inoculation. Microbial dependencies of single or co-inoculation of *R. irregularis* and *M. tianshanense* were significantly increased under drought stress, especially for *M. tianshanense* inoculation treatment, which shifted from negative to positive response. The shoots had a higher microbial dependency compared with roots.



**Figure 2.** The microbial dependency calculated according to van der Heijden (2002) based on shoot (a) and root (b) dry weights. F represents inoculation with *R. irregularis*, B represents inoculation with *M. tianshanense* treatments respectively, and FB represents the co-inoculation treatments.

### 3.4. Drought Tolerance Indices

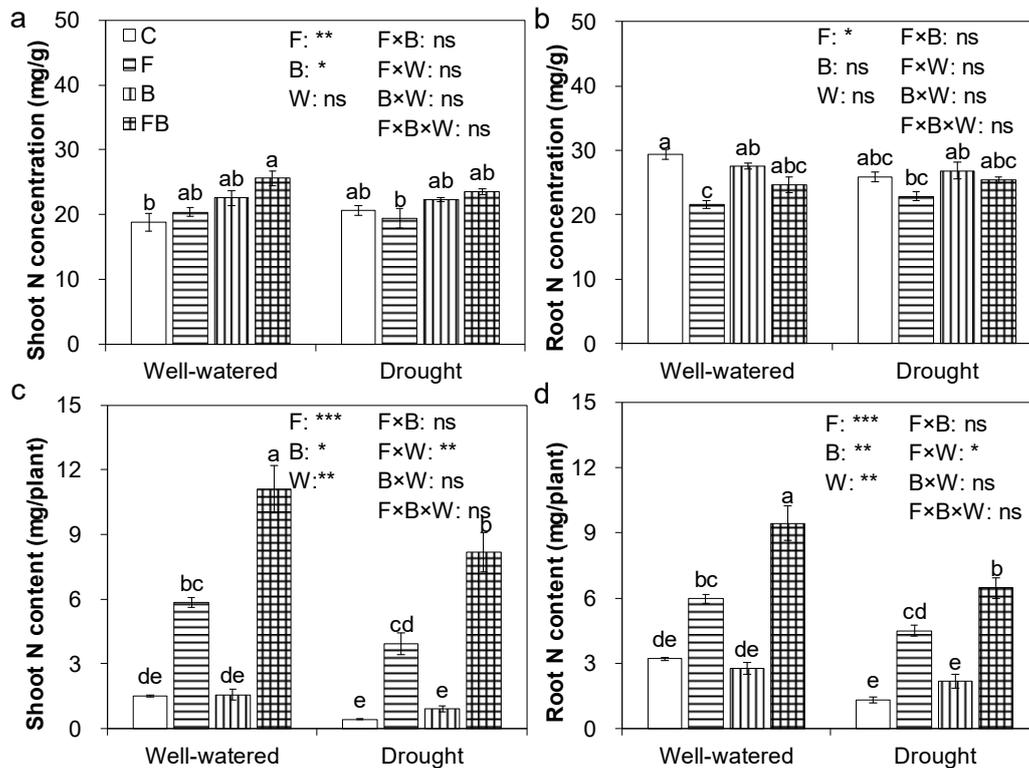
Plants inoculated with *R. irregularis* or *M. tianshanense* alone had a higher STI value compared with non-mycorrhizal control, with *R. irregularis* and *M. tianshanense* showing approximately 8.5-fold and 41.2% increase, respectively (Figure 3). Co-inoculation with *R. irregularis* and *M. tianshanense* showed the highest STI value, indicating a positive synergetic effect on plant drought tolerance.



**Figure 3.** Stress tolerance index (STI) of plants calculated based on plant shoot (a) and root (b) dry weights. C represents non-inoculation control, F represents inoculation with *R. irregularis*, B represents inoculation with *M. tianshanense* treatments respectively, and FB represents the co-inoculation treatments.

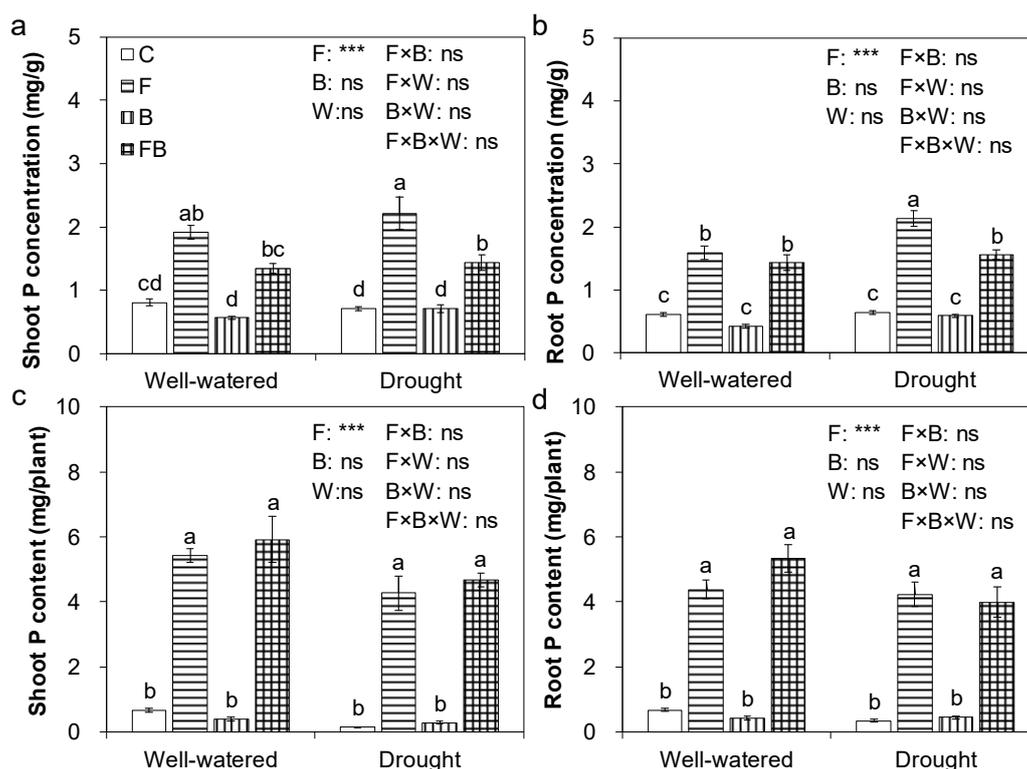
3.5. Plant Nutrient Uptake

*R. irregularis* single inoculation decreased root N concentration under well-watered condition, and increased shoot and root N contents under drought stress (Figure 4). The promotion effects induced by *M. tianshanense* inoculation were observed both in shoot and root N contents, compared with non-inoculated plants under drought stress. Co-inoculation with *R. irregularis* and *M. tianshanense* showed a positive synergistic effect on shoot and root N content, irrespective of water regimes.



**Figure 4.** Shoot (a) and root (b) N concentrations; shoot (c) and root (d) N contents of plants inoculated with/without AM fungus *Rhizophagus irregularis* and rhizobium *Mesorhizobium tianshanense* under well-watered and drought stress conditions (mean ± SE, n = 4). C represents non-inoculation control, F represents inoculation with *R. irregularis*, B represents inoculation with *M. tianshanense* treatments respectively, and FB represents the co-inoculation treatments. W represents water regime. The same letter above the error bars indicates no significant difference at  $p < 0.05$ . ns, not significant; \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

*R. irregularis* inoculation significantly increased plant shoot and root P concentration and contents. These promotion effects were independent from *M. tianshanense* inoculation and water regimes (Figure 5).



**Figure 5.** Shoot (a) and root (b) P concentrations; shoot (c) and root (d) P contents of plants inoculated with/without AM fungus *Rhizophagus irregularis* and rhizobium *Mesorhizobium tianshanense* under well-watered and drought stress conditions (mean  $\pm$  SE,  $n = 4$ ). C represents non-inoculation control, F represents inoculation with *R. irregularis*, B represents inoculation with *M. tianshanense* treatments respectively, and FB represents the co-inoculation treatments. W represents water regime. The same letter above the error bars indicates no significant difference at  $p < 0.05$ . ns, not significant; \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

#### 4. Discussion

Legumes can form symbiosis both with rhizobia and AM fungi. However, the presence of these microbial symbionts in roots is not always beneficial for plant growth [20,22,23]. In the present study, single inoculation with *M. tianshanense* promoted seedling growth and N content under drought stress conditions, whereas no effect was observed under well-watered conditions. This result confirmed that symbiotic microbes are more effective with a substantially improved plant root and shoot growth under drought stress conditions, which has also been previously observed both in meta-analytical and empirical studies [6,32,33]. Furthermore, *R. irregularis* promoted *Glycyrrhiza* plant growth and increased N and P compensating for the growth suppression induced by rhizobium under well-watered conditions.

*Glycyrrhiza* plants could effectively form symbiosis with both rhizobia and AM fungi, and rhizobia or AM fungi inoculation could substantially improve *Glycyrrhiza* growth [3,9,34]. Unsurprisingly, our results showed that *R. irregularis* stimulated *Glycyrrhiza* seedling growth, which was more pronounced under drought stress conditions that increased AM fungal colonization. Such pronounced plant growth response to mycorrhizal colonization under water-deficit conditions has been observed previously [33] and was likely attributed to the improvement of phosphorus nutrition content and the combination of physical and cellular effects induced by AM inoculation [35,36]. Our results indicated that *Glycyrrhiza* seedling P acquisition were dependent on mycorrhizal inoculation especially under drought stress conditions.

A plant's photosynthetic products were portioned out to support plant growth and reproduction of symbiotic microbes. Approximately 15–20% carbon is supporting the growth and respiration of

nodules [37], whereas approximately 4–20% carbon goes to mycorrhizal fungi [38]. In our study, under well-watered conditions, there was a negative rhizobial growth dependency, which might be due to the net cost of the symbiosis exceeding the net benefits for the plant [23]. The plant's allocation of more resources to root nodules could reduce the benefits of rhizobia inoculation. However, a positive rhizobial dependency was marked for *M. tianshanense*-inoculated plants under drought stress, which confirmed the *Glycyrrhiza* plant–*M. tianshanense* relationships may vary from a positive to negative microbial dependency. In relation to underlying carbon and nutrient trade, the attention was mainly paid to carbon costs as causes of negative microbial growth responses [24]. However, the range of responses of plants to microbial colonization should be explored as well as the fitness of both plants and their microbial partners [39]. The rhizobial function can be determined by several factors, and our results showed plants had a higher rhizobial dependency to acquire nitrogen resources under drought stress.

The possibility of extending the beneficial effects of plant–rhizobia symbiosis by introducing AM fungus into the symbiont under stress conditions is becoming a popular research topic [40,41]. Our results showed AM fungus enhanced the nodulation and significantly improved plant growth compared to single rhizobial inoculation. Synergistic effects of mycorrhizal and rhizobial symbioses could be reflected by increased rhizobial infection rate, plant nutrient content, and plant growth [42]. Several common signals and processes were involved in the establishment of these two symbiotic associations [13]. An increase in P content by mycorrhizal symbiosis could increase nitrogenase enzyme activity, leading to a higher N<sub>2</sub> fixation of rhizobial symbiont and in return a better mycorrhizal development [43]. In our study, rhizobia infection rate in plants co-inoculated with *R. irregularis* was much higher compared with single inoculated plants under drought stress conditions, which indicated that AM colonization stimulated nodule formation under stress conditions [44]. AM fungi could also decrease oxidative stress occurring in the nodules [11,45] and improve carbon metabolism of nodules [46]. Our results also demonstrated that AM fungus could increase plant growth and root N content dependency on *M. tianshanense* under well-watered condition. These results indicated that AM inoculation might be a promising driver to regulate the tripartite symbiosis establishment.

The STI has been widely used to identify stress-sensitive and -resistant cultivars in plant cultivation [31,47]. Based on differences of plant growth decrease under drought stress with respect to favorable conditions, STI could identify plant drought tolerance among different treatments. In this study, *R. irregularis* symbiosis in combination with *M. tianshanense* inoculation resulted in synergistic effects on plant drought tolerance, thereby indicating that the tripartite symbiosis could adapt to a wide range of environmental stresses based on STI. AM fungi and rhizobia could help plants tolerate various environmental stresses [46]. Improved nutrient acquisition induced by symbiotic microbes has been postulated as a primary mechanism for enhancing plant drought tolerance. Several mechanisms have also been proposed to explain the mycorrhizal effects, including direct water uptake by mycorrhizal aquaporin, enhanced plant osmotic adjustment, and reduced oxidative damage [35,36]. Our results suggested that co-inoculation with rhizobia and AM fungi could be a useful strategy to enhance the drought tolerance of legume plants.

## 5. Conclusions

This study suggested that the combined inoculation of *R. irregularis* and *M. tianshanense* was more effective than the single inoculation in promoting plant growth and enhancing plant tolerance to drought stress. Further confirmation of plant stress tolerance induced by AM fungi and rhizobia inoculation is needed under field conditions. For sustainable *Glycyrrhiza uralensis* production systems, the interactive effects of *Rhizobium* and AM fungal co-inoculation should be elucidated in detail in the future so that the optimized combinations of microorganisms can be applied as effective soil inoculants for plant growth promotion and fitness.

**Author Contributions:** Conceptualization, Z.H. and B.C.; data analysis, Z.H., X.J. and Z.W.; methodology, X.Z.; writing—original draft preparation, Z.H.; writing—review and editing, Z.H., W.X. and B.C.; funding acquisition, Z.H. and B.C.

**Funding:** This research was funded by the National Natural Science Foundation of China, grant number 41571250, and the National Key Research and Development Program of China, grant number 2016YFC0500702.

**Acknowledgments:** The authors appreciate the assistance from Huifeng Liu and Chunmei Li. The authors are very grateful to Xinhua Sui for providing *Mesorhizobium tianshanense*.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Fiore, C.; Eisenhut, M.; Krausse, R.; Ragazzi, E.; Pellati, D.; Armanini, D.; Bielenberg, J. Antiviral effects of *Glycyrrhiza* species. *Phytother. Res.* **2008**, *22*, 141–148. [[CrossRef](#)] [[PubMed](#)]
2. Mochida, K.; Sakurai, T.; Seki, H.; Yoshida, T.; Takahagi, K.; Sawai, S.; Uchiyama, H.; Muranaka, T.; Saito, K. Draft genome assembly and annotation of *Glycyrrhiza uralensis*, a medicinal legume. *Plant J.* **2017**, *89*, 181–194. [[CrossRef](#)] [[PubMed](#)]
3. Chen, M.; Yang, G.; Sheng, Y.; Li, P.; Qiu, H.; Zhou, X.; Huang, L.; Chao, Z. *Glomus mosseae* inoculation improves the root system architecture, photosynthetic efficiency and flavonoids accumulation of liquorice under nutrient stress. *Front. Plant Sci.* **2017**, *8*, 931. [[CrossRef](#)] [[PubMed](#)]
4. Hayashi, H.; Sudo, H. Economic importance of licorice. *Plant Biotechnol.* **2009**, *26*, 101–104. [[CrossRef](#)]
5. Shantz, A.A.; Lemoine, N.P.; Burkepile, D.E. Nutrient loading alters the performance of key nutrient exchange mutualisms. *Ecol. Lett.* **2016**, *19*, 20–28. [[CrossRef](#)] [[PubMed](#)]
6. Ossler, J.N.; Zielinski, C.A.; Heath, K.D. Tripartite mutualism: Facilitation or trade-offs between rhizobial and mycorrhizal symbionts of legume hosts. *Am. J. Bot.* **2015**, *102*, 1332–1341. [[CrossRef](#)] [[PubMed](#)]
7. Chen, W.X.; Wang, E.; Wang, S.Y.; Li, Y.B.; Chen, X.Q. Characteristics of *Rhizobium tianshanense* sp. nov., a moderately and slowly growing root-nodule bacterium isolated from an arid saline environment in Xinjiang, Peoples Republic of China. *Int. J. Syst. Bacteriol.* **1995**, *45*, 153–159. [[CrossRef](#)] [[PubMed](#)]
8. Liu, J.; Wu, L.; Wei, S.; Xiao, X.; Su, C.; Jiang, P.; Song, Z.; Wang, T.; Yu, Z. Effects of arbuscular mycorrhizal fungi on the growth, nutrient uptake and glycyrrhizin production of licorice (*Glycyrrhiza uralensis* Fisch). *Plant Growth Regul.* **2007**, *52*, 29–39. [[CrossRef](#)]
9. Xie, W.; Hao, Z.P.; Zhou, X.F.; Jiang, X.L.; Xu, L.J.; Wu, S.L.; Zhao, A.H.; Zhang, X.; Chen, B.D. Arbuscular mycorrhiza facilitates the accumulation of glycyrrhizin and liquiritin in *Glycyrrhiza uralensis* under drought stress. *Mycorrhiza* **2018**, *28*, 285–300. [[CrossRef](#)] [[PubMed](#)]
10. Xie, W.; Hao, Z.; Yu, M.; Wu, Z.; Zhao, A.; Li, J.; Zhang, X.; Chen, B. Improved phosphorus nutrition by arbuscular mycorrhizal symbiosis as a key factor facilitating glycyrrhizin and liquiritin accumulation in *Glycyrrhiza uralensis*. *Plant Soil* **2019**, *439*, 243–257. [[CrossRef](#)]
11. Porcel, R.; Barea, J.M.; Ruiz-Lozano, J.M. Antioxidant activities in mycorrhizal soybean plants under drought stress and their possible relationship to the process of nodule senescence. *New Phytol.* **2003**, *157*, 135–143. [[CrossRef](#)]
12. Takacs, T.; Cseresnyes, I.; Kovacs, R.; Paradi, I.; Kelemen, B.; Szili-Kovacs, T.; Fuzy, A. Symbiotic effectivity of dual and tripartite associations on soybean (*Glycine max* L. Merr.) cultivars inoculated with *Bradyrhizobium japonicum* and AM fungi. *Front. Plant Sci.* **2018**, *9*. [[CrossRef](#)] [[PubMed](#)]
13. Parniske, M. Arbuscular mycorrhiza: The mother of plant root endosymbioses. *Nat. Rev. Microbiol.* **2008**, *6*, 763–775. [[CrossRef](#)] [[PubMed](#)]
14. Vernie, T.; Camut, S.; Camps, C.; Rembliere, C.; de Carvalho-Niebel, F.; Mbengue, M.; Timmers, T.; Gascioli, V.; Thompson, R.; le Signor, C.; et al. PUB1 interacts with the receptor kinase DMI2 and negatively regulates rhizobial and arbuscular mycorrhizal symbioses through its ubiquitination activity in *Medicago truncatula*. *Plant Physiol.* **2016**, *170*, 2312–2324. [[CrossRef](#)] [[PubMed](#)]
15. Smith, S.E.; Nicholas, D.J.D.; Smith, F.A. Effect of early mycorrhizal infection on nodulation and nitrogen-fixation in *Trifolium subterraneum* L. *Aust. J. Plant Physiol.* **1979**, *6*, 305–316. [[CrossRef](#)]
16. Bianciotto, V.; Minerdi, D.; Perotto, S.; Bonfante, P. Cellular interactions between arbuscular mycorrhizal fungi and rhizosphere bacteria. *Protoplasma* **1996**, *193*, 123–131. [[CrossRef](#)]

17. Jou, M.H.H.; Besalatpour, A.A. Interactive effects of co-inoculation of *Bradyrhizobium japonicum* strains and mycorrhiza species on soybean growth and nutrient contents in plant. *J. Plant Nutr.* **2018**, *41*, 10–18.
18. Johansson, J.F.; Paul, L.R.; Finlay, R.D. Microbial interactions in the mycorrhizosphere and their significance for sustainable agriculture. *FEMS Microbiol. Ecol.* **2004**, *48*, 1–13. [[CrossRef](#)]
19. Jones, J.M.C.; Clairmont, L.; Macdonald, E.S.; Weiner, C.A.; Emery, R.J.N.; Guinel, F.C. E151 (sym15), a pleiotropic mutant of pea (*Pisum sativum* L.), displays low nodule number, enhanced mycorrhizae, delayed lateral root emergence, and high root cytokinin levels. *J. Exp. Bot.* **2015**, *66*, 4047–4059. [[CrossRef](#)]
20. West, S.A.; Kiers, E.T.; Simms, E.L.; Denison, R.F. Sanctions and mutualism stability: Why do rhizobia fix nitrogen? *Proc. R. Soc. Lond. Ser. B Biol. Sci.* **2002**, *269*, 685–694. [[CrossRef](#)] [[PubMed](#)]
21. Smith, S.E.; Jakobsen, I.; Gronlund, M.; Smith, F.A. Roles of Arbuscular Mycorrhizas in plant phosphorus nutrition: Interactions between pathways of phosphorus uptake in arbuscular mycorrhizal roots have important implications for understanding and manipulating plant phosphorus acquisition. *Plant Physiol.* **2011**, *156*, 1050–1057. [[CrossRef](#)] [[PubMed](#)]
22. Graham, J.H.; Abbott, L.K. Wheat responses to aggressive and non-aggressive arbuscular mycorrhizal fungi. *Plant Soil* **2000**, *220*, 207–218. [[CrossRef](#)]
23. Kiers, E.T.; Denison, R.F. Sanctions, cooperation, and the stability of plant-rhizosphere mutualisms. *Annu. Rev. Ecol. Evol. Syst.* **2008**, *39*, 215–236. [[CrossRef](#)]
24. Smith, F.A.; Smith, S.E. How useful is the mutualism-parasitism continuum of arbuscular mycorrhizal functioning? *Plant Soil* **2013**, *363*, 7–18. [[CrossRef](#)]
25. Wyatt, G.A.K.; Kiers, E.T.; Gardner, A.; West, S.A. Restricting mutualistic partners to enforce trade reliance. *Nat. Commun.* **2016**, *7*, 10322. [[CrossRef](#)] [[PubMed](#)]
26. Helgason, T.; Merryweather, J.W.; Young, J.P.W.; Fitter, A.H. Specificity and resilience in the arbuscular mycorrhizal fungi of a natural woodland community. *J. Ecol.* **2007**, *95*, 623–630. [[CrossRef](#)]
27. Grace, E.J.; Cotsaftis, O.; Tester, M.; Smith, F.A.; Smith, S.E. Arbuscular mycorrhizal inhibition of growth in barley cannot be attributed to extent of colonization, fungal phosphorus uptake or effects on expression of plant phosphate transporter genes. *New Phytol.* **2009**, *181*, 938–949. [[CrossRef](#)]
28. Phillips, J.M.; Hayman, D.S. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans. Br. Mycol. Soc.* **1970**, *55*. [[CrossRef](#)]
29. Trouvelot, A.; Kough, J.L.; Gianinazzi-Pearson, V. Mesure du taux de mycorrhization VA d'un système racinaire. Recherche de méthodes d'estimation ayant une signification fonctionnelle. In *Physiological and Genetical Aspects of Mycorrhizae*; Gianinazzi-Pearson, V., Gianinazzi, S., Eds.; INRA: Paris, France, 1986; pp. 217–221.
30. Van der Heijden, M.G.A. Arbuscular mycorrhizal fungi as a determinant of plant diversity: In search of underlying mechanisms and general principles. In *Mycorrhizal Ecology*; van der Heijden, M.G.A., Sanders, I.R., Eds.; Springer: Berlin/Heidelberg, Germany, 2003; pp. 243–265.
31. Clarke, J.M.; Depauw, R.M.; Townleysmith, T.F. Evaluation of methods for quantification of drought tolerance in wheat. *Crop Sci.* **1992**, *32*, 723–728. [[CrossRef](#)]
32. Rubin, R.L.; van Groenigen, K.J.; Hungate, B.A. Plant growth promoting rhizobacteria are more effective under drought: A meta-analysis. *Plant Soil* **2017**, *416*, 309–323. [[CrossRef](#)]
33. Jayne, B.; Quigley, M. Influence of arbuscular mycorrhiza on growth and reproductive response of plants under water deficit: A meta-analysis. *Mycorrhiza* **2014**, *24*, 109–119. [[CrossRef](#)] [[PubMed](#)]
34. Egamberdieva, D.; Li, L.; Lindstrom, K.; Rasanen, L.A. A Synergistic interaction between salt-tolerant *Pseudomonas* and *Mesorhizobium* strains improves growth and symbiotic performance of liquorice (*Glycyrrhiza uralensis* Fish.) under salt stress. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 2829–2841. [[CrossRef](#)] [[PubMed](#)]
35. Ruiz-Lozano, J.M. Arbuscular mycorrhizal symbiosis and alleviation of osmotic stress. New perspectives for molecular studies. *Mycorrhiza* **2003**, *13*, 309–317. [[CrossRef](#)] [[PubMed](#)]
36. Li, T.; Hu, Y.J.; Hao, Z.P.; Li, H.; Wang, Y.S.; Chen, B.D. First cloning and characterization of two functional aquaporin genes from an arbuscular mycorrhizal fungus *Glomus intraradices*. *New Phytol.* **2013**, *197*, 617–630. [[CrossRef](#)] [[PubMed](#)]
37. Kiers, E.T.; Rousseau, R.A.; West, S.A.; Denison, R.F. Host sanctions and the legume-rhizobium mutualism. *Nature* **2003**, *425*, 78–81. [[CrossRef](#)] [[PubMed](#)]

38. Johnson, N.C.; Graham, J.H.; Smith, F.A. Functioning of mycorrhizal associations along the mutualism-parasitism continuum. *New Phytol.* **1997**, *135*, 575–586. [[CrossRef](#)]
39. Johnson, N.C.; Graham, J.H. The continuum concept remains a useful framework for studying mycorrhizal functioning. *Plant Soil* **2013**, *363*, 411–419. [[CrossRef](#)]
40. Ashrafi, E.; Zahedi, M.; Razmjoo, J. Co-inoculations of arbuscular mycorrhizal fungi and rhizobia under salinity in alfalfa. *Soil Sci. Plant Nutr.* **2014**, *60*, 619–629. [[CrossRef](#)]
41. Larimer, A.L.; Clay, K.; Bever, J.D. Synergism and context dependency of interactions between arbuscular mycorrhizal fungi and rhizobia with a prairie legume. *Ecology* **2014**, *95*, 1045–1054. [[CrossRef](#)]
42. Chalk, P.M.; Souza, R.d.F.; Urquiaga, S.; Alves, B.J.R.; Boddey, R.M. The role of arbuscular mycorrhiza in legume symbiotic performance. *Soil Biol. Biochem.* **2006**, *38*, 2944–2951. [[CrossRef](#)]
43. Ren, C.G.; Bai, Y.J.; Kong, C.C.; Bian, B.; Xie, Z.H. Synergistic interactions between salt-tolerant rhizobia and arbuscular mycorrhizal fungi on salinity tolerance of *Sesbania cannabina* plants. *J. Plant Growth Regul.* **2016**, *35*, 1098–1107. [[CrossRef](#)]
44. Azcon, R.; Elatrach, F.; Barea, J.M. Influence of mycorrhiza vs soluble phosphate on growth, nodulation, and N<sub>2</sub> fixation (15 N) in alfalfa under different levels of water potential. *Biol. Fertil. Soils* **1988**, *7*, 28–31. [[CrossRef](#)]
45. Buecking, H.; Kafle, A. Role of Arbuscular Mycorrhizal fungi in the nitrogen uptake of plants: Current knowledge and research gaps. *Agronomy-Basel* **2015**, *5*, 587–612. [[CrossRef](#)]
46. Goicoechea, N.; Merino, S.; Sanchez-Diaz, M. Contribution of arbuscular mycorrhizal fungi (AMF) to the adaptations exhibited by the deciduous shrub *Anthyllis cytisoides* L. under water deficit. *Physiol. Plant.* **2004**, *122*, 453–464. [[CrossRef](#)]
47. Winter, S.R.; Musick, J.T.; Porter, K.B. Evaluation of screening techniques for breeding drought-resistant winter-wheat. *Crop Sci.* **1988**, *28*, 512–516. [[CrossRef](#)]



© 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).