

Physiological responses of mycorrhizal symbiosis to drought stress in white clover

Sheng-Min LIANG¹, Dao-Ju JIANG², Miao-Miao XIE¹,
Ying-Ning ZOU^{1*}, Qiang-Sheng WU^{1,3*}, Kamil KUČA³

¹Yangtze University, College of Horticulture and Gardening, Jingzhou, Hubei 434025, China; 894703552@qq.com; 972948575@qq.com; zouyingning@163.com (*corresponding author); wuqiangsh@163.com (*corresponding author)

²Jingzhou Natural Resources and Planning Bureau, Shashi Substation, Jingzhou, Hubei 434000, China; 281605243@qq.com

³University of Hradec Kralove, Faculty of Science, Department of Chemistry, Hradec Kralove 50003, Czech Republic; kamil.kuca@uhk.cz

Abstract

The aim of the present study was to analyze the effects of two arbuscular mycorrhizal fungi (AMF), *Funneliformis mosseae* and *Paraglomus occultum*, on leaf water status, root morphology, root sugar accumulation, root abscisic acid (ABA) levels, root malondialdehyde (MDA) content, and root antioxidant enzyme activities in white clover (*Trifolium repens* L.) exposed to well-watered (WW) and drought stress (DS) conditions. The results showed that root colonization by *F. mosseae* and *P. occultum* was significantly decreased by 7-week soil drought treatment. Under drought stress conditions, mycorrhizal fungal treatment considerably stimulated root total length, surface area and volume, as compared with non-mycorrhizal controls. In addition, inoculation with arbuscular mycorrhizal fungi also increased leaf relative water content and accelerated the accumulation of root glucose and fructose under drought stress. Mycorrhizal plants under drought stress registered higher activities of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) and ABA levels in roots, while lower MDA contents, relative to non-mycorrhizal plants. As a result, mycorrhiza-inoculated plants represented better physiological activities (e.g. antioxidant defense systems, root morphology, and sugar accumulation) than non-inoculated plants in response to soil drought, whilst *P. occultum* had superior effects than *F. mosseae*.

Keywords: ABA; antioxidant; drought stress; root architecture; white clover

Introduction

Soil drought is one of the severe abiotic stresses in the world, with global arid and semi-arid areas accounting for about 1/3 of the total land area (Kunert *et al.*, 2016; Zou *et al.*, 2020). As an important environment for human survival, grasslands are characterized with a fragile ecosystem, and global warming reduced the water utilization efficiency of grasslands (Asner *et al.*, 2004). White clover (*Trifolium repens* L.) is a perennial leguminous plant, which is used as a high-quality forage grass because of its tender grass quality, high yield and high nutritional value. Plant rhizosphere inhabits various microorganisms including endophytic fungi (Yang *et al.*, 2021). Earlier studies have confirmed that a single inoculation of AMF or rhizobia, or double

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inoculation on white clover accelerated plant growth and nutrient uptake, especially N (Xie *et al.*, 2020). However, white clover has a shallow root system and is very sensitive to soil water deficit. Therefore, it is an urgent task to enhance the drought resistance of white clover.

AMF are a kind of beneficial soil microorganisms that can form mutualistic symbiosis with more than 80% of terrestrial plants including white clover. Under environmental stresses including DS, AMF can induce various responses in plants to improve stress tolerance (He *et al.*, 2020a; Meng *et al.*, 2020; Zou *et al.*, 2021). Studies indicated that AMF promoted plant growth and enhanced the unsaturated fatty acid concentrations under DS conditions, thus, inducing higher drought tolerance of mycorrhizal plants versus non-mycorrhizal plants (Wu *et al.*, 2019; Zhang *et al.*, 2019). Arbuscular mycorrhizas possessed developed mycorrhizal extraradical hyphal network that could extend to the ineffective soil outside the root system, contributing 20% water of the host (Ruth *et al.*, 2011; Zhang *et al.*, 2018). In Greek olive cultivars, AMF improved root biomass, total root length, root surface area, root volume, root branch number and root tip number under soil drought conditions (Chatzistathis *et al.*, 2013). In addition, AMF also maintained the osmotic balance of plant cells by regulating osmotic solutes such as proline and soluble sugar in watermelon seedlings, so as to magnify the external water into plant cells (Mo *et al.*, 2016). Marulanda *et al.* (2007) showed that inoculation of *F. mosseae* and *Glomus intraradices* could reduce the accumulation of hydrogen peroxide in lavender plants under drought conditions, thereby, maintaining low oxidative burst of host plants exposed to DS (Zou *et al.*, 2020). AMF also regulated the level of endogenous ABA in plants, which could affect stomatal closure, activate the plant defense system, and thus affect the water relation of plants (Wu *et al.*, 2013). These results indicated that the mechanism of mycorrhizal fungi enhancing drought tolerance of plants is very complex and needs further study (Wu *et al.*, 2013). Nevertheless, it is difficult to know whether AMF enhance the drought resistance of white clover.

This study aimed to analyze the effect of two AMF species on root morphology, leaf relative water content, root carbohydrate content, endogenous ABA levels, and antioxidant enzyme activities of white clover exposed to WW and DS, and also to clarify the underlying mechanisms.

Materials and Methods

Experimental design

The experiment used a completely randomized block design with two water regimes (WW and DS) and three inoculation treatments (*F. mosseae*, *P. occultum* and non-AMF), with a total of six treatments. Each treatment was replicated 5 times, a total of 30 pots.

Plant culture

Seeds of white clover were disinfected with 75% alcohol for 10 min, rinsed with distilled water for 4 times, and sown in a 1.8-L plastic pot preloaded with autoclaved sterilized (121°C, 0.11 MPa, 2 h) substrate of soil and sand (1:1, v/v). Thirty seeds were enclosed in a pot, and the seedlings were thinned to 15 plants/pot after 20 days.

Funneliformis mosseae (Nicol. & Gerd.) Gerdemann & Trappe and *Paraglomus occultum* Morton & Redecker (Walker) were provided by the Bank of Glomeromycota in China, Beijing, China. The AMF strains were propagated with white clover in pots for three months, and the mycorrhizal inoculums contained spores, colonized roots, soil mycorrhizal hyphae and growth substrates. At the time of sowing, 1600 spores of each AMF fungus were applied to the pot as AMF treatment, and the non-AMF-treated pots received equivalent autoclaved inoculums.

After sowing, soil water content of pots was kept under 75% of the maximum field water capacity (WW), for 12 weeks. Subsequently, half of seedlings continued to grow under WW conditions for 7 weeks, and the other half changed their water status to 55% of the maximum field water capacity (DS) for 7 weeks.

The soil water of pots was controlled by weighing method every day, and plants were harvested after drought treatment for 7 weeks. All the plants were randomly placed in the glass greenhouse of College of Horticulture and Gardening, Yangtze University, without any temperature equipment.

Variable determinations

The root system was scanned by the Epson scanner, and the total length, projected area, surface area and volume of the root system were analyzed by the WinRHIZO software.

The root mycorrhizal colonization rate was carried out by Phillips and Hayman (Phillips and Hayman, 1970) with the staining of trypan blue (0.05%). Root mycorrhizal colonization was calculated as the percentage of AMF-infected root length against total root length.

The relative water content (RWC) of leaves was determined according to Bajji *et al.* (2001) using the fourth leaf on the tip.

The contents of glucose, fructose and sucrose in roots were determined by the protocol described by Wu *et al.* (2015).

The extraction of root ABA was performed in accordance with Chen *et al.* (2009), and was assayed as per the Enzyme-Linked Immunosorbent Assay (ELISA), provided by the Crop Chemical Control Research Center of China Agricultural University, Beijing, China, according to user's manual.

A 0.2 g fresh samples of leaf were ground with 5 mL 50 mM phosphate buffer (pH 7.8) and centrifuged at 4000 g for 10 min at 4 °C. The supernatant was collected for the analysis of antioxidant enzyme activities. SOD activity was assayed as per the protocol described by Giannopolitis and Ries (1977), based on the photochemical inhibition of nitroblue tetrazolium. CAT activity was monitored by Goldblith and Proctor (1950) with the titration of 0.1 M KMnO₄. POD activity was determined by the method described by Chance and Maehly (1955).

MDA, the by-product of lipid peroxidation of cell membranes, was analyzed using the protocol outlined by Sudhakar *et al.* (2001).

Statistical analysis

The experimental data were subjected to the analysis of variance with SAS software, and the Duncan's Multiple Range Test was used to conduct significant ($P < 0.05$) differences among treatments.

Results

Root mycorrhizal colonization and root morphology

Drought treatment significantly reduced the colonization of *F. mosseae* and *P. occultum* in white clover roots (Table 1). Under WW condition, the mycorrhizal colonization treated by *P. occultum* was significantly higher than that treated by *F. mosseae*, but there was no significant difference under DS condition. Compared to non-AM fungal treatment, AM fungal inoculation significantly improved root projection area, root surface area, root length, root volume: 12%, 11%, 13% and 12% higher under *F. mosseae*-inoculation condition and 14%, 26%, 23% and 24% higher under *P. occultum*-inoculation condition under WW; 16%, 12%, 13% and 9% higher under *F. mosseae*-inoculation condition and by 19%, 16%, 16% and 24% under *P. occultum*-inoculation under DS condition (Table 1).

Table 1. Effects of *Funneliformis mosseae* and *Paraglomus occultum* on root morphology and mycorrhizal colonization of white clover under well-watered and drought stress conditions

Water treatments	Inoculated treatments	Root total length (cm)	Root projected area (cm ²)	Root surface area (cm ²)	Root volume (cm ³)	Mycorrhizal colonization (%)
Well-watered	Non-AMF	789±40c	28.3±3.0c	84.8±8.8cd	0.78±0.05c	0e
	<i>F. mosseae</i>	884±28ab	31.3±0.9b	95.8±5.7ab	0.87±0.04b	66.73±5.21b
	<i>P. occultum</i>	914±77a	35.6±1.9a	104.0±10.5a	0.97±0.05a	76.95±8.78a
Drought stress	Non-AMF	687±79d	24.8±1.9d	77.5±4.0d	0.68±0.05d	0e
	<i>F. mosseae</i>	795±58c	28.8±2.5c	87.3±7.9bc	0.74±0.04c	54.21±3.42c
	<i>P. occultum</i>	819±78bc	28.8±1.6bc	90.2±4.6bc	0.84±0.01b	52.32±3.65c

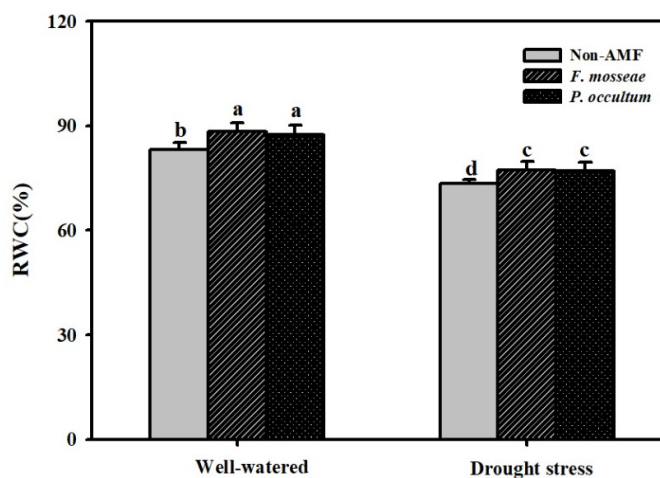
Note: Data (means ± SD, $n = 4$) followed by different letter were significantly different between treatments at $P < 0.05$.

Leaf RWC

Drought stress significantly reduced the leaf RWC, while mycorrhizal fungal treatment significantly increased it (Figure 1). Compared with non-AMF inoculation, inoculation of *F. mosseae* and *P. occultum* significantly increased the leaf RWC by 6% and 5% under WW condition, and by 5% and 5% under DS condition, respectively.

Root sugar contents

The contents of fructose and glucose in the roots of AMF-inoculated seedlings were significantly higher than those of non-AMF-inoculated seedlings, independent on AMF species (Figure 2a-c). Compared to non-AMF treatment, *F. mosseae* and *P. occultum* significantly increased root fructose and glucose contents by 60% and 136% under WW condition and by 30% and 11% under DS condition, respectively (Figure 2b-c). In addition, inoculation of *F. mosseae* did not significantly change the sucrose content in roots under WW and DS conditions, while inoculation of *P. occultum* significantly increased the sucrose content in roots by 88% and 49% under WW and DS conditions, respectively (Figure 2a).

**Figure 1.** Effects of *Funneliformis mosseae* and *Paraglomus occultum* on leaf RWC of white clover under well-watered and drought stress conditions

Data (means ± SD, $n = 4$) with different letters above the bars indicated the significant difference among treatments at 0.05 levels.

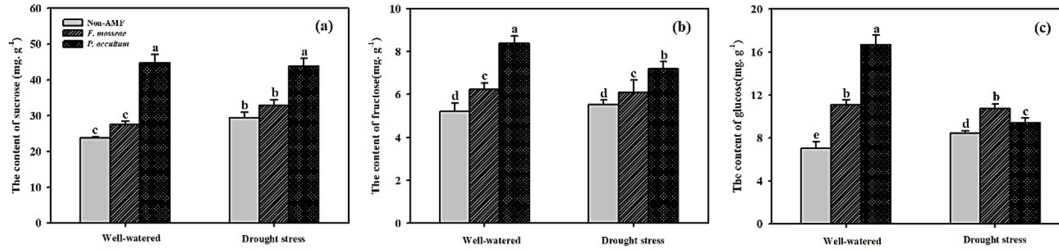


Figure 2. Effects of *Funneliformis mosseae* and *Paraglomus occultum* on sucrose (a), fructose (b) and glucose (c) contents in roots of white clover under well-watered and drought stress conditions. Data (means ± SD, n = 4) with different letters above the bars indicated the significant difference among treatments at 0.05 levels.

Root antioxidant enzyme activities

Under WW condition, *F. mosseae* and *P. occultum* treatment collectively significantly increased root SOD activities by 57% and 47%, root POD activities by 27% and 53%, and root CAT activities by 41% and 49%, respectively, compared with no-AMF-inoculated treatment (Figure 3a-c). Under DS condition, the activity of roots of antioxidant enzymes (SOD, POD, and CAT) in white clover was also significantly increased after AMF-inoculation, and the SOD and POD activities of *P. occultum*-colonized plants were considerably higher than those of *F. mosseae*-colonized plants.

Root MDA levels

Drought treatment significantly increased root MDA content in white clover, while inoculation of AM fungi significantly decreased MDA content in roots, with no significant difference between AM fungal treatments (Figure 4). *F. mosseae* and *P. occultum* treatment significantly reduced MDA content in roots by 20% and 26% under WW condition and by 15% and 17% under DS condition, compared with non-AMF treatment.

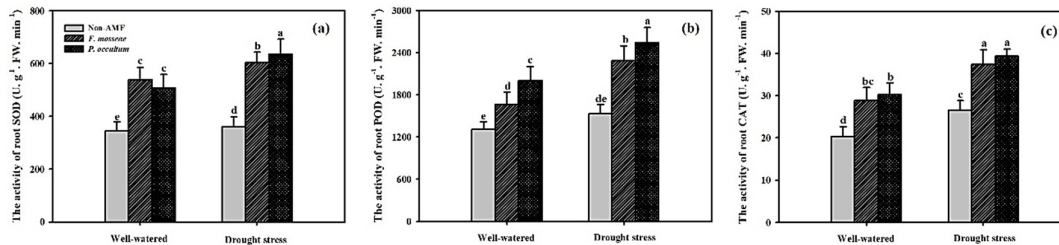


Figure 3. Effects of *Funneliformis mosseae* and *Paraglomus occultum* on SOD (a), POD (b) and CAT (c) activities in roots of white clover under well-watered and drought stress conditions. Data (means ± SD, n = 4) with different letters above the bars indicated the significant difference among treatments at 0.05 levels.

Root ABA levels

Except for plants treated with *F. mosseae*, ABA contents in roots of non-AMF-treated and *P. occultum*-colonized plants were significantly increased by soil drought treatment (Figure 5). Root ABA levels of *F. mosseae*- and *P. occultum*-colonized plants were distinctly increased when compared with those of non-AMF plants, with 31% and 11% increases under WW condition and 13% and 12% increases under DS condition, respectively.

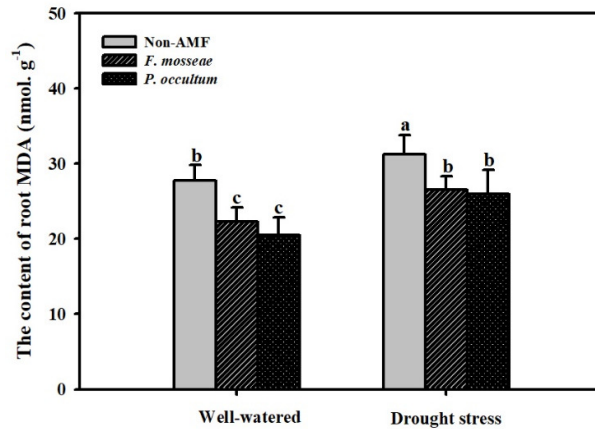


Figure 4. Effects of *Funneliformis mosseae* and *Paraglomus occultum* on MDA levels in roots of white clover under well-watered and drought stress conditions

Data (means \pm SD, $n = 4$) with different letters above the bars indicated the significant difference among treatments at 0.05 levels.

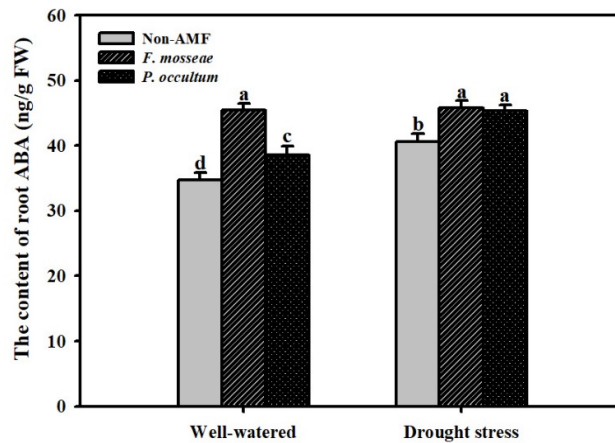


Figure 5. Effects of *Funneliformis mosseae* and *Paraglomus occultum* on ABA levels in roots of white clover under well-watered and drought stress conditions

Data (means \pm SD, $n = 4$) with different letter above the bars indicated the significant difference among treatments at 0.05 levels.

Discussion

In this study, compared with WW treatment, DS treatment significantly inhibited the root mycorrhizal infection of white clover, which was consistent with Zhang *et al.* (2014) on *Quercus acutissima*. The lower mycorrhizal infection rate may be related to the lower carbon utilization rate of host plants under DS (Francis, 1992; Wu *et al.*, 2013). The present study also showed that inoculation of *F. mosseae* and *P. occultum* significantly promoted the root total length, projected area, surface area and volume of white clover under both WW and DS conditions. Good root morphology is conducive to the uptake of soil water, and has a beneficial effect on enhancing drought tolerance of plants (Chatzistathis *et al.*, 2013). Greater root morphology of AMF

versus non-AMF plants may be related to the induction of hosts by mycorrhization to produce more auxins, salicylic acids, polyamines, and nitric oxide (Francis, 1992; Wu *et al.*, 2019; Zhang *et al.*, 2020).

The present study also indicated that inoculation with AMF dramatically increased the leaf RWC of white clover, which is consistent with the results of Yadav *et al.* (2015) in peas. AMF formed a developed mycorrhizal hyphal network on the root surface (Zhang *et al.*, 2018), and mycorrhizal hypha was directly involved in the absorption and transport of plant water (Ruth *et al.*, 2011). On the one hand, mycorrhizal symbiosis improved the root morphology and increased the surface area for the host root to contact with more water (Wu *et al.*, 2013).

Early studies indicated that inoculation of AMF could significantly increase the soluble sugar content in tree bean (Qiao *et al.*, 2011) and lettuce (Baslam and Goicoechea, 2012). Our study showed that inoculation with *F. mosseae* significantly increased the root glucose and fructose content of white clover under WW and DS conditions, and inoculation with *P. occultum* significantly increased the root sucrose, glucose and fructose content of white clover under WW and DS conditions. This indicated that AMF, especially *P. occultum*, induced the accumulation of sugars in the root of the host. The accumulation of a large amount of sugar further improved cell osmotic potential, protected organelles, and enhanced the drought tolerance of plants (Yooyongwech *et al.*, 2013). In addition, high sugar content of mycorrhiza plants supports the development of roots and mycorrhizas, thus facilitating the uptake of water and mineral elements by plants (Kozłowski and Pallardy, 2002).

Drought stress usually disrupts the balance of endogenous hormones in plants, resulting in the disorder of normal plant growth and metabolism, and thus inhibiting plant growth. AMF regulate stomatal behavior, reduce the transpiration rate, and reduce water loss by changing endogenous hormone levels, mainly ABA, so as to reduce drought damage of host plants (Zhang *et al.*, 2014). Our study showed that inoculation of AMF substantially increased ABA content in the roots of white clover under both WW and DS conditions. ABA is an important factor in the transmission of stress signals and can also induce the expression of antioxidant enzyme genes (Ruiz-Sánchez *et al.*, 2010), so as to enhance the scavenging capacity of reactive oxygen species and enhance the drought tolerance of plants (He *et al.*, 2020b). Our study indicated that inoculation of *F. mosseae* and *P. occultum* could significantly improve the activity of POD, SOD and CAT in roots under WW and DS conditions, among which *P. occultum* had a more obvious effect. Ruiz-lozano *et al.* (2001) also showed that AMF could induce mycorrhizal plants to produce new SOD isoenzyme (e.g. Mn-SODII) in response to DS. MDA as an indicator of plant oxidative stress significantly was increased in the root of white clover under DS condition, but was significantly decreased after inoculation with AMF, which was consistent with the results of Mirshad and Puthur (2016) on *Saccharum arundinaceum*. These results indicated that AMF increased the activity of antioxidant enzymes in the host under DS condition to reduce the accumulation of reactive oxygen species in the host, thus mitigating the degree of membrane lipid peroxidation and enhancing the drought tolerance of the host (Marulanda *et al.*, 2007; Zou *et al.*, 2020).

Conclusions

In a word, mycorrhizal plants had superior tolerance in response to soil drought through high physiological activities such as improving root morphology, accelerating sugar accumulation, and stimulating antioxidant enzyme activities and ABA levels.

Authors' Contributions

SML, MMX, YNZ and QSW conceived and designed the study. SML, DJJ and MMX performed the experiments and interpreted the data. SML and MMX wrote the manuscript, YNZ, QSW and KK provided

critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript.

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Conflict of Interests

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

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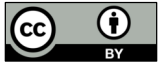
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