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RESEARCH ARTICLE

Wu Qiangsheng, Xia Renxue, Hu Zhengjia

Effect of Arbuscular Mycorrhiza on the Drought Tolerance of *Poncirus trifoliata* Seedlings

Abstract The effects of *Glomus mosseae* colonization on the plant growth and drought tolerance of 1-year-old trifoliate *Poncirus trifoliata* seedlings in potted culture were studied in natural water stress and rewatering conditions. Results showed that arbuscular mycorrhizal (AM) inoculation significantly improved the height, stem diameter, and fresh weight of *P. trifoliata* seedlings before natural water stress. By the end of the experiment, the survival percentage of AM-transplanted seedlings was 8% higher than those of non-AM ones. During water stress and rewatering, AM significantly increased the contents of soluble sugars and proteins in leaves, and enhanced the activities of superoxide dismutase (SOD), guaiacol peroxidase (G-POD), and catalase (CAT) in either seedling leaves or roots, which indicated that AM colonization could improve the osmotic adjustment response of *P. trifoliata*, enhance its defense system, and alleviate oxidative damages to membrane lipids and proteins. These results demonstrated that the drought tolerance of *P. trifoliata* seedlings was increased by inoculation with AM fungi. The functional mechanism underlying the observation that mycorrhizas increased the host's drought tolerance was closely related to enzymatic and non-enzymatic antioxidant defense systems such as SOD, G-POD, CAT, and soluble protein.

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Wu Qiangsheng (✉), Xia Renxue
Key Laboratory of Horticultural Plant Biology,
Ministry of Education,
College of Horticulture and Forestry,
Huazhong Agricultural University,
Wuhan 430070, China
E-mail: wuqiangsh@163.com

Hu Zhengjia
College of Life Science and Technology,
Huazhong Agricultural University,
Wuhan 430070, China

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1 Introduction

Citrus (*Poncirus trifoliata*) is an important economic tree species native to subtropical and tropical areas in eastern Asia; however, its growth and output are often affected by water deficiency in China, which has drawn much concern [1].

Arbuscular mycorrhiza (AM), popular in fruit trees, is a symbiotic association between arbuscular mycorrhizal fungi (AMF) and the roots of fruit trees [2]. Previous studies have shown that the inoculation of AMF could affect the water metabolism of hosts and consequently increase their drought tolerance [3–8]. In order to reduce the water consumption of citrus, AM colonization is necessary in citrus fields. Wu and Xia [7] reported that the inoculation of *Glomus mosseae* promoted active absorbing areas of trifoliate *P. trifoliata* roots and increased plant water use efficiency when the soil water contents were 20%, 16%, and 12%. Wang et al. [9] reported that the inoculation of *G. mosseae* reduced the production rate of O_2^-, improved the contents of superoxide dismutase (SOD) and catalase (CAT) in sugarcane leaves, and enhanced the removing ability of reactive oxygen, consequently reducing the peroxidation of membrane lipids and improving drought tolerance during water stress. To date, most studies on AMF have focused on its effects on the nutrition of plants (such as soybeans, lettuce, tobacco, and sugarcane), and little has been reported on the activities of antioxidant enzymes in AM and non-AM plants. Research works involving citrus plants are quite rare. Moreover, there is still an ongoing debate about AM's effects on the water relations and mechanisms involved.

The present study attempted to evaluate the activities of antioxidant enzymes and the contents of soluble elements in the leaves and roots of trifoliate orange seedlings colonized by *G. mosseae* in water stress and rewatering conditions.

Table 1 Root colonization and plant growth characteristics of AM and non-AM *P. trifoliata* seedlings before and after water treatments

Treatment	Root colonization /%	Leaf number per plant	Seedling height /cm	Stem diameter /cm	Fresh weight /g/plant	Survival /%
AMF	38.51±10.00[a]	18.3±4.1[a]	18.18±0.68[a]	0.212±0.025[a]	1.47±0.05[a]	96.4
Non-AMF	4.70±2.59[b]	15.6±4.2[a]	16.99±0.96[b]	0.190±0.005[b]	1.20±0.15[b]	89.3

Mean ± SD, followed by the same superscript letter within a column, shows nonsignificant difference ($LSD_{0.05}$).

2 Materials and methods

2.1 Plant and fungal materials

One-year-old trifoliate *P. trifoliata* seedlings of the same size were chosen for the experiment. These seedlings were transplanted into plastic pots (15 cm×20 cm) on 15 April 2003. Each pot was filled with 3.14 kg of growth substrate sterilized with 0.5% (v/v) formaldehyde for 7 days to eliminate indigenous fungi and was air-dried. The growth substrate in pots was a mixture of yellow soil and clean sand (9:1, v/v) sampled from a river, with the following conditions: pH 5.61, 8.4 g/kg organic matter, 11.85 mg/kg available phosphorus, 1.26 g/kg total nitrogen, 31.85 mg/kg potassium, and 26.3% maximum field capacity. Soil was collected from the Fruit Sample Garden, Huazhong Agricultural University (Wuhan, China).

Seedlings were inoculated with either *G. mosseae* or non-AM fungal inocula as control before transplanting. For AM inoculation, 770 spores were placed 5 cm below citrus seedlings. Inocula were provided by the Institute of Plant Nutrition and Resources, Beijing Academy of Agriculture and Forestry Sciences (Beijing, China).

All AM and non-AM seedlings were grown in a greenhouse with natural light and watered everyday (from April to November 2003).

2.2 Experimental design

A completely randomized design method was adopted in the experiment with two mycorrhizal treatments: AMF and non-AMF. Each of the two treatments had seven repeats for a total of 14 pots. Four seedlings were planted in each pot.

2.3 Water treatment and tissue harvest

All AM and non-AM seedlings were subjected to natural water stress 188 days after transplanting. By weighing before water stress, the relative soil water content of each treatment was maintained at 80%. Leaf tissues were harvested 8 and 12 days after water stress, quickly frozen by liquid nitrogen, and then stored at −20°C. Rewatering began 12 days after water stress. Leaf tissues were harvested 12 h, 1 day, and 4 days after rewatering, and then whole seedlings were harvested.

2.4 Parameter analysis

Leaf number per seedling, seedling height, stem diameter, and survival percentage were recorded before water stress. Fresh weights were measured for all plants in each pot after all seedlings had been harvested.

A small fraction of the fresh root system in each pot was cut into 1- to 2-cm-long pieces and fixed in formalin–acetic acid–alcohol solution (for at least 24 h). These root samples were made clear with 10% (w/v) KOH solution, stained with 0.05% (v/v) trypan blue in lactophenol [10], and observed under a microscope for root colonization.

Soluble sugars were determined by anthrone method, as described by Li [11], using sucrose as standard.

To prepare the extraction of enzymes and soluble proteins, 0.5 g of fresh samples of leaf or root tissues was homogenized in 5 mL of 0.1 mol/L phosphate buffer (pH 7.8) and centrifuged at 4,000 r/min for 10 min at 4°C, and then the supernatant was collected for assays.

Soluble proteins were evaluated by the method of Bradford [12] using bovine serum albumin as standard. The activities of SOD, guaiacol peroxidase (G-POD), and CAT were measured using the method of Li [11]. One unit of SOD was defined as the amount of enzyme that inhibited 50% nitro blue tetrazolium by light, and SOD activity was expressed as SOD units per gram of fresh weight.

2.5 Statistical analysis

Experimental data obtained were subjected to analysis of variance using SAS (8.1), and means were evaluated by Fisher's protected least significant difference (LSD). Differences at $p<0.05$ were considered significant.

Table 2 Defense system status of AM and non-AM roots in *P. trifoliata* seedlings 4 days after rewatering

Treatment	Soluble protein (mg/g fresh weight)	SOD (U/g fresh weight)	G-POD (U/g fresh weight)	CAT (mg/min per g fresh weight)
AMF	3.96±0.24[a]	687.33±81.82[a]	138.84±14.49[a]	2.03±0.65[a]
Non-AMF	2.94±0.31[b]	648.51±76.34[a]	91.72±9.47[b]	0.43±0.23[b]

Mean ± SD, followed by the same superscript letter within a column, shows nonsignificant difference ($LSD_{0.05}$).

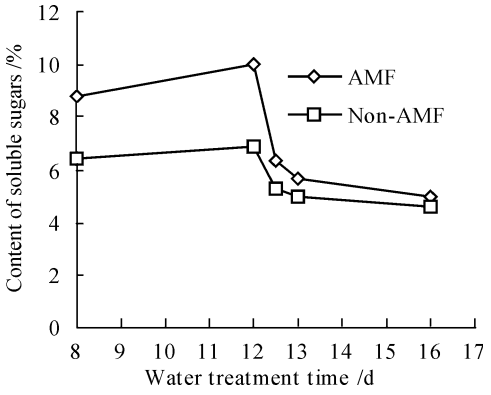

Fig. 1 Effect of AM colonization on the content of soluble sugars in *P. trifoliata* seedling leaves during water stress and rewatering

3 Results

3.1 Root colonization and plant growth

Arbuscular mycorrhizal inoculation increased root colonization (Table 1). The root colonization of *G. mosseae*-inoculated seedlings was 39%. The roots of non-AM seedlings were also infected by native AMF, while their root colonization was less than 5%.

Table 1 also shows that seedling height, stem diameter, fresh weight, and survival percentage were 12%, 7%, 23%, and 8% higher in AM seedlings than in non-AM seedlings, respectively. AM inoculation had no marked effect on leaf number per seedling.

3.2 Antioxidant defense system characteristics of rewatered roots in AM and non-AM seedlings

The inoculation of AM 4 days after rewatering significantly improved the contents of soluble proteins and the activities of G-POD and CAT in seedling roots, which were 34.7%, 51.4%, and 372.1% higher than those of control (Table 2). It also accelerated, to some extent, the activity of SOD in the root system, but the effect was not marked. These

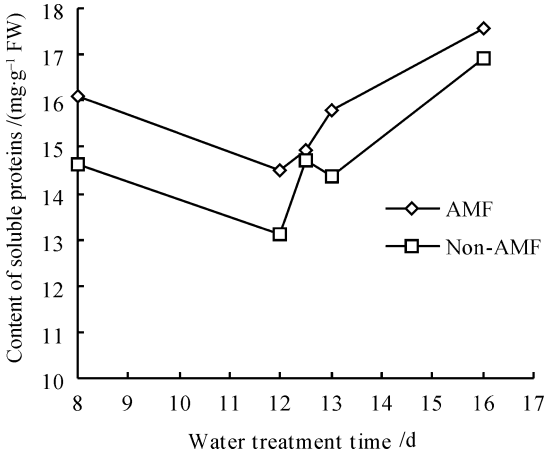

Fig. 2 Effect of AM colonization on the content of soluble proteins in *P. trifoliata* seedling leaves during water stress and rewatering

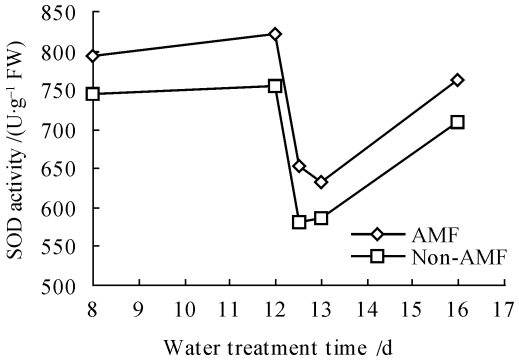

Fig. 3 Effect of AM colonization on SOD activity in *P. trifoliata* seedling leaves during water stress and rewatering

indicated that the inoculation of AM enhanced the defense system of roots and prevented damages to root cells after rewatering treatment.

3.3 Effect of AM colonization on soluble sugars in leaves

Fig. 1 shows that water stress increased the content of soluble sugars in leaves, which was 36% and 45% higher in AM seedlings than in non-AM seedlings at 8 and 12 days after water stress, respectively. The content of soluble sugars in AM and non-AM seedling leaves sharply dropped by 57% and 31% within 12 h after the rewatering treatment and then gently decreased. In addition, AM seedlings consistently had a higher content of soluble sugars in leaves than non-AM ones during the whole rewatering period. The greatest difference in the content of soluble sugars between AM and non-AM seedlings was observed on the 12th day after water stress.

3.4 Effects of AM colonization on enzymatic and nonenzymatic antioxidant defense systems in leaves

It was generally observed that the content of soluble proteins decreased markedly in both AM and non-AM leaves during water stress. The contents of soluble proteins

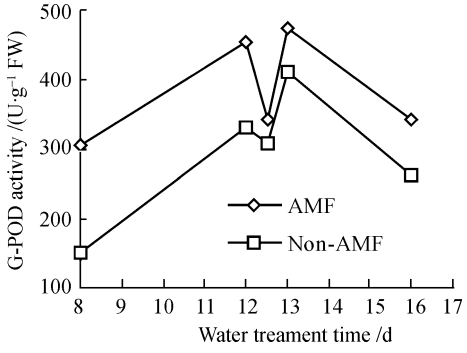

Fig. 4 Effect of AM colonization on G-POD activity in *P. trifoliata* seedling leaves during water stress and rewatering

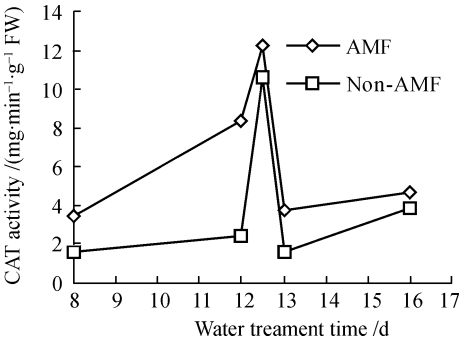

Fig. 5 Effect of AM colonization on CAT activity in *P. trifoliata* seedling leaves during water stress and rewatering

in AM leaves were 7.5% and 10.5% higher than those in non-AM leaves. They increased gradually to a level that was even higher than the original ones during subsequent rewatering treatment (Fig. 2). It could also be noted that AM leaves consistently showed a higher content of soluble proteins compared with non-AM leaves during the whole water treatment.

In leaves, water stress increased the activity of SOD, but the rewatering treatment reduced it sharply within 12 h (Fig. 3). Subsequently, the activity of SOD in AM leaves had a weak decline, but that in non-AM left a minor increment. From 1 to 4 days of rewatering, the activity of SOD in both AM and non-AM leaves increased roughly to the level before water stress.

Water stress greatly increased the activity of G-POD in both AM and non-AM leaves, and those in AM leaves were 61% and 29% higher than those in non-AM leaves in 8 and 12 days of water stress, respectively (Fig. 4). When seedlings were subjected to rewatering, the activity of G-POD began to decline in 12 h and increased in the next 12 h. From 1 to 4 days of rewatering, the activity of G-POD was decreased by rewatering. Although water treatments induced different responses to the activity of G-POD in leaves, AM leaves tended to have a greater activity of G-POD during water treatments. The greatest difference between AM and non-AM seedlings was seen in 8 days of water stress.

Fig. 5 shows that the activity of CAT in both AM and non-AM leaves increased from 8 days of water stress to 12 days of rewatering, and decreased from 12 to 1 day of rewatering. However, CAT activity increased a little in the next 3 days. The values of CAT in AM leaves were higher than those in non-AM leaves during water treatments. The greatest difference between AM and non-AM seedlings was seen in 12 days of water stress.

4 Discussion

It is well-documented that the inoculation of low phosphorus with AMF stimulated the growth of citrus seedlings. In our work, AM inoculation significantly stimulated the growth (e.g., stem diameter, plant height, and plant fresh weight) of trifoliate orange seedlings before water treatments. This is consistent with previous findings, where AM seedlings grew more vigorously than non-AM seedlings [13]. AM's effect on host plant growth has been related to improved mineral nutrition, especially phosphorus.

Our data indicated that the content of soluble sugar in AM leaves was higher than that in non-AM leaves during water treatments, confirming earlier findings [14] and suggesting that natural physiological metabolisms of non-AM leaves were less than those of AM leaves during water stress and rewatering. On the other hand, this result also suggested that AM inoculation was propitious to the accumulation of carbohydrates, especially soluble sugars, in adversity conditions, resulting in a decrease of osmotic potentials in host cells. Thus, AM seedlings could take up more water from the soil in water stress conditions and could rapidly resume plant growth after rewatering.

Cells continuously produce free radicals and reactive oxygen species as part of metabolic processes [15]. These free radicals are neutralized by an elaborate antioxidant defense system, including enzymatic and nonenzymatic antioxidants. The balance is steady in ample water conditions. When plants are subjected to water stress, the balance is broken, resulting in oxidative damage to proteins, DNA, and lipids. On the other hand, to counteract the toxicity of reactive oxygen species, a highly efficient antioxidant defense system, including both enzymatic and nonenzymatic constituents, is present in plant cells [16]. In the present study, AM seedlings tended to have a higher content of soluble proteins in the leaves and roots during water treatments, indicating that AM infection might alleviate or decrease RNA disassembly and might enhance the ability of nonenzymatic antioxidant defense systems by means of soluble proteins. Compared with non-AM leaves, AM leaves showed superior values of SOD, G-POD, and CAT, and maintained the difference up to the end of water treatments, suggesting that AM colonization could enhance the ability of enzymatic antioxidant defense systems. Thus, in adversity conditions, there was less peroxidation of membrane lipids in leaves inoculated with AMF. This agrees with previous reports obtained from tobacco [17,18], soybeans [19], lettuce [20], *Hippophae rhamnoides* [21], sugarcane [9], and three shrub species [22]. Mycorrhiza-induced increases in the activity of several antioxidant enzymes were not associated with mycorrhiza-induced increases in phosphorus content [23].

Li and Feng [24] reported that AMF might increase the drought tolerance of plants by means of mycorrhiza-induced increases in enzymatic antioxidants. In our experiment, AM colonization induced increments of several antioxidant enzymes (e.g., SOD, POD, and CAT) in leaves and roots, resulting in the greater drought tolerance of AM seedlings in water stress and rewatering conditions. Moreover, our data also showed that AM seedlings tended to have a greater nonenzymatic antioxidant defense system (e.g., soluble protein), keeping a higher physiological metabolism in AM seedlings during adversity. Thus, we concluded that the functional mechanism of AMF in enhancing the drought tolerance of the host plant was closely related to enzymatic and nonenzymatic antioxidant

defense systems, including mycorrhiza-induced increases in both the activities of SOD, G-POD, and CAT, and the content of soluble proteins.

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References

1. Ruiz-Sanchez M.-C., Domingo R., Save R., Biel C. and Torrecillas A., Effects of water stress and rewatering on leaf water relations of lemon plants, Biol. Plant., 1997, 39: 623–631
2. Gadkar V., David-Schwartz R., Kunik T. and Kapulnik Y., Arbuscular mycorrhizal fungal colonization. Factors involved in host recognition, Plant Physiol., 2001, 127: 1493–1499
3. Lu J.-Y., Mao Y.-M., Shen L.-Y., Peng S.-Q. and Li X.-L., Effects of VA mycorrhizal fungi inoculated on drought tolerance of wild jujube (*Zizyphus spinosus* Hu) seedlings, Acta Hortic. Sin., 2003, 20: 29–33 (in Chinese)
4. Johnson C.-R. and Hummel R.-L., Influence of mycorrhizae and drought stress on growth of *Poncirus* × *Citrus* seedlings, HortScience, 1985, 20: 754–755
5. Auge R.-M., Water relations, drought and vesicular–arbuscular mycorrhizal symbiosis, Mycorrhiza, 2001, 11: 3–42
6. Wu Q.-S. and Xia R.-X., Research and application on vesicular–arbuscular mycorrhiza of fruit trees, Plant Physiol. Commun., 2003, 39: 536–540 (in Chinese)
7. Wu Q.-S. and Xia R.-X., Effects of arbuscular mycorrhizal fungi on plant growth and osmotic adjustment matter content of trifoliate orange seedlings under water stress, J. Plant Physiol. Mol. Biol., 2004, 30: 583–588 (in Chinese)
8. Wu Q.-S. and Xia R.-X., The relation between vesicular–arbuscular mycorrhizae and water metabolism in plants, Chin. Agric. Sci. Bull., 2004, 20: 188–192 (in Chinese)
9. Wang Y.-Z., Zhang M.-Q., Ke Y.-Q., Pan T.-G., Huang Z.-P. and Liu W., Effects of mycorrhiza preinoculation on the active oxygen metabolism of sugarcane leaves under water stress conditions. Eco-Agric. Res., 1995, 10: 10–15 (in Chinese)
10. Phillips J.-M. and Hayman D.-S., Improve procedures for clearing roots and staining parasitic and vesicular–arbuscular mycorrhizal fungi for rapid assessment of infection, Trans. Br. Mycol. Soc., 1970, 55: 158–161
11. Li H.-S., Principles and Techniques of Plant Physiological Biochemical Experiment, Beijing: Higher Education Press, 2000 (in Chinese)
12. Bradford M.-M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem., 1976, 72: 248–254
13. Ishii T. and Kadoya K., Effect of VA mycorrhiza on citrus growth and carbohydrate contents, J. Jpn. Soc. Hortic. Sci., 1989, 58(Suppl. 1): 30–31
14. Subramanian K.-S., Charest C., Dwyer L.-M. and Hamilton R.-I., Arbuscular mycorrhizas and water relations in maize under drought stress at tasselling, New Phytol., 1995, 129: 643–650
15. Urso M.-L. and Clarkson P.-M., Oxidative stress, exercise, and antioxidant supplementation, Toxicology, 2003, 189: 41–54
16. Sundar D., Perianayaguy B. and Reddy R., Localization of antioxidant enzymes in the cellular compartments of sorghum leaves, Plant Growth Regul., 2004, 44: 157–163
17. Li Y.-P., Sun W., Zhao L.-L. and He X.-L., Effects of AM fungi on the growth and leaf protective enzymes of tobacco under K deficiency, Agric. Res. Arid Areas, 2003, 21: 51–53 (in Chinese)
18. Wang Y.-Z., Ke Y.-Q. and Pan T.-G., Effects of different mycorrhizal fungi on physiological metabolism of tobacco seedlings, Chin. J. Appl. Ecol., 2002, 13: 87–90 (in Chinese)
19. Porcel R., Barea J.-M. and 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
20. Ruiz-Lozano J.-M., Azcon R. and Palma J.-M., Superoxide dismutase activity in arbuscular mycorrhizal *Lactuca sativa* plants subjected to drought stress, New Phytol., 1996, 134: 327–333
21. Tang M., Xue S., Ren J.-H., Hu J.-J. and Liu J.-C., Mechanism of the promotion of drought resistance of *Hippophae rhamnoides* with arbuscular mycorrhizal fungi, J Northwest Forest Univ., 2003, 18: 29–31 (in Chinese)
22. Alguacil M.-M., Hernandez J.-A., Caravaea F., Portillo B. and Roldan A., Antioxidant enzyme activities in shoots from three mycorrhizal shrub species afforested in a degraded semi-arid soil, Physiol. Plant., 2003, 118: 562–570
23. Ruiz-Lozano J.-M., Arbuscular mycorrhizal symbiosis and alleviation of osmotic stress. New perspectives for molecular studies, Mycorrhiza, 2003, 13: 309–317
24. Li X.-L. and Feng G., Arbuscular Mycorrhizal Ecology and Physiology, Beijing: Huawen Press, 2001 (in Chinese)