Mycorrhizal efficacy of trifoliate orange seedlings on alleviating temperature stress

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ABSTRACT

Citrus plants often suffer from temperature stress, which seriously inhibits tree growth and even results in tree death. The present experiment was conducted to evaluate the effects of *Glomus mosseae* on growth, root morphology, superoxide dismutase (SOD) and catalase (CAT) activities, and soluble protein content of trifoliate orange (*Poncirus trifoliata*) seedlings at low (15°C), optimum (25°C) and high (35°C) temperatures. Sixty-eight days after temperature stresses, mycorrhizal colonization and number of both entry points and vesicles were significantly inhibited by low or high temperature. Mycorrhizal seedlings recorded significantly higher growth characteristics than non-mycorrhizal seedlings at both optimum and high temperatures, but the beneficial effects were almost lost at low temperature. Generally, mycorrhizal seedlings presented notably higher root traits (projected area, surface area, number of forks and volume) than non-mycorrhizal seedlings regardless of temperature levels. Mycorrhizal colonization significantly increased SOD and CAT activities and soluble protein content at high temperature, increased only SOD activity at optimum temperature, and decreased only soluble protein content at low temperature. It suggests that mycorrhizal alleviation of temperature stress in trifoliate orange seedlings was at high temperature, but the alleviation was obviously weakened at low temperature.

Keywords: antioxidant enzyme; arbuscular mycorrhiza; root morphology; temperature stress; trifoliate orange

Citrus, a most widely produced fruit, has grown commercially in more than 50 countries around the world. Hereinto, China's citrus production holds an important fresh fruit market in the world. Currently, citrus production faces various problems, especially temperature stress in China. The optimum temperature for the growth of citrus species is 22–30°C (Guo et al. 2006). However, south regions of China often suffer from either long-drawn high temperature (35–42°C) during summer or durative cryogenic freezing rain during January to February 2008, which seriously affects normal growth of citrus trees. Therefore, enhancing temperature tolerance of citrus plants appears particularly important and urgent.

Arbuscular mycorrhizal fungi (AMF), the common soil inhabitant fungi, can form symbiotic associations with the roots of ~90% of terrestrial plants, in which plant photosynthates are exchanged for water and mineral resources acquired by the fungi from the rhizosphere. It was previously reported that AM symbiosis can regulate the responses of plants to temperature stress (Matsubara et al. 2004, Ruotsalainen and Kytöviita 2004, Zhu et al. 2009, 2010, Wu and Zou 2010). The strawberry infected with AMF (*Gigaspora margarita*, *Glomus fasciculatum*, *G. mosseae*, *G.* sp., and *G. aggregatum*) showed better growth characteristics (number of leaves and roots, leaf area, diameter of crown and dry weights of leaves and roots) than a non-AMF

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strawberry at high temperature stress (over 35°C) (Matsubara et al. 2004). When maize plants were subject to low temperature stress, AM symbiosis could enhance stressed tolerance of maize plants via alterations in host water status, carbohydrate, protein, antioxidant enzyme activities (Zhu et al. 2009, 2010). However, other studies observed that the mycorrhizal benefit for the host plant (e.g. *Citrus tangerine* and *Gnaphalium norvegicum*) was almost lost at a low temperature (Ruotsalainen and Kytöviita 2004, Wu and Zou 2010). Additionally, the effect of suboptimum temperature on mycorrhizal formation of citrus plants has received little attention.

Trifoliate orange (*Poncirus trifoliata* L. Raf.), a deciduous and inedible ornamental citrus relative, has been regarded as the main citrus rootstock used in China. It is not clear if mycorrhizas can improve growth of trifoliate orange seedlings at suboptimal temperatures, and the beneficial effects are dependent on temperature conditions. The objective of this study was to examine the interaction of temperature stress and AMF on plant growth, antioxidant enzyme activities and soluble protein content of deciduous trifoliate orange seedlings. Information gained would be beneficial for critically evaluating the potential of AMF to alleviate temperature stress.

MATERIAL AND METHODS

Experimental design. The experiment was a 2 × 3 completely factorial design, which comprised two mycorrhizal inoculations (*Glomus mosseae* and non-AMF) and three temperature treatments (low temperature 15°C; optimum temperature as control 25°C; high temperature 35°C). Each treatment was replicated three times in a randomized design, and each replicate comprised three seedlings.

Plant culture. The trifoliate orange (*Poncirus trifoliata* L. Raf.) seedlings, which had germinated on moistened filter papers in the dark at 25°C for seven days, were transferred into plastic pots (18 cm in depth and 20 cm in mouth diameter) containing 3.4 kg of autoclaved (121°C, 0.11 MPa, 2 h) soil mixture (yellow soil/vermiculite/sphagnum, 5/2/1, v/v/v), which had received either 15 g inocula of *Glomus mosseae* (495 spores, hyphae, and infected roots of *Sorghum vulgare*) or a filtrate of soil inocula free from *G. mosseae* as the non-AMF control, respectively. The characteristics of growth substrate were pH 6.2, 9.6 g/kg organic matter, 16.45 mg/kg available phosphorus and 0.51 g/kg total phosphorus. These pots were placed in a plastic greenhouse at the College of Horticulture and Gardening, Yangtze University (Jingzhou, China) from March 20 to June 8, 2009. The photo flux density ranged from 600 to 821 μmol/m^2/s during the entire experiment. The average day/night temperature was 23°C/16°C; the air relative humidity was 70–95%.

Temperature treatments. After acclimatization for 80 days, the seedlings were subjected to temperature stress on June 8, 2009. These mycorrhizal and non-mycorrhizal seedlings under the conditions of plastic greenhouse transferred to three controlled growth chambers (PQX, Life Apparatus, Ningbo Life Science and Technology Ltd., China) at 15, 25, or 35°C, 16:8 photoperiod, 80% air relative humidity and light intensity 1700 Lx. Each growth chamber was controlled at a designed temperature (15, 25 or 35°C) and divided into two layers, and six pots (three mycorrhizal and three non-mycorrhizal pots) per temperature treatment were placed there. To avoid chamber-specific effects, these pots were swapped among the three chambers every week. The temperature treatments ended at August 15, 2009.

Parameter measurements. Plant height, stem diameter and leaf number per plant were recorded before harvest. At harvest, the shoots were separated from the roots, and the shoot fresh weight was recorded. The fresh roots were washed free of all media, and then scanned by root automatism scan apparatus, EPSON Expression/STD 4800 scanner (Seiko Epson Corp., Nagano, Japan). Subsequently, the root morphological traits, including projected area, surface area, total volume, average diameter and number of tips and forks were determined based on the image analysis using WinRHIZO Pro 2007b software (Regent Instruments Inc., Quebec, Canada). The root fresh weight was recorded after scanning.

A small quantity of 1-cm fresh root pieces (30 root segments per pot) was cleared with 10% (w/v) KOH solution and stained with 0.05% (w/v) trypan blue in lactophenol (Phillips and Hayman 1970). The AM colonization was observed using a dissecting microscope (LEICA DME, Wetzlar, Germany) and quantified according to the following formula:

$$\text{AM colonization (\%)} = \frac{\text{root length infected}}{\text{root length observed}} \times 100$$

At the time of microscopical observation, entry points, vesicles and arbuscules were counted from the infected roots and described as the number per cm root.

Fresh leaf samples (0.3 g) were homogenized with 7 mL of 0.1 mmol/L phosphate buffer (pH 7.8)

and centrifuged at 4 000 g for 10 min at 4°C. The supernatant was collected and stored at 4°C for the analysis of soluble protein, superoxide dismutase (SOD, EC 1.15.1.1) and catalase (CAT, EC 1.11.1.6). SOD activity was measured using the method of Giannopolitis and Ries (1977). One unit of SOD was defined as the amount of enzyme that inhibited 50% nitro blue tetrazolium (NBT) by light, and SOD activity was expressed as SOD units per g fresh weight. CAT activity was performed as described by Wu et al. (2010). Soluble protein content was evaluated by the method of Bradford (1976) using bovine serum albumin as the standard.

Statistical analysis. The data were analyzed using variance analysis (ANOVA) with the SAS software. Fisher's Protected Least Significant Differences ($P < 0.05$) were used to compare the significant differences between treatments and interactions.

RESULTS AND DISCUSSION

Suboptimum temperature can influence an adversely mycorrhizal formation (Tommerup 1983, Daniels Hetrick and Bloom 1984). The present study indicated that high and low temperatures significantly inhibited mycorrhizal colonization and number of entry points compared to optimum temperature treatment (Table 1). It was interesting here that high temperature treatment also maintained 30% higher mycorrhizal colonization and 99% higher number of entry points compared to low temperature treatment. Low temperature, but not high temperature, significantly reduced number of vesicles by 15%. The number of arbuscules was increased by high temperature but decreased by low temperature. These results suggested that mycorrhizal establishment of *G. mosseae*-colonized trifoliate orange seedlings was more susceptible to low temperature than to high temperature, which may be the limited responses of spores of *G. mosseae* to suboptimum temperature (Tommerup 1983).

In general, low or high temperature treatment decreased plant growth characteristics of the seedlings such as leaf number per plant, stem diameter, plant height, shoot and root fresh weights, as compared to optimum temperature (Tables 2 and 3). This result is consistent with the previous report by Allen and Vu (2009). Inoculated seedlings with AMF at 25 and 35°C represented higher these growth characteristics compared with the corresponding un-inoculated ones (Tables 2 and 3). Under low temperature stress, mycorrhizal seedlings only recorded 9% higher stem diameter and 19% higher root fresh weight than non-mycorrhizal ones. The growth traits here indicated that mycorrhizal improvement of plant growth was more significant in high than in low temperature stress (Tables 2 and 3). Wu and Zou (2010) observed that the beneficial roles of AMF on growth of *C. tangerine* seedlings at 25°C were almost lost at 15°C. These results show that mycorrhizas can absolutely alleviate high temperature stress, but the growth improvement was strongly weakened by the low temperature.

Generally, root morphology is strongly affected by suboptimum temperature (Poerwanto et al. 1989, Hund et al. 2008). The present study showed that low temperature inhibited root development but high temperature stimulated root development of trifoliate orange seedlings, as compared with the optimum temperature (Tables 2 and 4). Previous studies also observed the limited root growth of citrus plants at a temperature below 20°C, as well as no root growth at a temperature below 12 or 14°C (Inoue and Harada 1988). Poerwanto et al. (1989) reported that trifoliate orange budded with satsuma mandarin at high temperature produced more fibrous root and higher length/dry weight ratios of fibrous roots. It seems that high temperature properly stimulated root growth but low temperature limited root growth of trifoliate orange seedlings. When these stressed seedlings were inoculated with exogenous AMF, mycorrhizal seedlings exhibited a marked influence on root characteristics. Mycorrhizal seedlings generally exhibited higher root projected area, root area, number of forks and root volume than non-mycorrhizal seedlings regardless of temperature levels

Table 1. Mycorrhizal colonization and number of vesicles, arbuscules and entry points of *Glomus mosseae*-colonized trifoliate orange roots under temperature stress

Temperature (°C)	Mycorrhizal colonization (%)	Vesicles	Arbuscules	Entry points
		(No./cm root)		
35	48^{b}	4.0^{ab}	4.0^{a}	1.5^{b}
25	61^{a}	4.6^{a}	2.8^{b}	2.1^{a}
15	37^{c}	3.9^{b}	2.3^{c}	0.9^{c}

Different letters within the same column mean significant differences at 5% level by *LSD*

Table 2. Significance of the sources of variation for several indexes in mycorrhizal and non-mycorrhizal trifoliate orange (*Poncirus trifoliata*) seedlings exposed to different temperatures

Indexes	Low temperature	High temperature	AMF	Interaction
Leaf number per plant	**	NS	*	NS
Stem diameter	*	**	**	NS
Plant height	**	**	**	*
Shoot fresh weight	**	**	**	**
Root fresh weight	**	**	**	*
Number of root tips	NS	NS	*	NS
Root projected area	*	**	**	**
Root surface area	**	**	**	**
Root average diameter	NS	NS	NS	NS
Number of root forks	NS	**	**	NS
Root volume	*	**	**	**
Superoxide dismutase	NS	NS	*	*
Catalase	**	**	**	**
Soluble protein	NS	NS	NS	**

NS – not significant; *$P < 0.05$; **$P < 0.01$

(Table 2 and Table 4). Number of root tips was higher in mycorrhizal than in non-mycorrhizal seedlings exposed to 15°C but not 25 and 35°C. Both mycorrhizal inoculation and temperature stress did not affect the average root diameter of the seedlings. A previous experiment showed fewer effects of *G. mosseae* on root morphology of *Citrus tangerine* at 15 and 25°C. Probably mycorrhizal effects on root morphology are independent of temperatures but dependent of citrus species. Better root morphology would benefit water and nutrient uptake of plants from soils (Hodge et al. 2009), thus alleviating temperature stress on mycorrhizal plants. Root morphological improvement due to mycorrhization may be related to a modified endogenous balance of growth regulators such as cytokinins and gibberellins, and the changes of carbohydrates (e.g. glucose, sucrose and polysaccharide) in plant tissues (Berta et al. 1993).

Oxidative stress is commonly induced when plants are subject to high or low temperature stress (Djanaguiraman et al. 2010). At the same time, plants also hold antioxidant defense systems to protect cells from oxidative stress (Tunc-Ozdemir et al. 2009). Herein, SOD, an antioxidant enzyme, can catalyse the dismutation of O_2^- to H_2O_2; CAT dissociates H_2O_2 to oxygen and water. Accumulation of soluble proteins, especially dehydrin proteins, can protect cells from dehydration (Mohammadkhani and Heidari 2008). Therefore, both SOD and CAT are important for plants to tolerate temperature stress. In our experiment, temperature stress did not alter the SOD activities of mycorrhizal seedlings but increased the SOD activities of non-mycorrhizal seedlings (Figure 1). The result suggests more tolerance for temperature stress in mycorrhizal than in non-mycorrhizal seedlings, because non-mycorrhizal seedlings

Table 3. Interacted effects of *Glomus mosseae* and temperature stress on growth characteristics of trifoliate orange (*Poncirus trifoliata*) seedlings

Temperature (°C)	AMF status	Leaf number per plant	Stem diameter (cm)	Plant height (cm)	Shoot fresh weight (g)	Root fresh weight (g)
15	AMF	10.9^{d}	0.198^{b}	9.5^{c}	0.35^{c}	0.25^{d}
	non-AMF	10.8^{d}	0.183^{cd}	8.9^{c}	0.31^{c}	0.21^{e}
25	AMF	15.9^{ab}	0.211^{a}	13.3^{a}	0.62^{a}	0.36^{a}
	non-AMF	13.4^{c}	0.194^{bc}	10.8^{b}	0.44^{b}	0.28^{c}
35	AMF	16.4^{a}	0.180^{d}	11.0^{b}	0.40^{b}	0.32^{b}
	non-AMF	14.3^{bc}	0.166^{e}	9.7^{c}	0.34^{c}	0.22^{ed}

The different letters indicate significant differences among treatments at 5% level by *LSD*

Table 4. Interacted effects of *Glomus mosseae* and temperature stress on root morphological traits of trifoliate orange (*Poncirus trifoliata*) seedlings

Temperature (°C)	AMF status	Number of tips	Projected area (cm^2)	Surface area (cm^2)	Average diameter (mm)	Number of forks	Volume (cm^3)
15	AMF	139^{a}	7.20^{bc}	22.72^{c}	0.483^{a}	296^{bc}	0.262^{bc}
	non-AMF	72^{b}	4.66^{d}	14.40^{d}	0.499^{a}	113^{d}	0.183^{d}
25	AMF	128^{a}	8.13^{b}	29.26^{b}	0.496^{a}	406^{b}	0.306^{b}
	non-AMF	136^{a}	6.54^{c}	20.98^{c}	0.472^{a}	186^{cd}	0.238^{cd}
35	AMF	174^{a}	13.67^{a}	42.48^{a}	0.462^{a}	778^{a}	0.540^{a}
	non-AMF	134^{a}	8.54^{b}	23.38^{c}	0.496^{a}	398^{b}	0.303^{b}

The different letters indicate significant differences among treatments at 5% level by *LSD*

are susceptible to temperature than mycorrhizal seedlings. The temperature stress increased the CAT activities in both mycorrhizal and non-mycorrhizal seedlings. However, low temperature stress induced the accumulation of soluble protein in non-mycorrhizal seedlings but reduced soluble protein content of mycorrhizal seedlings. Similarly, high temperature stress influenced soluble protein content: increased in mycorrhizal seedlings; decreased in non-mycorrhizal seedlings. The incompatible results imply that mycorrhizal symbiosis altered the adaptive responses of soluble protein to temperature stress. The further experiment will need to clarify the function of AMF on soluble protein of plants exposed to temperature stress.

Our study showed that at the low temperature, mycorrhizal presence did not affect the SOD and CAT activities but decreased the soluble protein content by 51% (Table 2; Figures 1 and 2). Therefore, it seems that mycorrhizal plants do not possess the antioxidant capacity to tolerate low temperature stress. At optimum temperature, mycorrhizal infection increased only the SOD activities by 75%, whereas at high temperature SOD and CAT activities and soluble protein content of leaves were 44%, 90%, and 212% higher in the mycorrhizal than in the non-mycorrhizal seedlings, respectively (Table 2; Figures 1 and 2). This is consistent with previous results obtained from Zhu et al. (2009), who reported the increases of SOD and CAT activities in temperature-stressed maize plants colonized by *G. etunicatum*. These results imply that mycorrhizal colonization could increase antioxidant enzymatic activities and soluble protein content to alleviate the damage of reactive oxygen species, thus increasing tolerance of high temperature stress.

In conclusion, inoculation with *G. mosseae* significantly improved plant growth performance and root morphological traits, enhanced SOD and CAT activities, and elevated soluble protein content of trifoliate orange seedling under high temperature stress, thus alleviating damage of high temperature stress. However, the alleviation was obviously weakened at low temperature. Therefore, mycorrhizal efficacy in alleviating temperature-stressed trifoliate orange seedlings was dependent on temperature conditions.

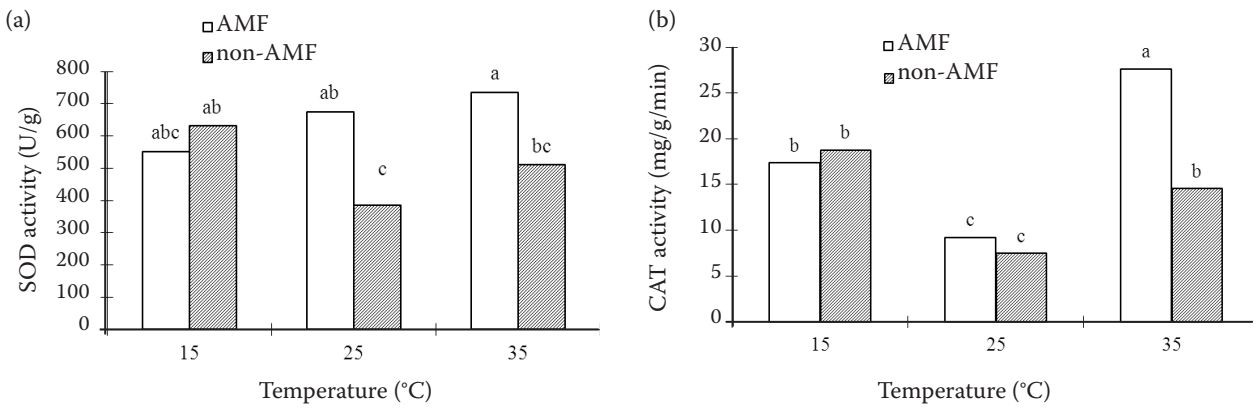

Figure 1. Interacted effect of *Glomus mosseae* and temperature stress on (a) superoxide dismutase (SOD) and (b) catalase (CAT) activity in leaves of trifoliate orange (*Poncirus trifoliata*) seedlings. Means followed by the different letters indicate significant differences among treatments at 5% level by *LSD*

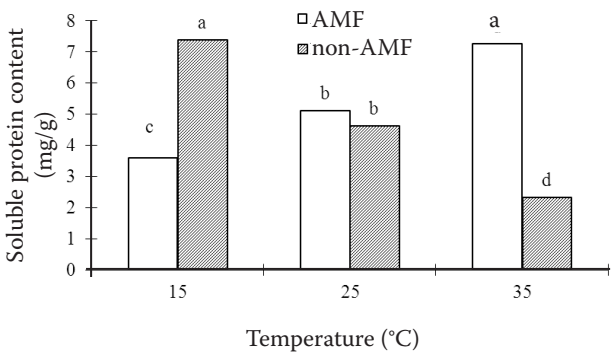

Figure 2. Interacted effect of *Glomus mosseae* and temperature stress on soluble protein content in leaves of trifoliate orange (*Poncirus trifoliata*) seedlings. Means followed by the different letters indicate significant differences among treatments at 5% level by *LSD*

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