Mycorrhiza has a direct effect on reactive oxygen metabolism of drought-stressed citrus

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ABSTRACT

A greenhouse experiment was conducted to investigate the dynamic trend of symbiotic development in *Citrus sinensis*/*Poncirus trifoliata* trees colonized by *Glomus versiforme* during 12-day lasting drought and to evaluate correlation between symbiotic development and reactive oxygen metabolism of citrus. One year after planting, water was withheld from all trees for 12 days. During the drought stress mycorrhizal colonization and arbuscles showed a durative reduction. Mycorrhizal trees maintained significantly lower contents of superoxide anion, hydrogen peroxide and malondialdehyde than non-mycorrhizal control throughout the experiment. There were significantly greater activities of superoxide dismutase, guaiacol peroxidase and catalase in mycorrhizal trees throughout the drought stress period. Ascorbate and glutathione contents of mycorrhizal trees were notably higher than those of uninoculated ones during 12 days of drought stress. Correlation analysis showed that not vesicles and entry points but mycorrhizal colonization and arbuscules had a substantive direct effect on reactive oxygen metabolism. These results suggest that mycorrhizal colonization and arbuscles play a major role in improving reactive oxygen metabolism of drought-stressed citrus, thus inducing a lower oxidative damage.

Keywords: arbuscular mycorrhizal fungi; drought; citrus; reactive oxygen species

Arbuscular mycorrhizal (AM) symbiosis is the most important mutualistic association between arbuscular mycorrhizal fungi (AMF) from soils and the roots of terrestrial plants (Gadkar et al. 2001). It is well documented that AM symbiosis can improve plant water relations or enhance drought resistance of host plants, thus protecting host plants against detrimental effects caused by drought stress (Augé 2001). It is now accepted that the contribution of AM symbiosis to plant drought resistance is the result of accumulative physical, nutritional, physiological and cellular effects (Aliasgharzad et al. 2006). Presence of AM symbiosis might increase the drought tolerance of citrus plants by promoting both the activities of antioxidant enzymes and the contents of antioxidants (Wu et al. 2006a, b). However, the exact role of the symbiosis in improving reactive oxygen metabolism of citrus remains unclear, though mycorrhiza-induced increases in the activities of several antioxidant enzymes are often associated with mycorrhiza-induced increases in shoot biomass and P or N content (Alguacil et al. 2003). Little attention in these literatures was paid to the correlation between symbiotic development and these biochemical variables, as well as the dynamic trend of symbiotic development during drought stress.

In field, most citrus plants lack root hairs and are strongly dependent on AM symbiosis, because AM symbiosis replaces partly the function of root hairs to uptake nutrition and water. Grafted citrus trees are usually used in produced orchard, but little is known about biochemical responses of grafted citrus trees colonized by AMF to drought stress. The objective of the present work was to examine the dynamic trend of symbiotic development of AM grafted citrus tree exposed to drought stress, and to evaluate the relationship between symbiotic development and reactive oxygen metabolism of grafted citrus tree.

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MATERIALS AND METHODS

The experiment was randomized in complete blocks with mycorrhizal treatments (*Glomus versiforme* (Karsten) Berch and non-AMF) and days of soil drying (0, 3, 6, 9, and 12 days of soil drying) to give a 2 × 5 factorial with three replications.

Seeds of trifoliate orange (*Poncirus trifoliata* (L.) Raf.) were surface-sterilized with 5% H_2O_2 for 5 min and sown into 15 × 20 cm plastic pots containing 2.78 kg of autoclaved medium (0.11 MPa, 121°C, 2 h) of yellow soil, vermiculite and sphagnum (2:1:1, v/v/v), whose characteristics were pH 5.9, available phosphorus 22.38 mg/kg, available potassium 141.72 mg/kg and ammonium nitrogen 300.90 mg/kg. Thirteen-gram inocula of *G. versiforme* containing approximately 845 spores were placed 5 cm below seeds in each pot at sowing. The mycorrhizal inocula were provided by the Institute of Plant Nutrition and Resources, Beijing Academy of Agriculture and Forestry Sciences. Non-AM treatments received 13 g of autoclaved medium per pot. The experimental pots were placed in a greenhouse without temperature-regulating equipment and conducted in Huazhong Agricultural University, Wuhan from September 2003 to September 2004. All of the seedlings were 'T' budded with Newhall navel orange (*Citrus sinensis* Osbeck) scion in September 2004.

The grafted citrus trees inoculated with *G. versiforme* or uninoculated were grown for about 1 year in the greenhouse under ample water conditions. The trees were watered to field capacity (32% volumetric soil moisture) with deionized water every third day. Soil water of pots was controlled to field capacity on September 8, 2005, and was then subjected to soil drying by withholding water for 12 days. The plant samples were collected from three pots per treatment at 0, 3, 6, 9, 12^{th} days of soil drying and then were used for assays.

Roots were carefully washed by tap water, cut into 1-cm root segments, cleared with 10% KOH solution, stained with 0.05% trypan blue in lactophenol (Phillips and Hayman 1970), and microscopically observed for root colonization. At the same time, the number of entry points, vesicules and arbuscules were directly recorded and expressed as number/cm root. Quantification of AM colonization was counted by the following formula:

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

Malondialdehyde (MDA) content was determined by the thiobarbituric acid reaction as previously described by Sudhakar et al. (2001). Hydrogen peroxide (H_2O_2) content was colorimetrically measured by the procedure described by Wang and Jiao (2000). Superoxide anion (O_2^-) content was assayed based on hydroxylamine oxidation reaction ($NH_2OH + 2O_2^- + H^+ \rightarrow NO_2^- + H_2O_2 + H_2O$). The production of NO_2^- was quantified using colorimetric method (Wang and Luo 1990).

Enzymatic extraction products were carried out at 0–4°C. Frozen sample (0.5 g) was homogenised in 5 ml of 0.1M phosphate buffer (pH 7.8) containing 0.1mM EDTA, 1mM ascorbate, 1mM 1,4-dithiothreitol and 2% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 4200 g for 10 min, the resulting supernatant was used for assays. Superoxide dismutase (SOD) activity was measured spectrophotometrically according to its ability to inhibit the photochemical reduction of nitro blue tetrazolim (Giannopolitis and Ries 1977). Guaiacol peroxidase (G-POD) activity was analyzed in 2.9 ml of 0.1M phosphate buffer containing 1.0 ml of 0.05M guaiacol and 1.0 ml of 2% H_2O_2 (Amako et al. 1994). The increase in absorbance at 470 nm was recorded after adding 2.0 ml 20% chloroacetic acid. Catalase (CAT) activity was measured according to the method of Li (2000).

The extract was prepared by grinding 0.5 g of frozen materials with 8 ml of 5% TCA and centrifuged at 15 000 g for 15 min at 4°C. The supernatant was used for the assays. Ascorbate (ASC) and glutathione (GSH) were measured according to the previous methods of Wu et al. (2006a).

Data were subjected to analysis of variance (ANOVA) with mycorrhizal treatment, day of soil drying, and mycorrhizal treatment-day of soil drying interaction as sources of variation, and followed by Fisher's protected least significant difference at a probability of $P < 0.05$. Correlation coefficients among variables were calculated with Proc Corr in SAS.

RESULT AND DISCUSSION

No mycorrhizal structure was observed in the roots of non-AM grafted citrus trees, but inoculated trees were successfully infected by *G. versiforme*. Under the drought stress, entry points and vesicles sharply decreased at an early stage (3 days of drought), quickly reached the maximum 6 days after treatment, and decreased significantly thereafter (Figure 1). Previous works showed a significant reduction of mycorrhizal colonization in citrus seedlings exposed to long-term (80 days) moderate drought (Wu et al. 2006a, b). In the

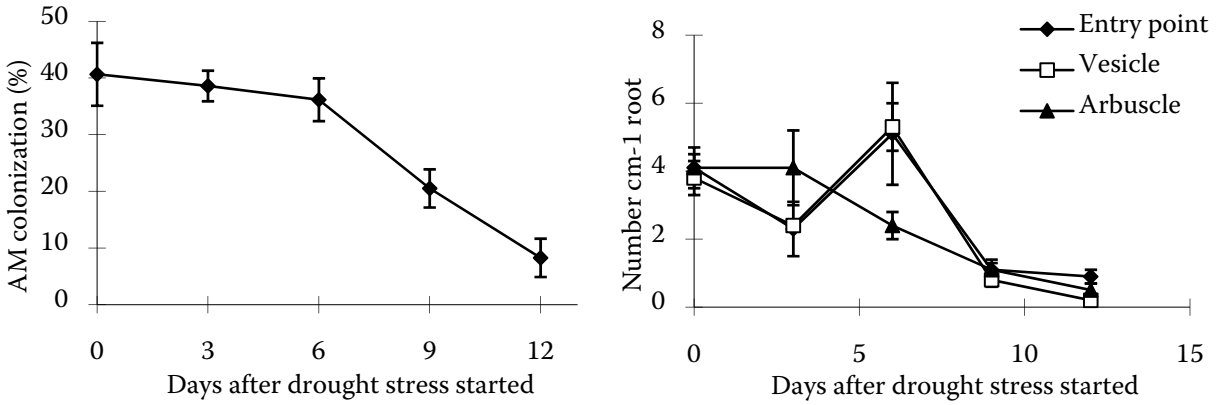

Figure 1. Changes in vesicle, arbuscle, entry point and AM colonization of mycorrhizal grafted citrus trees during 12 days of drought stress

present work, a non-significant reduction in AM colonization was observed only when the grafted citrus trees were subjected to short-term (up to 6 days) soil water deficits (Figure 1), implying that in such instances, low duration of drought does not appear to favor or discourage colonization (Augé 2001). The result is in accordance with the finding by Simpson and Daft (1990), who reported that AM infection was not affected by drought stress. The phenomena might attribute to the unchanged carbon availability from host plants. Once soil drought lasted 12 days, AM colonization and AM structures showed clear reduction in the present study (Figure 1). The results may ascribe to the reduced carbon availability from host plants and the lower spore germination by drought stress. Water availability of a spore depends on the characteristics of interpreting water availability the zone of contact between the spore and the soil. Large changes in the matric potential of the soil

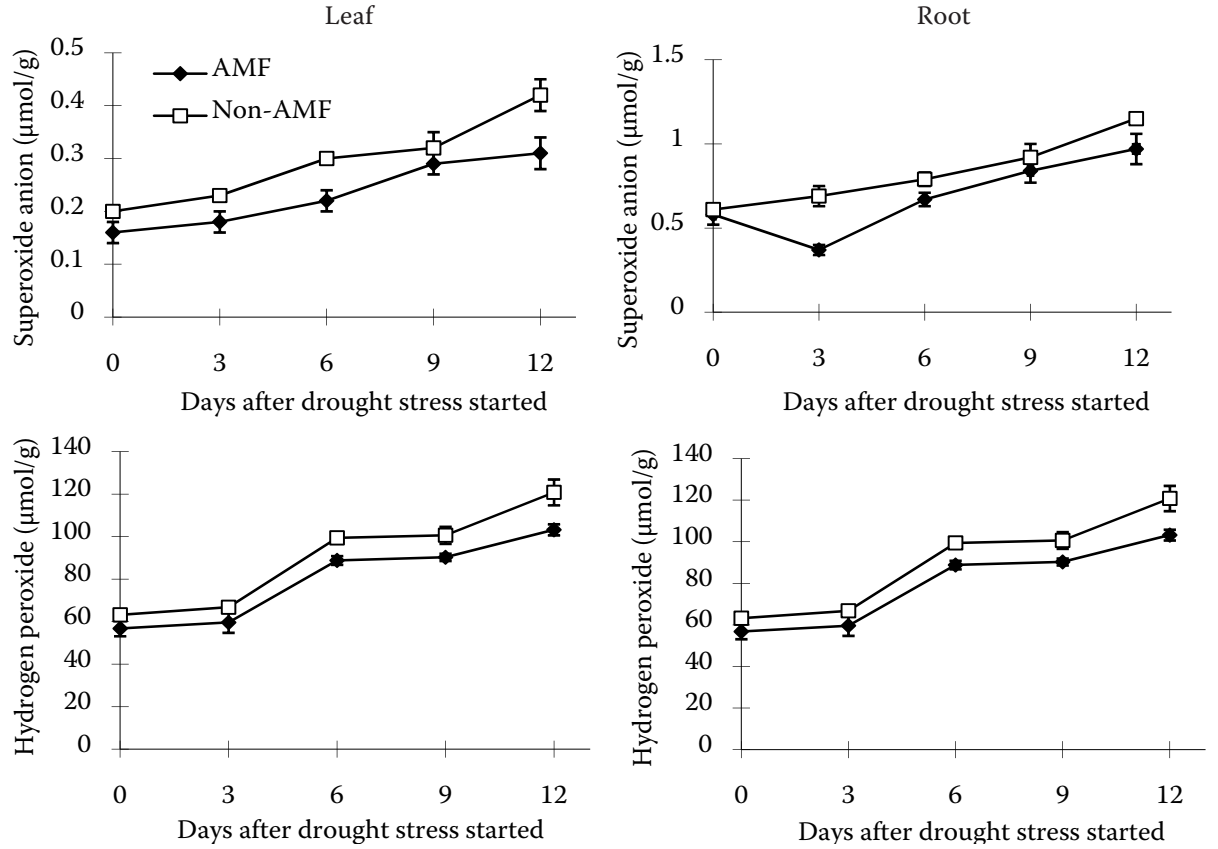

Figure 2. Changes in hydrogen peroxide and superoxide anion contents of AM and non-AM grafted citrus trees during 12 days of drought stress

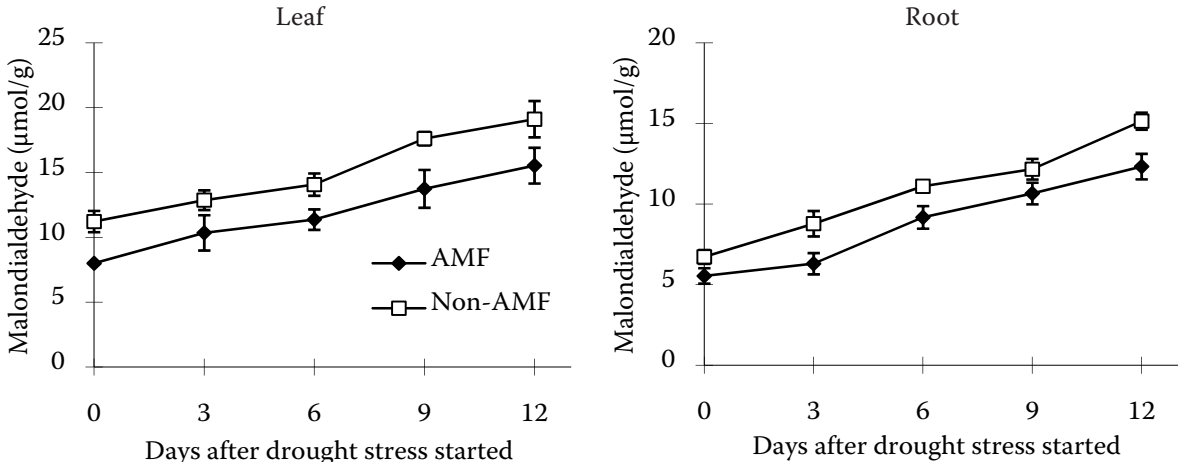

Figure 3. Changes in malondialdehyde contents of AM and non-AM grafted citrus trees during 12 days of drought stress

surrounding a spore result either in recession of water from smaller spores or movement into larger spores with consequential changes in the degree of contact between spores and soil water. As a result, the wetted surface areas of the spore wall are decreased, resulting in the decreases of spore germination. Thus, symbiotic development including entry points, vesicles and arbuscles is strongly repressed by severe drought, inducing the decrease of AM colonization. The AM colonization decreased from 40.65% for well-watered plants (day 0) to 8.27% in plants drought-stressed for 12 days. There was a positive correlation between AM colonization and arbuscles ($r = 0.9311$, $P < 0.05$), suggesting that arbuscles and AM colonization depend on each other during drought.

It was clear that AM trees always exhibited lower H_2O_2, O_2^- and MDA contents of leaves and roots than the corresponding non-AM ones (Figure 2 and Figure 3). H_2O_2 plays a dual role in plants: at low concentrations, it acts as a molecular messenger involved in acclamatory signal triggering tolerance to various stresses, and at high concentrations it orchestrates programmed cell death (Quan et al. 2008). It appears likely that low concentration of H_2O_2 acts as a signal molecule triggering drought tolerance of AM trees, and excess H_2O_2 accumulation of non-AM trees leads to oxidative stress, then inducing programmed cell death. H_2O_2 as a signal molecule is potentially localized in AM symbiosis and is related to arbuscle senescence. Correlation analysis indicated that H_2O_2 content of AM roots was negatively correlated with AM colonization, and H_2O_2 contents of AM leaves and roots were each significantly negatively correlated with arbuscles (Table 1). A diaminobenzidine staining technique confirmed that H_2O_2 accumulation was within cortical root cells in the space occupied by arbuscules, especially pronounced in cells containing clumped and less branched arbuscles

Table 1. Pearson's correlations (r) between symbiotic development and biochemical variables

		O_2^-	H_2O_2	MDA	SOD	G-POD	CAT	ASC	GSH
Leaf	AM colonization	–0.89*	–0.85	–0.95*	0.04	–0.89*	–0.86	0.87	0.91*
	entry points	–0.48	–0.41	–0.72	–0.09	–0.52	–0.71	0.52	0.71
	vesicles	–0.55	–0.43	–0.72	0.00	–0.53	–0.69	0.54	0.70
	arbuscles	–0.94*	–0.97**	–0.95*	–0.19	–0.96**	–0.87	0.99**	0.93*
Root	AM colonization	–0.96*	–0.97**	–0.92*	0.35	–0.87*	–0.94*	0.98**	0.70
	entry points	–0.67	–0.67	–0.56	0.24	–0.51	–0.59	0.67	0.62
	vesicles	–0.17	–0.71	–0.59	0.31	–0.52	–0.64	0.71	0.56
	arbuscles	–0.99**	–0.99**	–0.99**	0.07	–0.94*	–0.88*	0.91*	0.74

* and ** significant at $P < 0.05$ or $P < 0.01$, respectively. Correlation coefficients not followed by asterisk(s) indicate correlation was not significant. + and – signify positive or negative correlation

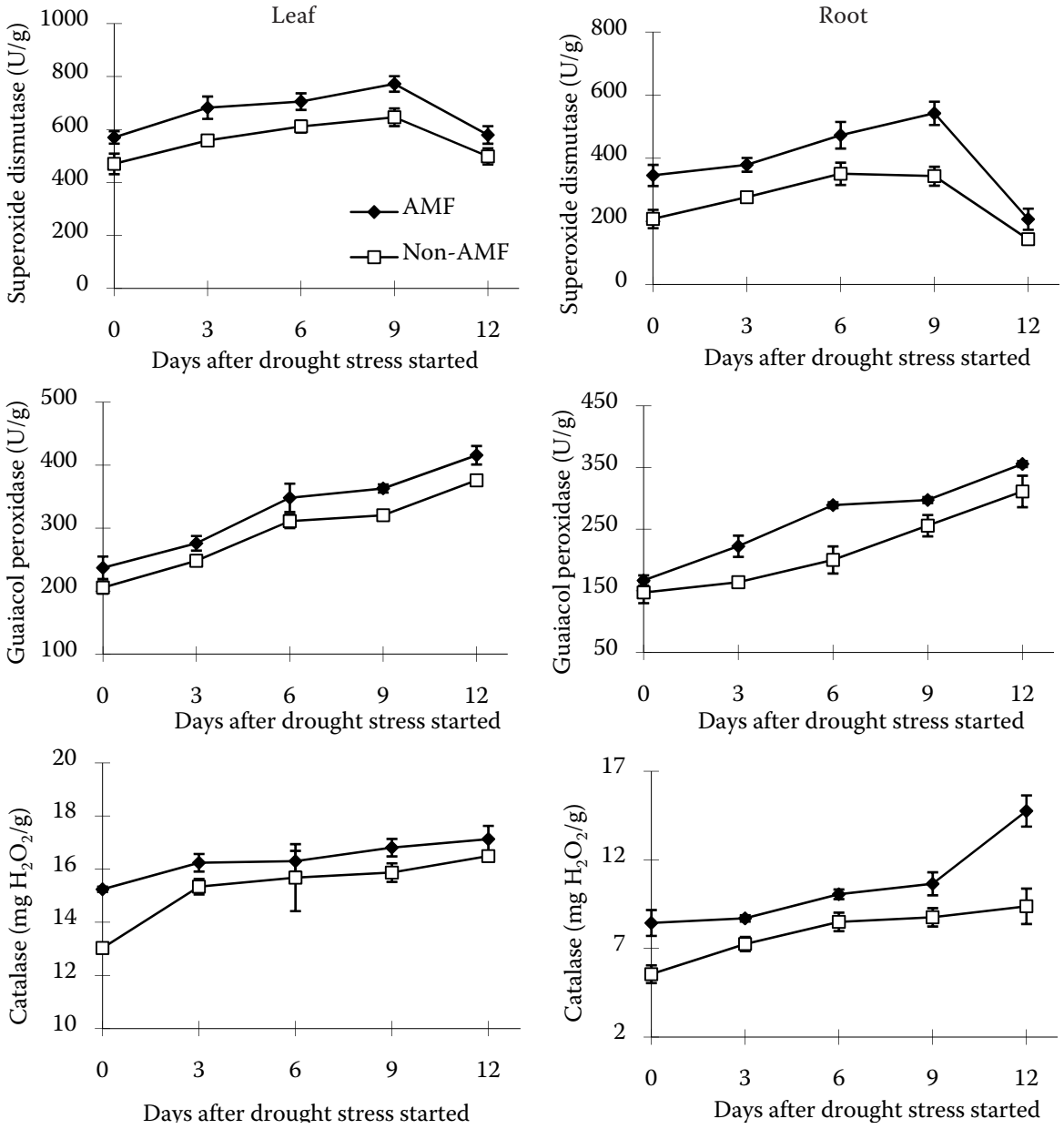

Figure 4. Changes in superoxide dismutase, guaiacol peroxidase and catalase activities of AM and non-AM grafted citrus trees during 12 days of drought stress

(Salzer et al. 1999). Hyphal tips growing along the middle lamella, appressoria and vesicles did not show H_2O_2 accumulation. The intracellular accumulation of H_2O_2 was found in the cytoplasm of *Zea mays* colonized by *G. intraradices* close to intact and collapsing fungal structures, whereas intercellular H_2O_2 was located on the surface of fungal hyphae (Fester and Hause 2005). These findings suggest that arbuscles can accumulate partly H_2O_2, thus alleviating the oxidative burst in host plants to some extent.

The drought stress with AMF treatment lead to the enhancement in SOD, G-POD and CAT activities when compared to non-AMF treatment (Figure 4). According to the correlation coefficients, there were good linear relationships between G- POD activities of AM trees and AM colonization or arbuscles, and CAT activities were significantly negatively correlated with AM colonization or arbuscles only in mycorrhizal roots (Table 1). It seemed that in the three antioxidant enzymes, only G-POD and CAT activities of AM trees would largely rely on AM colonization and arbuscles. The mechanism might be that AMF themselves possess several special genes encoding for antioxidant enzymes, whose expression patterns can regulate the activities of antioxidant enzymes independently, regardless of the mycorrhizal colonized levels. On the other hand, although the AM colonization decreased so much in only 12 days of drought, it does not exclude that the

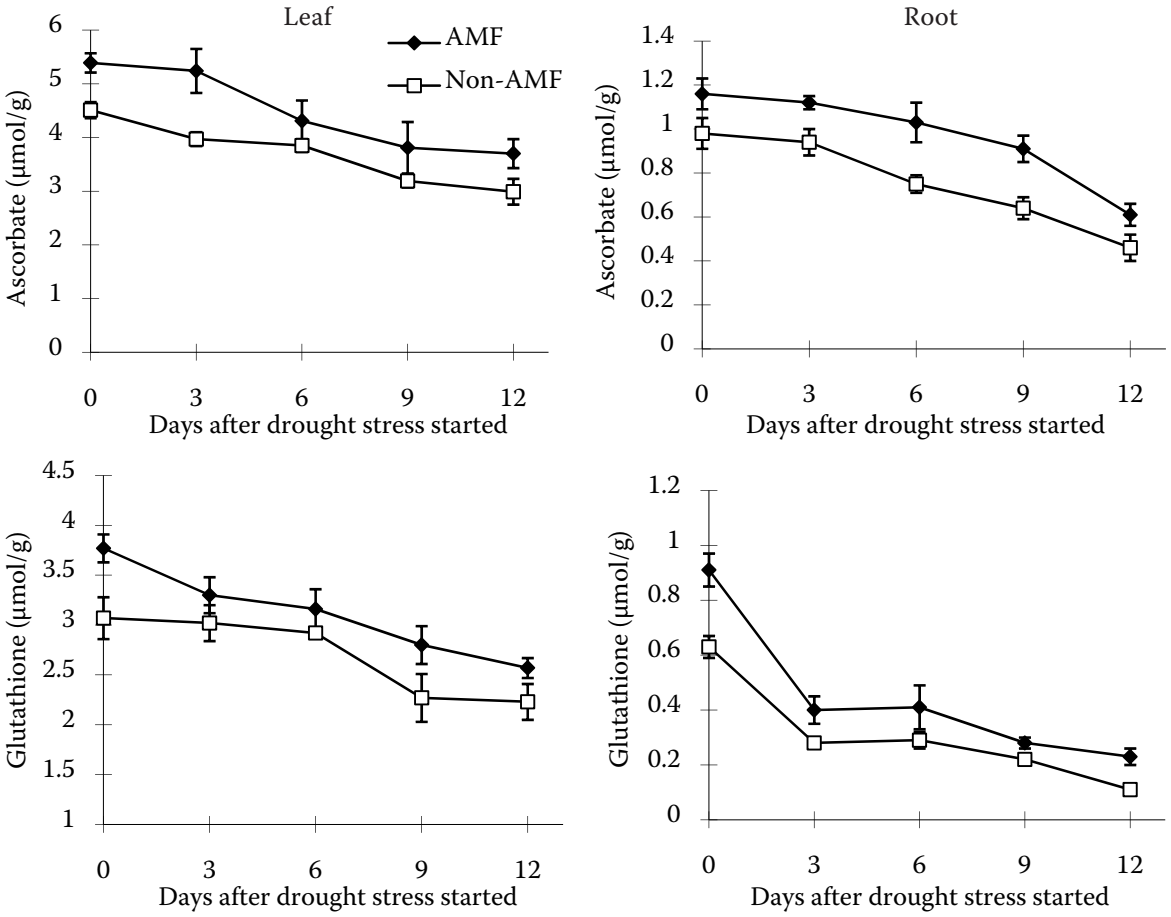

Figure 5. Changes in ascorbate and glutathione contents of AM and non-AM grafted citrus trees during 12 days of drought stress

amelioration of photosynthetic rate, plant biomass, and nutrient status due to mycorrhization might be linked with the increased activities of antioxidant enzymes in mycorrhizal plants (Alguacil et al. 2003, Huang et al. 2008, Roldán et al. 2008).

ASC and GSH are the two key non-enzymic antioxidants in Foyer-Halliwell-Asada cycles. On the basis of the results obtained, the contents of ASC and GSH were higher in AM leaves and roots of grafted citrus trees than in non-AM leaves and roots during drought stress (Figure 5), indicating that the cycle utilizes more ASC as an antioxidant and more GSH as a reductant to regenerate ASC. Therefore, H_2O_2 of AM trees was controlled in a lower level. These observations are in agreement with the findings by Wu et al. (2006a, b) in drought-stressed citrus seedlings and Huang et al. (2008) in *Avena nuda* seedlings exposed to SO_2. The contents of non-enzymatic antioxidants of AM citrus trees were positively correlated with AM colonization or arbuscles (Table 1). Rivera-Becerril et al. (2005) observed that mycorrhizal development in the presence of Cd could activate the expression of homoglutathione synthetase gene. It seemed that during drought, mycorrhizal development, and especially mycorrhizal colonization and arbuscles, might induce some genes coding for antioxidants that are involved in alleviation of damage caused by ROS. The viewpoint will be checked further in the future using molecular clone technique in mycorrhizal drought-stressed citrus plants.

From these investigations it concludes that reactive oxygen metabolism is improved in grafted citrus trees colonized by AMF subjected to drought. In AM symbiosis, only AM colonization and arbuscles have a substantive direct effect on improving reactive oxygen metabolisms.

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