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ORIGINAL PAPER

Influence of arbuscular mycorrhiza on lipid peroxidation and antioxidant enzyme activity of maize plants under temperature stress

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Abstract The influence of the arbuscular mycorrhizal (AM) fungus, *Glomus etunicatum*, on characteristics of growth, membrane lipid peroxidation, osmotic adjustment, and activity of antioxidant enzymes in leaves and roots of maize (*Zea mays* L.) plants was studied in pot culture under temperature stress. The maize plants were placed in a sand and soil mixture under normal temperature for 6 weeks and then exposed to five different temperature treatments (5ºC, 15ºC, 25ºC, 35ºC, and 40ºC) for 1 week. AM symbiosis decreased membrane relative permeability and malondialdehyde content in leaves and roots. The contents of soluble sugar content and proline in roots were higher, but leaf proline content was lower in mycorrhizal than nonmycorrhizal plants. AM colonization increased the activities of superoxide dismutase, catalase, and peroxidase in leaves and roots. The results indicate that the AM fungus is capable of alleviating the damage caused by temperature stress on maize plants by reducing membrane lipid peroxidation and membrane permeability and increasing the accumulation of osmotic adjustment compounds and antioxidant enzyme activity. Consequently, arbuscular mycorrhiza formation highly enhanced the extreme temperature tolerance of maize plant, which increased host biomass and promoted plant growth.

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Introduction

Temperature is one of the most important environmental factors that affect the growth and distribution of maize plants (Song and Wang 2005). Maize is a thermophilic plant that is susceptible to various temperature conditions. Both high and low temperatures will have an impact on the physiology and metabolism of maize plants, which accordingly handicaps growth and development. In the past few years, global temperatures have changed sharply due to climate change and have severely affected the production of maize. This has also impacted on the growth and activity of symbiotic arbuscular mycorrhizal (AM) fungi (Staddon et al. 2002; Martin and Stutz 2004; Kytöviita and Ruotsalainen 2007).

It is well-known that AM fungi not only stimulate the growth of plants but also contribute in enhancing plant tolerance to abiotic and biotic stresses such as high or low temperature (Charest et al. 1993) and drought (Augé 2001). The literature reports that AM fungi play a very important role in terms of plant growth (Anderson et al. 1987; Haugen and Smith 1992), nutrition uptake and transport (Gavito et al. 2005; Hawkes et al. 2008), water status (EI-Tohamy et al. 1999), chlorophyll content (Charest et al. 1993; Paradis et al. 1995), and mycorrhizal root colonization (Hayman 1974; Grey 1991) under high or low temperature. However, there is very little research on the influence of AM fungi on membrane lipid peroxidation and the activity of antioxidant enzymes under temperature stress.

When the plant is subjected to high or low temperature stress, the cell membrane is first affected with increased membrane permeability. At the same time, a variety of reactive oxygen species (ROS), such as superoxide anion radical (O_2^-), hydroxyl radicals ($OH^·$) and hydrogen peroxide (H_2O_2), are induced, causing a loss in balance between production and scavenging in the cell or organism, which causes membrane lipid peroxidation (Apel and Hirt 2004; Jaleel et al. 2009). As a consequence, plants protect themselves against oxidative injury by inducing osmotic adjustment and activity of antioxidant enzymes (Asada 1999). The AM symbiosis can alter plant physiology in a way to cope with stresses under stressful conditions (Miransari et al. 2008). However, the mechanisms by which the AM symbiosis influences the reactive oxygen metabolism of host plants under temperature stress are not clear. Therefore, understanding the effect of AM fungi on lipid peroxidation and antioxidant enzyme activity of plants under temperature stress is of importance.

The purpose of this study is to evaluate the effect of *Glomus etunicatum* on growth, membrane lipid peroxidation, osmotic adjustment, and antioxidant enzyme activity in the leaves and roots of maize plants under low and high temperature stresses, in order to further understand heat and chilling tolerance mechanisms in AM plants.

Materials and methods

Plant material and growth conditions

Seeds of maize hybrid Zhengdan 958 were surface sterilized with 0.5% NaClO for 30 min, rinsed four times with sterile-distilled water, and then germinated on wet filter paper in Petri dishes at 28ºC. Three pre-germinated seeds were sown in separate pots containing 4 kg of an autoclaved mixture (0.11 MPa, 121ºC, 2 h, Sheng et al. 2008) of black soil and sand (1:1.5, *v*/*v*). The characteristics of the soil were pH 6.6, 26.9 g/kg organic matter, 118.8 mg/kg available nitrogen, 18 mg/kg available phosphorus, and 111 mg/kg available potassium. The soil was collected from Dehui City, Jilin Province, China. Seedlings were thinned to two seedlings per pot 1 week after emergence.

AM fungal inoculum

Mycorrhizal fungal inoculum was provided by the Institute of Plant Nutrition and Resources, Beijing Academy of Agriculture and Forestry Sciences, China. The inoculum consisted of soil, spores (the spores density was 30,000 per 20 ml inoculums), hyphae, and infected root fragments from a stock culture of *G. etunicatum*. Each pot was inoculated with 30 g inoculum for mycorrhizal treatments or 30 g sterilized (autoclaved) inoculum plus 10 ml mycorrhizal fungus-free filtrate from a water suspension of inoculum for the nonmycorrhizal treatment. The mycorrhizal and sterilized inocula were placed 3 cm below the maize seeds at sowing time.

Experimental design

The experiment combined two treatments (nonmycorrhizal control and *G. etunicatum* inoculated) at five temperature treatments (5ºC, 15ºC, 25ºC, 35ºC, and 40ºC). A completely randomized block design with four replicates was used. Before being exposed to the five temperature treatments, the maize seedlings were grown in a greenhouse at 22–25ºC with 14 h daylight and 75–90% relative humidity for 6 weeks after emergence. Different temperature treatments were given to the seedlings for 1 week by placing them in different growth chambers.

Parameter measurements

After 7 weeks, maize plants were harvested. One plant from each pot was used for determination of fresh and dry weight and one for all other parameters. Root and shoot dry weights were determined after over-drying at 75ºC for 72 h. A fraction of the roots was carefully washed, cut into 1-cm long segments, dipped in 10% KOH at 90ºC for 20 min, acidified in 2% HCl for 5 min, and stained with 0.05% trypan blue in lactophenol (Phillips and Hayman 1970). Mycorrhizal colonization rate was measured using the gridline intercept method described by Giovannetti and Mosse (1980).

Membrane relative permeability was measured according to Bai et al. (1996). Leaf and root samples were washed with deionized water, followed by the introduction of small excisions and then incubated in deionized water at room temperature. After 1 h, the electrical conductivity (L1) of the immersion solution was measured using a conductivity meter (DDS-11A, China). The immersion solution was then placed in a boiling water bath for 10 min, and the electrical conductivity (L2) was measured after cooling. Membrane relative permeability was calculated by the formula $L1/L2 \times 100\%$.

Malondialdehyde (MDA) was measured according to the thiobarbituric acid (TBA) reaction as described by Zhang and Qu (2004). Leaf and root samples were homogenized with 5% trichloroacetic acid and centrifuged at 4,000×*g* for 10 min. Two milliliters of extract was added to 2 ml 0.6% TBA placed in a boiling water bath for 10 min, and absorbance was read at 532, 600, and 452 nm. The MDA concentration was calculated according to the formula: $6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}$.

Proline content was determined using the method of Zhang and Qu (2004). Pure proline was used as a standard. Fresh roots and leaves were extracted with 3% sulfosalicy-

clic acid, placed in a boiling water bath for 10 min, and filtered with filter paper. Two milliliters of extract was added to 6 ml assay media containing 2 ml ninhydrin solution and 2 ml acetic acid and incubated for 30 min at 100ºC and then cooled. The colored product formed was extracted with 4 ml toluene by shaking. The absorbance of the resultant organic layer was measured at 520 nm.

Soluble sugar content was determined by the anthrone method (Zhang and Qu 2004) using sucrose as the standard. Leaf and root samples were homogenized with distilled water, placed in a volumetric flask for 1 h, and filtered with filter paper. The reaction mixture contained 1 ml extract and 5 ml anthrone (100 mg anthrone+100 ml 72% H_2SO_4) and were placed in a boiling water bath for 10 min, and then absorbance was read at 620 nm.

Fresh leaves and roots were homogenized in 5 ml phosphate buffer (0.1 mol/l, pH 7.8) and centrifuged at 10,000×*g* for 20 min at 4ºC, and the supernatant was used for superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) assays. SOD activity was measured according to Bai et al. (1996) based on the ability of SOD to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide radicals generated photochemically. The reaction mixture contained 50 mM phosphate buffer, pH 7.8, 14 mM methionine, 75 μM NBT, 0.1 μM EDTA, 4 μM riboflavin, and the required amount of enzyme extract. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50% at 25ºC. CAT activity was measured by the disappearance of H_2O_2 (Zhang and Qu 2004). The reaction mixture (2.5 ml) contained 50 mM phosphate buffer, pH 7.0, and 12.5 mM H_2O_2. The reaction was initiated by adding 50 μl of the extract and monitoring the change in absorbance at 240 nm for 3 min. POD activity was determined using guaiacol oxidation (Bai et al. 1996) in a reaction mixture containing 50 mM phosphate buffer (pH 6.0), 20.1 mM guaiacol, 12.3 mM H_2O_2, and enzyme extract. The increase in absorbance was recorded by the addition of H_2O_2 at 470 nm for 3 min.

Statistical analysis

The experimental data were subjected to correlation analysis and two-way analysis of variance using ANOVA. Means were compared by Duncan's test at the 5% level (SPSS version 13.0).

Results

Plant growth and AM root colonization

Dry weight of shoots was higher in mycorrhizal than in nonmycorrhizal plants at 5ºC and high temperature conditions. At 5ºC, 25ºC, and 40ºC, root dry weight in mycorrhizal and nonmycorrhizal plants was similar (Fig. 1a, b). Under low temperature stress, both root and leaf growth suffered inhibition. The symptoms of temperature stress, such as the leaves becoming narrow, yellow, and sapless, were observed in maize plants treated with low- and high-temperature stresses. Under the same temperature conditions, leaf injury was more severe in nonmycorrhizal plants than in mycorrhizal plants according to the shoot fresh weight (Fig. 1c) and chlorophyll content (data not shown).

Non-inoculated maize plants were not colonized by *G. etunicatum*. Plants inoculated with *G. etunicatum* had root colonization levels of 75–84%. The highest root colonization was seen in the 25ºC treatment; the lowest was at 5ºC (Fig. 1d).

Membrane permeability and lipid peroxidation

Membrane relative permeability in the leaves and roots was lower in mycorrhizal than in nonmycorrhizal plants. Significant differences in membrane relative permeability between mycorrhizal and nonmycorrhizal plants were observed among temperature treatments, except at 25ºC for leaves and roots and at 15ºC for the leaves, (Fig. 2a, b). MDA content in the leaves and roots was lower in mycorrhizal than in nonmycorrhizal plants at all temperature treatments, although the difference between mycorrhizal and nonmycorrhizal plants was not significant for the root MDA content at 35ºC (Fig. 2c, d). As the temperature stress was increased, the membrane relative permeability and MDA content in the leaves and roots of both AM-inoculated and non-inoculated plants increased, and at similar conditions, the root membrane relative permeability was higher than that in leaves.

Osmotic adjustment

At all temperature treatments, proline content in the leaves was markedly lower in mycorrhizal than in nonmycorrhizal plants (Fig. 3a). However, the root proline content was higher in mycorrhizal than in nonmycorrhizal plants under low temperature stress (Fig. 3b). With the increase in temperature stress, proline content in both leaves and roots of AM-inoculated and non-inoculated maize plants increased. Moreover, proline content in the roots was generally higher than that in the leaves under the same temperature conditions.

Mycorrhizal plants had higher soluble sugar content in the roots at all temperature treatments, compared with nonmycorrhizal plants, although the difference was not significant at 25ºC and 35ºC. There was also no significant difference in soluble sugar content in leaves between

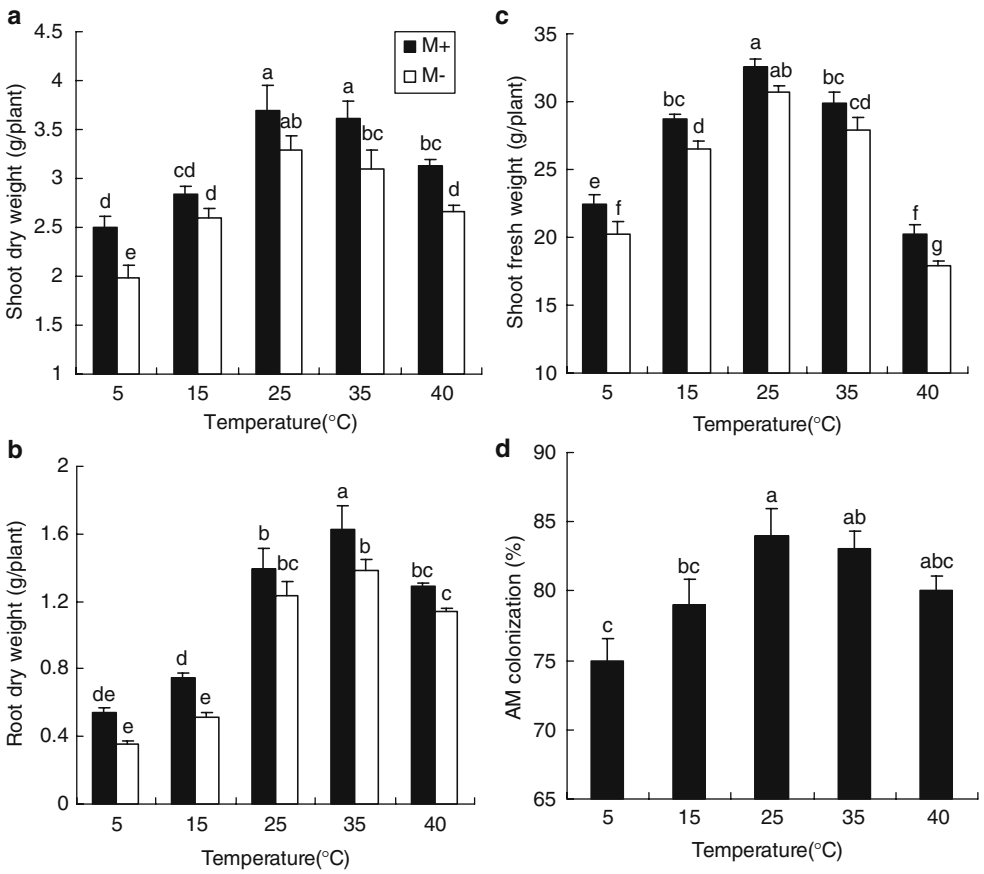

Fig. 1 Dry weight of shoot (**a**) and root (**b**), shoot fresh weight (**c**), and arbuscular mycorrhizal root colonization (**d**) of maize plants inoculated (M+) or not (M−) with *Glomus etunicatum* at five temperature treatments. *Mean pairs followed by different letters* are significantly different ($p<0.05$) by Duncan's test; $n=4$

mycorrhizal and nonmycorrhizal plants regardless of temperature treatments (Fig. 3c, d). Under low-temperature stress, soluble sugar content in leaves of both AM-inoculated and non-inoculated maize was decreased as the temperature decreased, but the root soluble sugar content increased significantly. Maize plants had similar leaf soluble sugar content at 35ºC and 40ºC, but the root soluble sugar content was higher at 40ºC than that at 35ºC.

Activities of antioxidant enzymes

Root colonization by *G. etunicatum* enhanced SOD (by 7–30%) and CAT (by 13–79%) activity in the leaves and in the roots (by 7–26% and by 72–340%, respectively) of maize plants grown at all temperature treatments (Fig. 4a–d). Activity of SOD in the leaves and roots of all maize plants decreased with the increase in temperature stress. The root CAT activity increased and then decreased with increased temperature but increased again at 40ºC. However, leaf CAT activities continued to decline after reaching the peak. Leaf POD activity in mycorrhizal and nonmycorrhizal plants was similar at all temperature treatments, and root POD activity was higher in mycorrhizal than in nonmycorrhizal plants at 5ºC, 35ºC, and 40ºC (Fig. 4e, f).

Discussion

Temperature stress affects the growth of AM plants. Many investigations report that root colonization is greatly restrained at low temperatures (Baon et al. 1994; Zhang et al. 1995; Liu et al. 2004). However, Volkmar and Woodbury (1989) found that low root temperature had no impact on root colonization of barley inoculated with indigenous AM fungi. Charest et al. (1993) also found that root colonization levels were independent of temperature. High-temperature soil conditions alter AM fungal activity (Martin and Stutz 2004). Root colonization by AM fungi often decreases when the temperature exceeds 30ºC (Bowen 1987), and it is always lethal to AM fungi if the temperature rises above 40ºC (Bendavid-Val et al. 1997). Our results showed that root colonization of maize plants can be lowered at low temperatures, but high temperatures do not have significant effects. This may be a result of different experimental design and biological materials.

Mycorrhizal symbiosis and environmental temperatures can affect the growth of plants (Entry et al. 2002). Our studies showed that maize plants inoculated with *G. etunicatum* grow better than nonmycorrhizal plants under high and low temperatures. This agrees with much of the

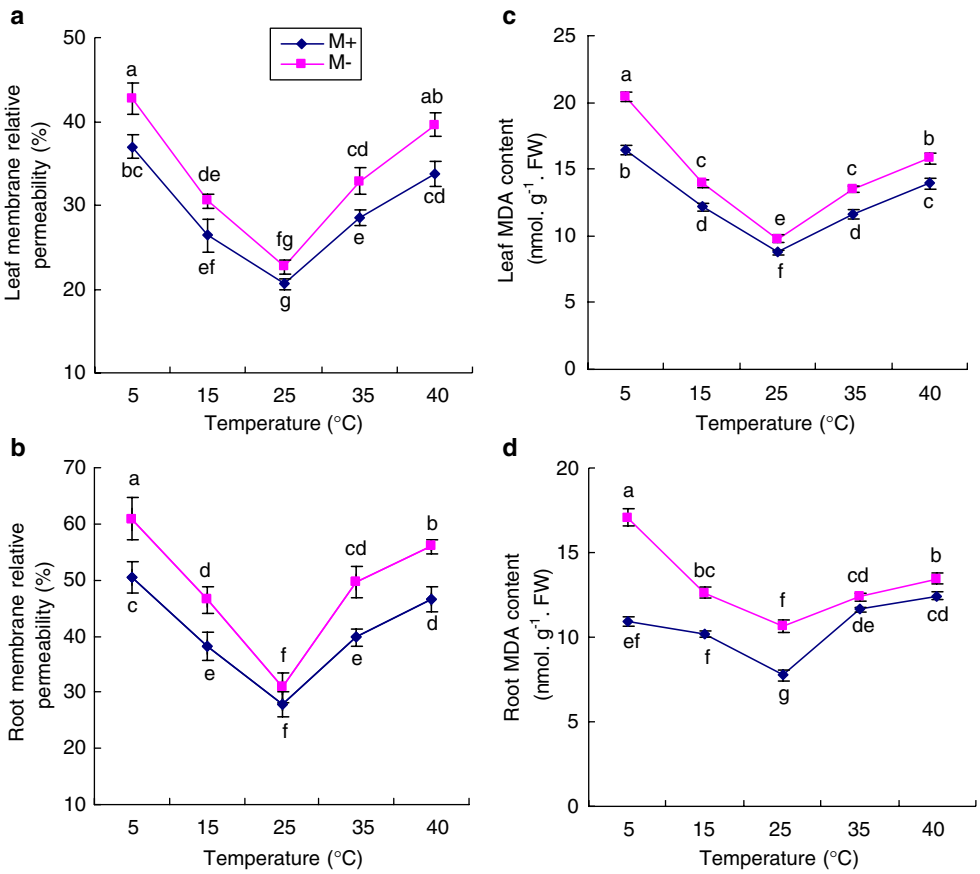

Fig. 2 Membrane relative permeability in the leaves (**a**) and roots (**b**) and malondialehyde (MDA) content in the leaves (**c**) and roots (**d**) of maize plants inoculated (M+) or not (M−) with *Glomus etunicatum* at five temperature treatments. *Mean pairs followed by different letters* are significantly different ($p<0.05$) by Duncan's test; $n=4$

greenhouse research reported in sorghum (Raju et al. 1990), mung bean (Haugen and Smith 1992), and pepper (Martin and Stutz 2004).

Temperature stress causes physiological disorders of plants and induces the production of ROS (Mittler 2002). The accumulation of ROS breaks the balance between ROS production and the capacity of plants to scavenge for them, which induces destructive oxidative processes such as membrane lipid peroxidation and protein oxidation (Herbinger et al. 2002). MDA is often regarded as the product and a reflection of the degree of membrane lipid peroxidation (Thompson et al. 1988; Ali et al. 2005). Therefore, MDA content in the leaves and roots of maize plants was measured under temperature stress. With the acceleration of temperature stress, both leaf and root MDA contents increased. However, the MDA content in mycorrhizal plants remained lower than that in nonmycorrhizal plants, which shows that the presence of the AM fungus could alleviate the peroxidation of membrane lipids. Enhancement of membrane lipid peroxidation causes an increase in membrane permeability, exosmosis of electrolytes, and finally injures the cell membrane system. There was a significant positive correlation between membrane permeability and MDA content in the leaves of mycorrhizal maize plants ($r=0.975$, $p<0.01$). EI-Tohamy et al. (1999) indicated no significant effects of electrolyte leakage in mycorrhizal and nonmycorrhizal kidney beans under low temperature stress. However, in this study, there were significant differences between mycorrhizal and nonmycorrhizal plants in both leaf and root membrane relative permeability under high- and low-temperature stresses. Moreover, membrane relative permeability of mycorrhizal plants was lower than that of nonmycorrhizal plants. Although the membrane relative permeability of both AM-inoculated and non-inoculated plants increased under temperature stress, the amplitude of the increase in mycorrhizal plants was lower than that of nonmycorrhizal plants. All this shows that AM fungi can lower the plant's membrane permeability and alleviate the damage on the cell membrane caused by temperature stress.

Osmotic adjustment is considered an important constitution of heat and chilling tolerance mechanisms for higher plants. Under temperature stress, higher plants acquire high osmotic adjustment through accumulation of small molecules including organic solutes and inorganic ions. Organic solutes include soluble

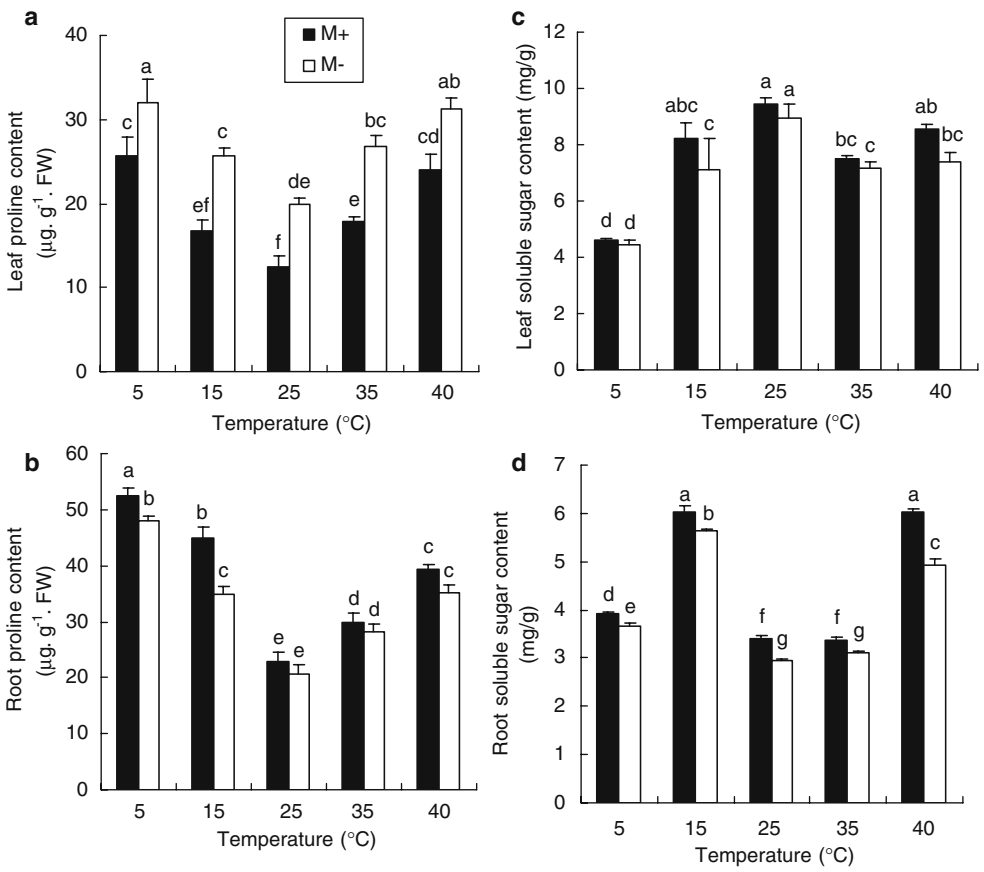

Fig. 3 Proline content in the leaves (**a**) and roots (**b**) and soluble sugar content in the leaves (**c**) and roots (**d**) of maize plants inoculated (M+) or not (M−) with *Glomus etunicatum* at five temperature treatments. *Mean pairs followed by different letters* are significantly different ($p<0.05$) by Duncan's test; $n=4$

sugar, proline, etc. The soluble sugar content in the roots of mycorrhizal plants is noticeably higher than that of nonmycorrhizal plants. This may be due to the sink effect of the AM fungus-demanding sugar from leaves (Porcel and Ruiz-Lozano 2004). Root-soluble sugar content increased under temperature stress, but leaf-soluble sugar content decreased. The reason for this could be that photosynthesis was weakened, so respiration depletion caused the reduction of leaf-soluble sugar. Moreover, proline serves as a sink for energy to regulate redox potentials, acting as a hydroxyl radical scavenger (Sharma and Dietz 2006), as a solute that protects macromolecules against denaturation, and as a means of reducing acidity in the cell (Kishor et al. 1995, 2005), and it is the most important organic osmotic adjustment. High- or low-temperature stress could cause large accumulation of proline. In this study, greater root proline contents were found in mycorrhizal plants at low temperature conditions, compared with nonmycorrhizal plants. In contrast, proline content in the leaves of mycorrhizal plants was lower than in nonmycorrhizal plants (Fig. 3a, b). This is in agreement with a study on soybeans under drought stress by Porcel and Ruiz-Lozano (2004). This may be due to the strengthening of the osmotic adjustment ability of mycorrhizal plants and alleviation of damage caused by temperature stress due to the accumulation of root proline. The changes in leaf proline suggest that the degree of injury in mycorrhizal plants was slighter so that there was no need to synthesize more proline for osmotic adjustment protection.

It is well-known that plants can induce effective antioxidant systems to protect themselves against oxidation damage. The antioxidant defense systems include enzymatic and non-enzymatic systems in which SOD, CAT, and POD are the important antioxidant enzymes because they can efficiently prevent the accumulation of O_2^- and H_2O_2 and minimize the deleterious effects of ROS (Jaleel et al. 2009). Present studies found that maize plants inoculated with an AM fungus had higher SOD, CAT, and POD activity in both leaves and roots compared to that of non-inoculated plants under temperature stress. This was consistent with previous reports obtained for shoots of *Glycine max* colonized by *G. etunicatum* and subjected to NaCl salinity (Ghorbanli et al. 2004) and from the leaves and roots of citrus seedlings colonized by *G. versiforme* during drought (Wu et al. 2006). This implies that AM colonization could alleviate the damage of ROS, protect the plants against damage by oxidation,

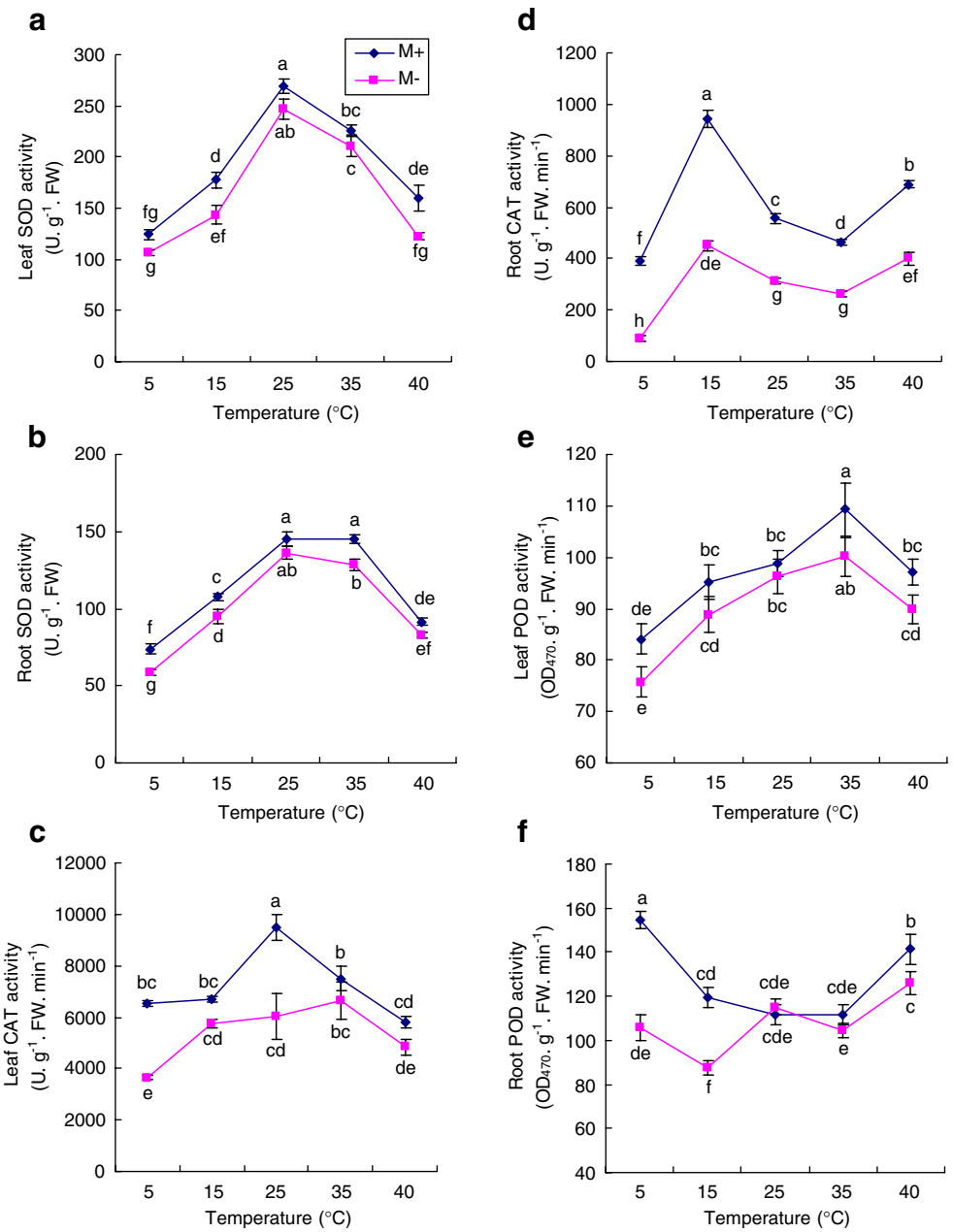

Fig. 4 Superoxide dismutase (SOD) activity in the leaves (**a**) and roots (**b**), catalase (CAT) activity in the leaves (**c**) and roots (**d**), and peroxidase (POD) activity in the leaves (**e**) and roots (**f**) of maize plants inoculated (M+) or not (M−) with *Glomus etunicatum* at five temperature treatments. *Mean pairs followed by different letters* are significantly different ($p<0.05$) by Duncan's test; $n=4$

and finally improve the extreme temperature tolerance of maize. Different responses in CAT and POD activities to temperature stress were similar to the previous investigation by Porcel and Ruiz-Lozano (2004) under drought stress. The increased activity of some antioxidant enzymes may be due to nonspecific plant defense responses under temperature stress. Therefore, it is essential to further investigate the regulation of other enzyme activities.

In conclusion, *G. etunicatum* inoculation could protect maize plants against high- and low-temperature stress. However, it is still not clear how the AM symbiosis affects the reactive oxygen metabolism, membrane lipid peroxidation, osmotic adjustment, and antioxidant production in plant leaves and roots. Thus, it is necessary to conduct further studies on the mechanism by which AM symbiosis affects oxygen metabolism, membrane lipid peroxidation, and antioxidant production of plants, in order to better understand their role in agricultural production.

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