

## Inoculation with *Septoglomus constrictum* improves tolerance to heat shock in tomato plants

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**Abstract:** Arbuscular mycorrhizal fungi (AMF) are symbiotic soil fungi colonizing roots of about 80% of vascular plants. This symbiosis enhances the growth and survival of numerous plant species including vegetables; moreover, it offers some other benefits for the plants. This work aimed to study the impact of AMF on tomato plant tolerance to heat shock. Tomato (*Solanum lycopersicum* L.) plants inoculated or not with *Septoglomus constrictum* were placed in a commercial potting media at 26/22°C with 16/8h photoperiod for six weeks, then exposed to normal (26°C for 6h) or high temperature (42°C for 6h). Arbuscular mycorrhizal (AM) colonization rate, level of lipid peroxidation (malondialdehyde – MDA), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) accumulation and antioxidative enzymes in roots and leaves were measured after the stress application. AM colonization rate of mycorrhizal plants was 73% under non-stress conditions and 68% under heat shock conditions while no mycorrhizal colonization found in non-AM treatments. MDA and H<sub>2</sub>O<sub>2</sub> content substantially increased in leaves of all plants after exposure to the heat shock. Leaf and root peroxidase (POD), leaf catalase (CAT) and root superoxide dismutase (SOD) activities of mycorrhizal plants were enhanced compared to those in non-AM ones while the activity of leaf SOD and root CAT in mycorrhizal plants remained unchanged. Furthermore, there were significant decreases in MDA and H<sub>2</sub>O<sub>2</sub> content in leaves of inoculated plants compared with non-AM ones under heat shock conditions. Our results indicate that AM inoculation can increase stress tolerance against heat shock by enhancing the activity of antioxidative enzymes. Further research is required to understand the mechanisms that contribute to heat tolerance.

**Abbreviations:** AM, arbuscular mycorrhizal; AMF, arbuscular mycorrhizal fungi; CAT, catalase; FW, fresh weight; MDA, malondialdehyde; POD, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase.

**Keywords:** arbuscular mycorrhizal fungi, *Septoglomus constrictum*, heat shock stress, stress tolerance, tomato plants

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### Introduction

Due to the effects of global warming, heat stress has become the major challenge for crop production on earth. Heat stress causes anatomical, morphological, physiological, biochemical, and genetic responses *in planta* (Camejo et al. 2005; Chen et al. 2012; Min et al. 2014), diminishing crop yield and quality. When plants are subjected to very high temperatures, severe injuries in the cell, even death, may take place within minutes. These could be ascribed to a devastating collapse of the cellular system (Schoffl et al. 1999). Nevertheless, such cell damages and death may take place merely after plants are exposed to moderately high temperatures in the long term (Wahid et al. 2007). As direct consequences of heat stress, proteins are denatured and aggregated while the fluidity of membrane lipids is elevated in plants. Indirectly

or slowly, high-temperature stress causes enzyme inactivation in mitochondria and chloroplast, protein synthesis prohibition, degradation of proteins and membrane integrity loss (Howarth 2005). These damages eventually lead to starvation, plant growth reduction, decreased ion flux, generation of toxic compounds as well as reactive oxygen species (ROS) (Howarth 2005).

ROS consist of peroxides, superoxide, hydroxyl radical, and singlet oxygen. ROS overproduction may result in cell death as a consequence of oxidative stress, such as peroxidation of membrane lipid, causing oxidative damage to nucleic acids (Tanou et al. 2009). Peroxidation of membrane lipid is detected by measuring malondialdehyde (MDA) which is a widely used marker of oxidative lipid injury caused by environmental stresses (Kong et al. 2016). To reduce oxidative damage under temperature

stresses, plants have evolved different antioxidative strategies to detoxify harmful ROS components by non-enzymatic and enzymatic antioxidant defence systems where peroxidase (POD), catalase (CAT), superoxide dismutase (SOD) are main enzymatic ROS scavengers (Wu et al., 2014). SOD is the first defense against ROS (Alscher et al. 2002) due to its ability to catalyze the dismutation of  $O_2^{\cdot-}$  to  $H_2O_2$  (Wu et al. 2014). Subsequently, CAT and other scavenging enzymes detoxicate  $H_2O_2$  to  $H_2O$  and  $O_2$  (Apel and Hirt 2004). POD can also generate and detoxify  $H_2O_2$  in the first and next phase, respectively (Siegel 2003).

Studies on plants under temperature stresses are extensively carried out to develop strategies to deal with the adverse effects of heat stress on crop productivity through breeding heat-tolerant varieties, suitable crop shifts and cultivation practices. Most of them are costly and time-consuming whereas noticeably, using beneficial microbes has been proved as a potential solution to improve plant tolerance to various abiotic and biotic stresses. Arbuscular mycorrhizal fungi (AMF), one of the common soil microbes, can form the symbiotic association with roots of 80% of terrestrial plant species. The application of AMF enhances not only nutrient and water uptake but plant tolerance to abiotic stress (Birhane et al. 2012).

Cabral and coworkers (2016) showed that inoculation of an AM mixture including *Rhizophagus irregularis* BEG140, *Rhizophagus irregularis*, *Funneliformis mosseae* BEG95, *Funneliformis geosporum*, *Claroideoglossum claroideum* in wheat plants mitigated adverse effects of temperature stress at 35°C (day) and 25°C (night) for seven days. In maize, inoculation of *Claroideoglossum etunicatum* reduced relative membrane permeability and MDA concentration in roots and leaves of plants while it increased soluble sugar and proline content in roots but lowered leaf proline content, relative to non-AM plants as exposure to 35°C and 40°C for one week (Zhu et al. 2010). Improved net photosynthetic rate ( $P_n$ ), stomatal conductance ( $g_s$ ), transpiration rate ( $E$ ), the maximum quantum efficiency of photosystem II (Fv/Fm) together with higher chlorophyll contents in leaves of mycorrhizal maize plants under such heat stress conditions were observed (Zhu et al. 2011). Enhanced

biomass of mycorrhizal plants under heat stress conditions was also detected (Zhu et al. 2010; Maya and Matsubara 2013; Matsubara et al. 2014; Maya et al. 2014). AM application triggered the higher activity of SOD, ascorbate peroxidase in whole plants (roots, tubers, and leaves) (Matsubara et al. 2014; Maya and Matsubara 2013) and resulted in heightened leaf ascorbic acid and polyphenol in cyclamen plants after exposure to heat stress (Maya and Matsubara 2013). Similarly, POD, SOD, CAT activities were elevated in roots and leaves in mycorrhizal plants under temperature stresses (Zhu et al. 2010). It is worth mentioning that the level and duration of stress (acute versus chronic) significantly affect plant responses (Tattersall et al. 2007; Pinheiro and Chaves 2011). Previous studies on mycorrhizal plants focused on the chronic heat stresses which usually lasted for one week. Therefore, mechanisms underlying the effect of AM inoculation on the ROS metabolism and antioxidative enzymes of host plants under heat shock representing acute stress remain unknown.

Tomato (*Solanum lycopersicum* L.) is a main vegetable crop in the world, widely cultivated optimally in agricultural areas with temperatures between 20°C and 30°C. Tomato is a primary dietary component in different countries because it contains a rich source of vitamins, antioxidant compounds, minerals, sugars, providing significant nutritional value for the human. Nevertheless, tomato productivity is substantially decreased by abiotic stresses (Schwarz et al. 2010).

The objective of this study was, hence, to contribute to the understanding of the effect of AM inoculation on ROS metabolism and the antioxidative activity in tomato plants under heat shock (42°C for 6h). *Septoglossum constrictum*, distributed around the world (Opik et al. 2010), was chosen as the fungal inoculant in our study. A degree of lipid peroxidation (estimated by MDA) and  $H_2O_2$  accumulation, together with POD, SOD, CAT activity in leaves and roots of mycorrhizal tomato plants and non-AM tomato plants under heat shock were examined.

## Material and methods

Tomato (*Solanum lycopersicum*) seeds, cultivar MoneyMaker, were soaked in 2.5% sodium hypochlorite for 20 minutes, then washed with

distilled water five times and placed on wet papers in Petri dishes for germination for three days at room temperature. Germinated seeds were put in plastic pots (0.5-lit volume) with 0.5 kg of the sterile mixture of sand and soil (4:1, v/v). The loamy soil with pH 7.1, 1.61% organic matter, N 15.6 mg kg<sup>-1</sup>, available P 36 mg kg<sup>-1</sup>, available K 60 mg kg<sup>-1</sup> (Duc et al., 2017) was used.

The experiment consisted of 12 plants without AM inoculation and 12 plants inoculated by *Septoglomus constrictum* (formerly *Glomus constrictum* Trappe.). The AM inoculum was cultured in the sterile sand with *Zea mays* as host plants for four months. Thirty grams of the AM inoculum (27 spores g<sup>-1</sup>) were utilised for each pot in AM treatment while plants in non-AM treatment were added the same amount of autoclaved inoculum and 3 ml aliquot of a filtrate (< 20 µm) of the AM inoculum in order to provide a general microbial population free AMF propagules. Pots were put randomly in a growth chamber (EKOCHIL 1500), and the pot positions were changed weekly. Growing conditions, 26/20°C with 16/8 hours photoperiod, light intensity 600 µmol m<sup>-2</sup> s<sup>-1</sup> and 60% humidity were applied. Pots were watered twice and fertilized with Long Ashton nutrient solution (Hewitt, 1966) with low phosphorus level (3.2 µM Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O) once a week. After six weeks of plant growth, heat shock was carried out by transferring six non-AM plants and six AM plants to 42°C for 6h (Zhou et al. 2014) whereas six plants without AMF and six mycorrhizal plants were kept under non-stress conditions. Then all leaf and root samples were collected for further analysis.

#### *Assessment of arbuscular mycorrhizal colonization*

Root samples were washed by tap water and cleaned before staining according to Vierheilig et al. (1998). AM colonization was examined by visual inspection of fungal structures under a stereomicroscope at x 100 magnification. AM colonization rate was determined by the gridline intersect method (Giovanetti and Mosse 1980).

#### *Measurement of hydrogen peroxide accumulation and oxidative damage to lipids*

The H<sub>2</sub>O<sub>2</sub> content was determined by the method

of Alexieva et al. (2001). Shortly, leaf samples (500 mg) were ground with a cold 0.1% (w/v) trichloroacetic acid (TCA) (5 ml), subsequently, centrifuged at 12,000×g at 4°C for 15 min. The reaction mixture consisted of 100 mM potassium phosphate buffer (pH 7.0, 0.5 ml), 1 M KI (1 ml) and the leaf extract supernatant (0.5 ml). The reaction occurred in the dark for 1h. Its absorbance was recorded at 390 nm.

The leaf lipid peroxidation was estimated by the method of Heath and Packer (1969). In detail, leaf samples (200 mg) were ground in 0.1% TCA (5 ml), then centrifuged at 10,000×g for 5 min. A 1 ml of leaf supernatant was mixed with 20% TCA (4 ml) containing 0.5% 2-thiobarbituric acid (TBA). Then the mixture was heated at 95°C for 15 min and immediately cooled. Absorbances of the mixture at 532 nm and 600 nm were recorded for MDA estimation. The content of MDA was estimated using an extinction coefficient of 155 mM<sup>-1</sup> cm<sup>-1</sup>.

#### *Measurement of antioxidant enzymatic activities*

Leaf and root samples (500 mg) were frozen in liquid nitrogen and ground with 50 mM Tris-HCl buffer pH 7.8 (3 ml) containing 1 mM Na<sub>2</sub>EDTA and 7.5% (w/v) polyvinylpyrrolidone K25. Then, crude extracts were centrifuged at 10,000 x g for 20 minutes at 4°C. The supernatants were collected to examine enzyme activities by U-2900 UV-VIS spectrophotometer (Hitachi). Soluble protein contents were estimated according to the method of Bradford (1976).

The activity of peroxidase (POD, EC 1.11.1.7) was tested according to Rathmell and Sequeira (1974). A 2.2 ml reaction mixture included 10 µl plant extract, 12mM H<sub>2</sub>O<sub>2</sub> (100 µl), 50 mM Guaiacol (100 µl) and 0.1 M sodium phosphate buffer (pH 6.0). Changes in the absorption at 436 nm for 5 minutes were recorded. The activity of POD was presented by the changes in absorbance per mg protein per minute.

The activity of superoxide dismutase (SOD, EC 1.15.1.1) was examined by the method of Beyer and Fridovich (1987). A 2 ml 50 mM phosphate buffer (pH 7.8) containing 55 µM NBT, 2 mM EDTA, 9.9 mM L-methionine and 0.025% Triton X-100 and leaf extract (20 µl), 1 mM riboflavin (20 µl) was used for the reaction

mixture. Absorbance changes of the reaction at 560 nm were recorded.

The activity of catalase (CAT, EC 1.11.1.6) was assayed by the method described by Aebi and Lester (1984). The reaction mixture (3 ml) consisted of 10 mM H<sub>2</sub>O<sub>2</sub>, 50 mM potassium phosphate buffer (pH 7.0) and the enzyme extract. Absorbance decreases of the reaction at 240 nm were recorded. CAT activity was presented as absorbance changes per mg protein per minute.

#### Statistical analysis

All data were tested by two-way factorial analysis of variance (ANOVA) using SAS 9.1 (SAS Institute, Cary, North Carolina). The means were compared at the 5% level by Duncan posthoc test.

#### Results

No dead plants were observed in our experiment after heat shock treatment. AM colonization rate of plants colonized by *S. constrictum* was 73% under non-stress conditions and 68% under heat shock conditions whereas no AM colonization was observed in plants without mycorrhiza (Figure 1A). Also, heat shock (42°C in 6 hours) did not change the AM colonization significantly

although a slight decrease occurred. Under non-stress conditions, both AM and non-AM plants had similar MDA values, however; when plants were subjected to heat shock, MDA levels considerably increased in non-AM plants (by 42%) but not in AM plants (Figure 1B).

In addition, AM plants also showed a significant lower MDA (17% lower) than non-AM plants under heat shock conditions. Similarly, there was no significant difference in H<sub>2</sub>O<sub>2</sub> level in AM and non-AM plants under non-stress conditions (Figure 1C). Heat shock increased H<sub>2</sub>O<sub>2</sub> level by six-fold in non-AM plants and by over three-fold in AM plants. H<sub>2</sub>O<sub>2</sub> accumulation reduced by 40% in AM plants, as compared to the corresponding in non-AM plants under heat shock.

As shown in Figure 2A, POD activity in leaves of mycorrhizal plants was not significantly different from that of non-AM plants under non-stress conditions. Nonetheless, exposure to heat shock substantially increased (61-76%) leaf POD level in plants although AM symbiosis induced a considerable higher (9%) POD activity than plants without AM inoculation. Notably, heat shock substantially heightened (19-26%) SOD activity of leaves in plants but there was

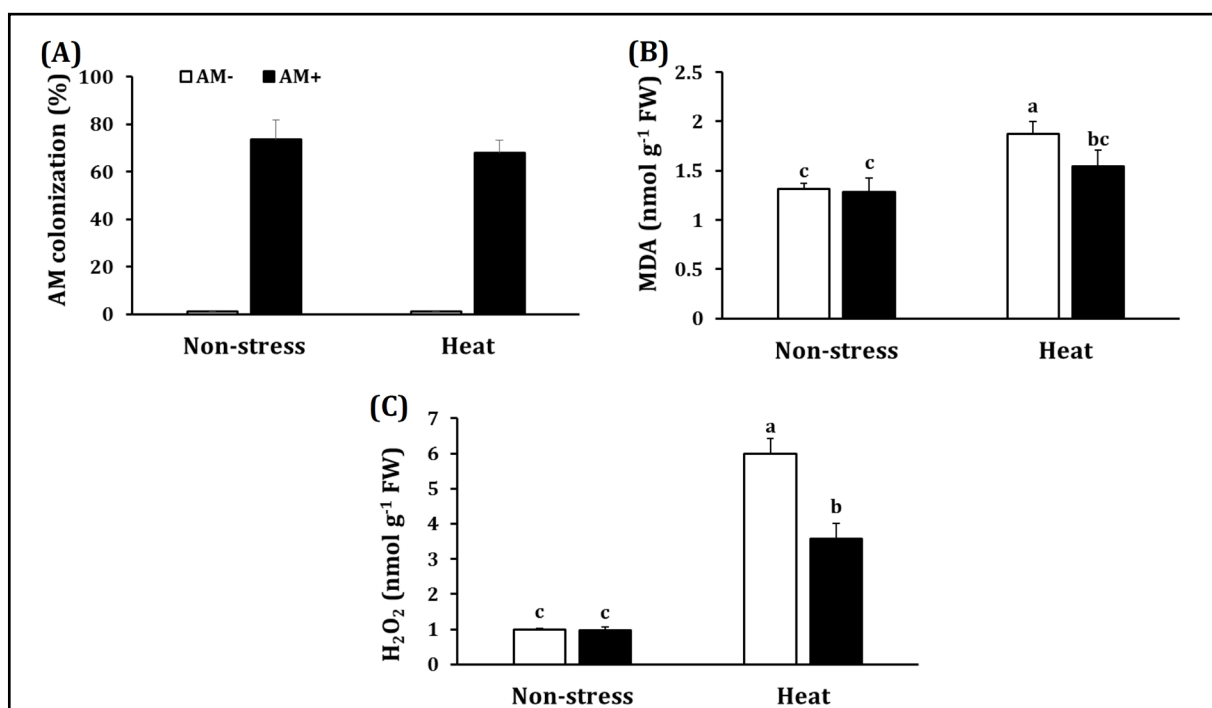


Figure 1. Mycorrhizal colonization rate (A), malondialdehyde (MDA) (B) and H<sub>2</sub>O<sub>2</sub> (C) accumulation in leaves of non-AM plants (AM-) and plants colonized by *Septoglomus constrictum* (AM+) exposed to non-stress, heat-shock conditions. Bars present mean ± standard deviation (n = 4). Different letters present significant differences among treatments according to Duncan posthoc test (P < 0.05).

no significant difference in leaf SOD activity between AM and non-AM plants (Figure 2B). Likewise, leaf CAT activity of colonized plants was considerably improved (increased by 18%), in comparison to that of uncolonized plants under heat shock despite no significant differences in this enzyme between both AM and non-AM plants under non-stress conditions (Figure 2C).

In roots, POD activity had the same pattern as in leaves (Figure 2D). Root POD activity in AM and non-AM plants under non-stress conditions did not differ considerably, nevertheless; under heat shock conditions AM plants expressed an improved POD activity (increased by 22%), relative to non-AM plants. In contrast to leaf SOD activity, this enzyme was enhanced significantly

(increased by 87%) in AM plants, as compared to non-AM plants under heat stress conditions although no substantial differences between AM and non-AM plants under normal conditions were recorded (Figure 2E). Regarding root CAT activity, no considerable differences between AM and non-AM plants were found under non-stress as well as heat shock conditions although the level of CAT was elevated by 75-102% in plants subjected to the heat stress (Figure 2F).

## Discussion

AMF can enhance the host tolerance to temperature stresses in maize (Zhu et al. 2010; 2011), in cyclamen (Maya and Matsubara 2013), in citrus (Wu, 2011), in tomato (Abdel Latif and

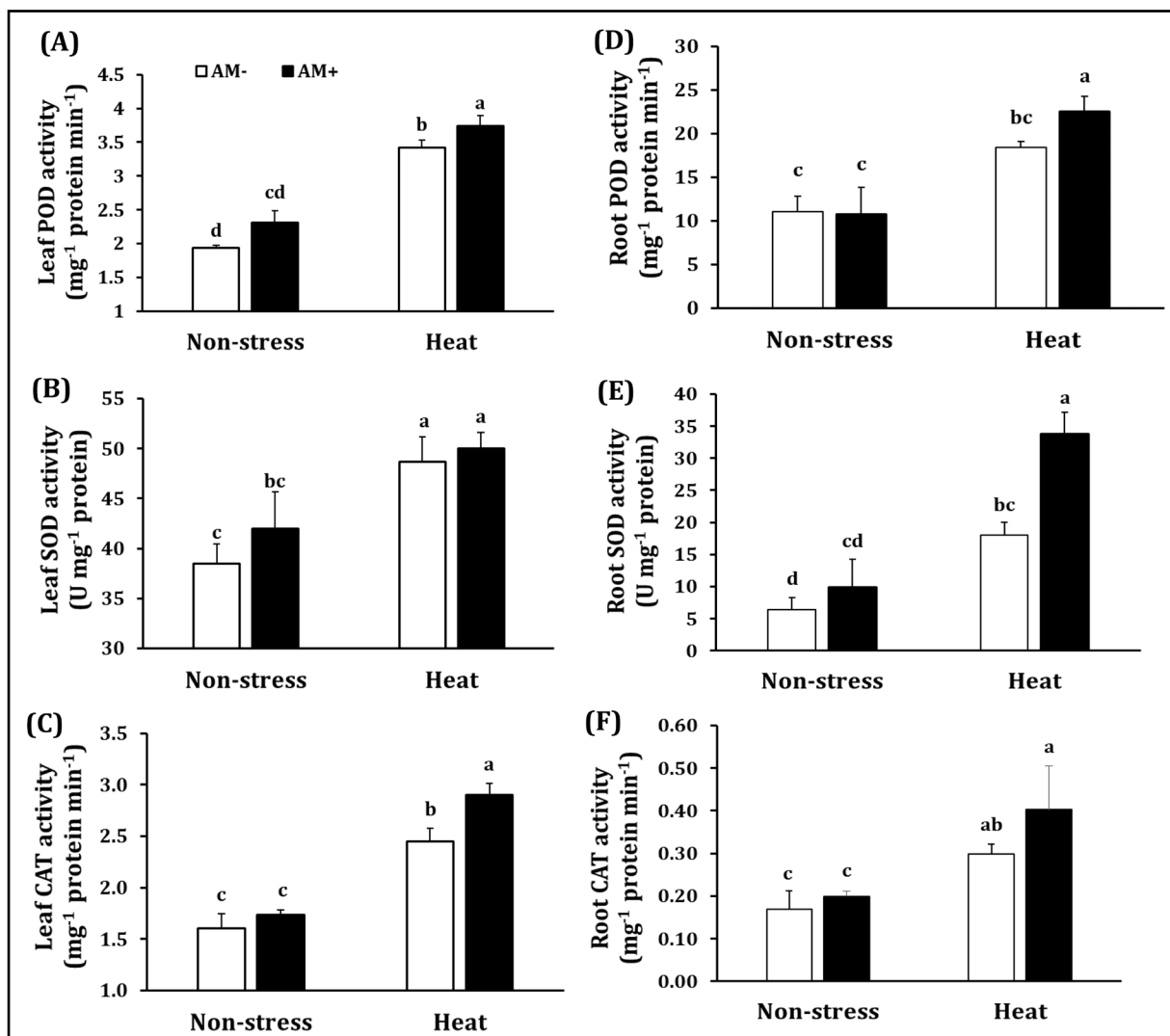


Figure 2. Peroxidase (POD) (A), superoxide dismutase (SOD) (B), catalase (CAT) (C) activity in leaves and POD (D), SOD (E) and CAT (F) in roots of non-AM plants and plants colonized by *Septoglomus constrictum* exposed to non-stress, heat-shock conditions. Bars present mean  $\pm$  standard deviation ( $n = 4$ ). Different letters present significant differences among treatments according to Duncan posthoc test ( $P < 0.05$ ).

Chaoxing 2011), however; little information on how AM symbiosis responds to heat shock was explored. In this study, the effect of AM inoculation with *Septoglomus constrictum*, an uncommon AM isolate in previous studies under heat stress, on plant tolerance to heat shock in tomato plants was investigated.

Temperature stresses can negatively impact on the growth and development of AM symbiosis (Zhu et al. 2010; 2011). Earlier studies showed that the development of AMF was inhibited by low temperatures (Zhu et al. 2010) whereas high temperatures influenced detrimentally the activity of AMF (Martin and Stutz 2004) and negative or neutral AM colonization (Compant et al. 2010). In the present study, heat shock had no significant effect on mycorrhizal colonization of tomato plants, which might be due to the length of heat stress applied. This result is in line with the observations in previous studies under heat stress in cyclamen (Maya and Matsubara, 2013), in maize (Zhu et al. 2010).

Extreme temperatures cause unbalanced cellular homeostasis, resulting in overproduction of ROS, membrane lipid peroxidation, and damage plant cells. In this study, we observed that there was an elevated MDA and H<sub>2</sub>O<sub>2</sub> accumulation in leaves of tomato plants exposed to heat shock, nevertheless, substantially lower MDA and H<sub>2</sub>O<sub>2</sub> contents were found in colonized plants than in plants without AM inoculation, suggesting that the presence of the AMF could alleviate the oxidative stress and peroxidation. The present results concur with observations in tomato plants (Abdel Latef and Chaoxing 2011). The authors illustrated that inoculation of *Funneliformis mosseae* decreased MDA in plants as compared to non-AM plants under cold stress (8°C) for 1 week. Zhu et al. (2010) also showed that maize plants colonized by *Claroideoglossum etunicatum* reduced substantially MDA in roots and leaves when plants were subjected to various temperature stresses (5, 15, 35, 40°C) for one week.

Plant tolerance to high-temperature stress has been linked with an increase in activities of antioxidant

enzymes (Sairam et al. 2000). Under heat shock, AM plants showed an enhanced POD, CAT activity in leaves, relative to non-AM plants, which is consistent with lower MDA and H<sub>2</sub>O<sub>2</sub> contents observed in leaves of AM plants in our study. In roots, POD and SOD activity in mycorrhizal plants were significantly higher than those in uncolonized plants. These findings suggest that AMF treatment improved the effectiveness of antioxidant systems to protect the host plant against oxidation damage under heat shock conditions. Our results are in accordance with earlier mycorrhiza studies in tomato plants under cold stress (Abdel Latef and Chaoxing 2011), in maize under high-temperature stresses (Zhu et al., 2010). However, leaf SOD and root CAT were not improved in AM plants in our study, which was inconsistent with observations of Abdel Latef and Chaoxing (2011), Zhu et al. (2010) and Maya and Matsubara (2013). The findings may indicate that different AMF isolate could induce differently antioxidant systems in the host plant under heat stress conditions. Similarly, Cekic et al. (2012) reported that pepper plants colonized by *Rhizophagus irregularis* enhanced substantially CAT activity in leaves whereas the leaf SOD level remained unchanged in both AM and non-AM plants when plants exposed to 1 mM NaCl stress.

In conclusion, our results point out that inoculation of AMF, *Septoglomus constrictum*, could enhance tomato plant tolerance against heat shock by decreasing oxidative stress (reduced H<sub>2</sub>O<sub>2</sub> and MDA content) and increasing activities of main ROS scavengers such as leaf and root POD, root SOD and leaf CAT. Nonetheless, further studies are necessary to elucidate mechanisms by which AMF influence antioxidant production, proline, photosynthesis, respiration, and water status in plants to better understand their benefits under heat stress for agricultural application.

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