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RELATIONSHIPS BETWEEN MYCORRHIZAS AND ANTIOXIDANT ENZYMES IN CITRUS (*CITRUS TANGERINA*) SEEDLINGS INOCULATED WITH *GLOMUS MOSSEAE*

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Abstract

A potted experiment was conducted to evaluate the effects of an arbuscular mycorrhizal fungus (AMF), *Glomus mosseae*, on growth performance and superoxide dismutase (SOD) and catalase (CAT) activities of citrus (*Citrus tangerina*) seedlings. After five months of AMF inoculation, mycorrhizal colonization and vesicles, but not arbuscules and entry points, increased with the increase of inoculated mycorrhizal dosages among 5 40 g (32 spores/g dosage). Mycorrhizal inoculation with 10 40 g dosages significantly increased plant growth traits, including plant height, stem diameter, and shoot, root and total fresh weights. Higher leaf chlorophyll content was found in all the mycorrhizal plants, compared with the non-mycorrhizal plants. Inoculation with *G. mosseae* markedly decreased SOD and CAT activities of leaf and root, except an increase of either root CAT with the 20 g mycorrhizal treatment or root SOD with the 20 and 40 g mycorrhizal treatments. In addition, mycorrhizal colonization and vesicles significantly positively correlated with root SOD and without root CAT. We also discussed the relationships between mycorrhizal effects on antioxidant enzymes and growth environment of host plants.

Introduction

Arbuscular mycorrhizal fungi (AMF), the most common mycorrhizal fungi inhabited in the soil, can colonize into cortical cells of roots, thus forming intracellular structures, vesicles and arbuscules in the root cortex (Gaude *et al*., 2012). AMF can establish symbiotic associations with the roots of 90% terrestrial plants, namely arbuscular mycorrhizas (AMs) (Gadkar *et al*., 2001). AMF can inhabit various natural ecosystems and thereby present lots of physiological and ecological functions (Burni *et al*., 2011). It was well documented that inoculation with AMF obviously enhanced growth of the host plant by improving mineral nutritions, especially phosphorus, as well as water uptake (Porras-Soriano *et al*., 2009; Smith *et al*., 2011; Abdul-Wasea & Elhindi, 2011).

Various organelles of plants, such as chloroplasts, mitochondria, and peroxisomes have antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), etc., which protect the cell against membrane lipid peroxidation by reactive oxygen species (ROS) (Masoumi *et al*., 2010). Therefore, antioxidant enzymes act as a primary antioxidant defense system during adverse stresses. Hereinto, SOD can catalyze the dismutation of O_2 to both O_2 and H_2O_2 and thereby plays a vital role in antioxidant defense systems in plant organisms. CAT, an ubiquitous tetrameric heme-containing enzyme, catalyzes the dismutation of two molecules of H_2O_2 into H_2O and O_2 (Sharma *et al*., 2012). Interestingly, Lanfranco *et al*., (2005) identified a *GmarCuZnSOD* gene isolated from an arbuscular mycorrhizal fungus, *Gigaspora margarita*, which was up-regulated during the fungal life cycle. In addition, H_2O_2, one of ROS, gave priority to accumulate in the cells containing clumped and less branched arbuscules in the roots of *Medicago truncatula* infected by *Glomus intraradices* (Salzer *et al*., 1999). Wu & Zou (2009) observed significantly higher SOD and CAT activities in leaf and root of *G. versiforme*-colonized *Citrus sinensis* grafted on *Poncirus trifoliata* exposed to 12 days drought stress, compared with the non-AMF infected control. However, in *Juniperus oxycedrus* plants, AMF inoculation significantly decreased shoot SOD activity in AM than in non-AM plants under drought stress (Roldán *et al*., 2008). The results suggest that the relationships of AMF with antioxidant enzymes are complex. But, it is unclear whether the relation is also in citrus seedlings and whose structures of mycorrhizas play the key role.

Citrus as one of the most important fruit trees in southern regions of China shows few root hairs in field and thus strongly depends on AMF, especially *Glomus* species. Red tangerine (*Citrus tangerina* Hort. ex Tanaka) is regarded as a major citrus rootstock used in the central and southwestern regions of China. The present work was to analyze the influences of AMF on growth and antioxidant enzymatic activities of citrus seedlings and thus establish the correlation of mycorrhizal structures with antioxidant enzymes.

Materials and Methods

Experimental design: A completely randomized design with AMF dosage as the factor was used. The design consisted of 0, 5, 10, 20 and 40 g inocula of *Glomus mosseae* per pot, respectively described as GM-0, GM-5, GM-10, GM-20 and GM-40. Each treatment replicated five times, for a total of 25 pots.

Experimental setup: The experiment was conducted at Yangtze University, Jingzhou, China, from March to September, 2010. At March 27, 2010, the seeds of red tangerine were surface-sterilized with 70% ethanol for 10 min and then germinated on the sterilized filter papers at 25ºC at darkness. After 9 days, the six two-leaf-old seedlings (~2 cm tall) were transplanted into a plastic pot supplied with 2.7 kg autoclaved (121ºC, 2h) substrate with xanthi-udic ferralsols, vermiculite, and perlite (5 : 1 : 1, v/v/v, pH 5.8). At transplantation of seedlings, the different dosages of *Glomus mosseae* inocula were given

at 5 cm depth below the growth substrate of each pot for AMF treatments. The inocula from the Bank of Glomeromycota in China consisted of hyphae, infected root segments of *Sorghum vulgare*, and spores (32 spores/g). The mycorrhizal and non-mycorrhizal plants were placed in a plastic greenhouse without any environmentally controlled equipment at Jingzhou until the plants were harvested at September 7, 2010. There were no additional nutrient solutions supplied during the entire experiment.

Parameter measurement: After harvested, the plants were divided into shoot and root, and then the fresh weights of shoot and root were recorded. Root mycorrhizal colonization was determined by randomly selected ten 1-cm-length root fragments per plant after the clearance with 10% KOH at 90ºC for 1.5 h and staining with 0.05% trypan blue for 5 min (Phillips & Hayman, 1970). The root colonization was observed under a microscope. At the time of microscopical observation, mycorrhizal structures including entry points, vesicles, and arbuscules were counted in the colonized root segments. The root mycorrhizal colonization was expressed by the following formula:

$$\text{Root colonization (\%)} = \frac{\text{Infected root length}}{\text{Observed root length}} \times 100$$

SOD activity was measured according to the method of Giannopolitis & Ries (1977). Meanwhile, one unit of SOD is defined as the amount of enzyme that inhibits 50% nitro blue tetrazolium (NBT) by light. CAT activity was performed as described by Wu *et al.,* (2010). Leaf chlorophyll content was determined by the method of Lichtenthaler & Wellburn (1983) in terms of extraction with 80% acetone.

Statistical analysis: Data were analyzed by the SAS software, and one-factor analysis of variance (ANOVA) was used to compare the significant differences among treatments with the LSD test at the level of 0.05. Pearson's correlation coefficients between mycorrhizal development and SOD or CAT were performed using the Proc Corr's procedure in the SAS.

Results and Discussion

Mycorrhizal development: The mycorrhizal development of red tangerine seedlings grown in different inoculum levels of *G. mosseae* is shown in Table 1. Mycorrhizal colonization, vesicles and arbuscules were the best in the seedlings grown with GM-40 treatment. The entry points of red tangerine seedlings grown in GM-5, GM-10, and GM-20 treatments were not significantly different but significantly higher than those in GM-40. Mycorrhizal colonization and number of vesicules, but not arbuscules and entry points, increased with the increase of inoculated mycorrhizal dosages, suggesting that mycorrhizal development was not dependent on mycorrhizal dosages. Although AMs are usually mutualistic with host plants, Garrido *et al*., (2010) found that at high AM fungal inoculum concentrations, AMs may consider to be parasitic but not symbiotic on plants when net cost of the symbiosis exceeds net benefits.

Plant growth performance: Table 2 showed that plant height, stem diameter, shoot, root, and total fresh weights, and chlorophyll content in mycorrhizal plants were notably higher than those in non-mycorrhizal plants. Wu *et al*., (2011) also reported that after AMF colonization, the biomass of bermudagrass was significantly improved. The fresh weights were the highest in the seedlings grown with GM-20. The chlorophyll content was the highest in the seedlings grown with GM-10, and no significant differences were observed among the seedlings grown with GM-5, GM-10 and GM-20. Plant height, stem diameter, and shoot fresh weight were higher in mycorrhizal plants than those in non-mycorrhizal plants, while no significant differences were detected among the seedlings grown with GM-10, GM-20 and GM-40. All results suggest that 20 g dosage was more effective than other inoculated dosages in increasing growth variates. The mycorrhizal-mediated growth improvement may be due to water and nutrient uptake throught extraradical hyphae of AMs (Smith & Smith, 2011), which expand the areas of nutrient and water absorption in roots from soils (Henrike *et al.*, 2007). So it is reasonable that growth of the seedlings in the present study was increased with mycorrhizal inoculation.

Activities of SOD and CAT in leaf and root: Our previous study showed that *G. mosseae* and *G. versiforme*-inoculations, especially *G. mosseae* significantly increased the SOD and CAT activities of trifoliate orange grown in salt stress (Wu *et al*., 2010). Similar results were also found in *G. versiforme*-colonized *Citrus sinensis* grafted on *Poncirus trifoliata* during 12 days of drought stress (Wu & Zou, 2009). AMF inoculation can also stimulate SOD, peroxidase, and CAT activities in leaf and root of micropropagated trifoliate orange seedlings during acclimation (Wu *et al*., 2006). Zhu *et al.,* (2011) reported that activities of SOD and CAT were significantly higher in *Zea mays* plants inoculated with AMF than with non-AMF under drought stress. In the present study, inoculation with *G. mosseae* markedly decreased CAT activities of leaf and root, except an increase of root CAT with GM-20 treatment (Fig. 1). Lowest leaf CAT activity was found in the seedlings with GM-5 treatment, and lowest root CAT activity with GM-10 treatment. The results were in agreement with the findings of Porcel *et al*., (2003), who observed that CAT activity in nodules was lower in mycorrhizal soybean plants than in non-mycorrhizal plants under drought stress. In leaf, inoculated treatments except GM-20 and GM-40 significantly decreased SOD activity (Fig. 1). In root, GM-5 and GM-10 treatments did not alter SOD activity but GM-20 and GM-40 treatments significantly increased SOD activity (Fig. 1). Since the dyanmic balance between ROS production and elimination is kept in AM and non-AM plants exposed to optimum environment, AM plants do not induce antioxidant enzymatic defense system to scavenge ROS. It suggests that under normal environments, mycorrhizal seedlings did not show higher antioxidant capacity than non-mycorrhizal seedlings.

Table 1. Root mycorrhizal development of red tangerine (*Citrus tangerina*) seedlings grown in different inoculum dosages of *Glomus mosseae*.

G. mosseae inocula (g)	Root colonization (%)	Arbuscules (num./cm root)	Entry points (num./cm root)	Vesicles (num./cm root)
GM-0	0.0 ± 0.0e	0.0 ± 0.0b	0.0 ± 0.0c	0.0 ± 0.0d
GM-5	26.6 ± 7.6d	2.6 ± 0.9a	1.6 ± 0.7a	3.7 ± 0.9c
GM-10	38.6 ± 4.2c	2.7 ± 1.2a	1.6 ± 0.4a	5.2 ± 0.6b
GM-20	46.4 ± 6.1b	2.5 ± 0.9a	2.0 ± 0.9a	5.7 ± 0.8ab
GM-40	54.2 ± 5.1a	2.9 ± 0.2a	0.8 ± 0.3b	6.9 ± 2.0a

Note: Means± SE (n=5) followed by different letter with the same column mean significant differences at 0.05 level by LSD

Table 2. Effects of different inoculum dosages of *Glomus mosseae* on growth performance and chlorophyll content of red tangerine (*Citrus tangerina*) seedlings.

G. mosseae inocula (g)	Chlorophyll content (mg/g)	Plant height (cm)	Stem diameter (cm)	Fresh weight (g/plant)		
				Shoot	Root	Total
GM-0	38.88 ± 6.81c	6.84 ± 2.97b	0.183 ± 0.013c	0.42 ± 0.23b	0.34 ± 0.01d	0.76 ± 0.22c
GM-5	51.26 ± 4.29ab	7.20 ± 0.85b	0.204 ± 0.012b	0.58 ± 0.27b	0.40 ± 0.06cd	0.98 ± 0.11c
GM-10	53.87 ± 5.42a	13.02 ± 2.10a	0.234 ± 0.016a	1.11 ± 0.50a	0.48 ± 0.27c	1.59 ± 0.52b
GM-20	52.20 ± 5.83a	12.96 ± 0.30a	0.236 ± 0.009a	1.31 ± 0.12a	0.86 ± 0.10a	2.05 ± 0.14a
GM-40	45.76 ± 7.87b	11.03 ± 3.80a	0.239 ± 0.0045a	1.17 ± 0.42a	0.68 ± 0.25b	1.85 ± 0.67ab

Note: Means ± SE (n=5) followed by different letter with the same column mean significant differences at 0.05 level by LSD

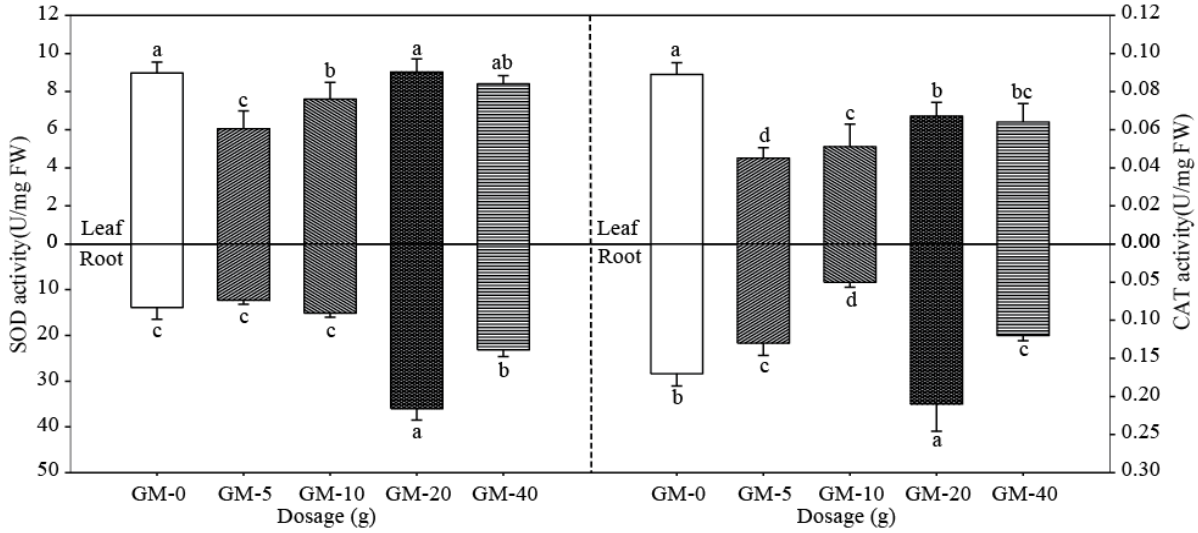

Fig. 1. SOD and CAT activities of red tangerine (*Citrus tangerina*) seedlings grown in different inoculum dosages of *Glomus mosseae*. Means ± SE (n=3) followed by different letter with the bar mean significant differences at 0.05 level by LSD.

Table 3. Pearson correlation coeffcients between mycorrhizal development and antioxidant enzymatic activities of roots (n=5).

	SOD	CAT
Root colonization	0.588*	-0.134
Arbuscules	0.317	-0.053
Entry points	0.342	0.114
Vesicles	0.528*	-0.290

Note: *, p<0.05

Correlation between mycorrhizal development and root antioxidant enzyme activities: Table 3 showed that root CAT was not significantly correlated with root mycorrhizal colonization, arbuscules, entry points, and vesicles, suggesting that mycorrhizal sysmbiosis did not directly alter root CAT activity. However, root SOD was significantly positively correlated with root colonization and vesicles, implying that mycorrhizal symbiosis has the directly positive effect on root SOD. Ruiz-Lozano *et al*., (2001) reported that under well-watered conditions mycorrhizal symbiosis down-regulated the expression pattern of *Fe-sod*, *Mn-sod I*, and *Mn-sod II* genes cloned from luttuce plants colonized by *G. mosseae* and *G. intraradices*, whereas under drought stress conditions mycorrhizal symbiosis significantly increased the expression of the *Mn-sod II* gene. Wu & Zou (2009) observed that root mycorrhizal colonization and arbuscules had a substantive direct effect on root CAT and peroxidase activities of citrus plants grown in drought stress. Diaminobenzidine staining showed that H_2O_2 could accumulate in fungal cytosol, hyphal surface, spore and hyphal wall, arbuscule, and root cells containing clumped or less-branched arbuscules (Fester & Hause, 2005). The

special structures of AMs, to some extent, limit the ROS burst. Combined with the previous reports, the present result suggests that mycorrhizal sysbiosis could affect root antioxidant enzyme activities, which is dependent on growth environment of host plants.

Conclusions

The present study showed that mycorrhizal colonization and vesicles, but not arbuscules and entry points, increased with increase of inoculated mycorrhizal dosages. Mycorrhizal inoculation with 10–40 g dosages significantly increased plant growth traits, including plant height, stem diameter, and shoot, root and total fresh weights. Inoculation with *G. mosseae* markedly decreased SOD and CAT activities of leaf and root, except an increase of root CAT with GM-20 treatment and root SOD with GM-20 and GM-40 treatments. Correlation analysis revealed that root mycorrhizal colonization and vesicles were significantly positively correlated with root SOD, but not with root CAT.

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