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Effects of high growth-medium temperature under controlled conditions on characteristics of tomato leaves

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Abstract

High temperatures have become a major threat that seriously affects crop growth and yield. The present work aimed to investigate the acclimation process in adjusting plant responses to high root temperatures. Tomato (*Solanum lycopersicum* L., cv. Micro-Tom) during the flowering time was subjected to heat treatments (day/night temperatures at the root level of 40 or 45 °C for 4 d) while control plants were maintained at 25 °C, and the heat-stress treatment effects were analysed in the tomato leaves. The results showed a reduction in the content of chlorophylls *a* and *b* as well as chlorophyll *a*/*b* ratio at both high temperatures. Further, the increase in the amount of malondialdehyde as an indicator of lipid peroxidation was greater at 45 °C. The leaf content of hydrogen peroxide was induced in tomato plants subjected to 45 °C whereas it was markedly decreased in plants maintained at 40 °C as compared to control plants. Antioxidant enzymes showed higher activity in tomatoes treated at 45 °C compared to those treated at 40 °C. Moreover, the highest amount of antioxidants such as carotenoids and ascorbate in tomato plants were found at a temperature of 45 °C. Collectively, we provide evidence that physiological and biochemical components can be altered depending on the heat level, exposure time, and developmental stage. The interaction of root and shoot under high temperatures must be further characterized in terms of understanding the challenging climate changes.

Keywords: antioxidant enzymes, heat stress, oxidative stress, reactive oxygen species, *Solanum lycopersicum*, tomato.

Introduction

Tomatoes (*Solanum lycopersicum* L.) are known as an excellent source of healthy micronutrients such as carotenoids, ascorbic acid, and flavonoids (Tieman *et al.* 2017). Moreover, it is one of the most important horticultural crops produced worldwide. In Tunisia, tomato is grown throughout the year and ranks among the most extended crops based on cultivated area and exported products, hence its economic importance. Yet, the growing environment, the cultivation system, seasons, and locations have different influences on plant growth and development. Under the prospective climate change associated with global warming, the temperature increase is the most limiting factor for plant cultivation and crop productivity throughout the world (Li *et al.* 2013). Although many environmental factors, such as drought, wounding, and salinity may already be limited by specific field practices (irrigation, anti-wind barriers, modification of the control system), the temperature is one of the most

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Abbreviations: APX - ascorbate peroxidase; AsA - ascorbic acid; CAT - catalase, DHA - dehydroascorbic acid; DTT - dithiothreitol; GPX - guaiacol peroxidase; GR - glutathione reductase; GSH - glutathione; MDA - malondialdehyde; NEM - N-ethylmaleimide; PRX - peroxidase; ROS - reactive oxygen species; SOD - superoxide dismutase; TBA - thiobarbituric acid; TCA - trichloroacetic acid.
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difficult parameters to manage especially in the field conditions. In addition, frequent episodes of intensified heat waves are expected in the future due to the longer duration of hot days during the crop-growing season, which can cause harmful effects in agriculture (Wang *et al.* 2016). Thereby, plants may be damaged to a great extent by either high day or high night temperatures and by either high air or soil temperatures. Commonly, plants are grown in open fields or in greenhouses where high temperatures can affect shoot as well as pots, media and roots to above ambient temperatures (Cocetta *et al.* 2018). At the point when sunlight-based radiation is high and day lengths are long, the subterranean part of plants can similarly warm out to levels causing physiological disorders that might compromise their survival. In the same way, aeroponic and hydroponic systems in greenhouses can be heated, resulting in altered nutrient uptake and decreased plant growth (Falah *et al.* 2010, Benlloch-González *et al.* 2017). Further, given that most of the tomato roots are found in the top of 10 - 20 cm of soil, soil temperatures can frequently exceed optimal levels, particularly in field conditions (*e.g.*, exceed 34 - 35 °C at 10 - 15 cm), with some cultivation practices that can favour soil warming (Díaz-Pérez and Batal 2002).

In Tunisia, tomato crops already experience periods of potentially unfavourable temperatures when growth media receives direct sunlight that can lead to elevated root zone temperatures particularly during the hot summer when temperatures may reach 50 °C. Further, and to extend the growing season, greenhouses in the south part of the country are often heated with geothermal water to compensate for nocturnal thermal deficits observed during the coldest months of winter and which can afflict physiological behaviour of plants. However, the heating remains determined by the control of the ambient and root zone temperatures since this operation allows the use of water at high temperatures ranging from 40 to 80 °C. This water circulates through pipelines lying on the ground or elevated off the floor according to the cultivation system that provides heat directly to the growth medium. Therefore, the root system could be altered especially when the temperature exceeds 38 °C due to occurring defects in the cooling system (Mougou and Verlodt 1991).

While most studies have focused on plant responses to raised air temperatures (Wahid *et al.* 2007), limited understanding exists regarding the influence of an abrupt high root temperature of media on the shoot growth, which formed the basis of this work. Despite several published papers related to root zone temperature effects, information regarding the impact of high root temperature on tomato plants is very limited (Moorby and Graves 1980, Nkansah and Tadashi 1995). Furthermore, damaging effects caused by high root temperatures and associated consequences on plant development are not well characterised, especially in its reproductive phase, and knowledge on this factor is still scarce (Fei *et al.* 2017).

Heat stress creates physiological, metabolic, and cellular imbalances, leading to severe retardation in plant growth and development, reduced yield and quality, and even plant death (Fahad *et al.* 2016, Rykaczewska 2017). Both shoots and roots are sensitive to heat stress, but extensive analysis has shown that roots can manifest higher sensitivity to supra-optimal temperature than the top part of the plant (Sailaja *et al.* 2014, Giri *et al.* 2017). As a primary barrier against soil adverse conditions, the root system has the ability to adjust the rate of physiological and molecular processes such as water and nutrient uptake, leaf growth, and metabolite content in the shoots (Aidoo *et al.* 2016). In fact, root metabolism and functions could be disrupted in several ways under high temperatures, with direct effects on root growth (elongation, number, and biomass) (Huang *et al.* 2012). Also, a rise in soil temperature may change the retention surface area of roots restricting the supply of water and mineral nutrients (Wang *et al.* 2015, Wu *et al.* 2017), and in consequence suppress the stomatal conductance (Tahir *et al.* 2008). Additionally, high root zone temperatures have been recognized to affect the production of hormones synthesized in the roots and transported to the shoots thereby altering the sink-source relationship between shoots and roots (Wahid *et al.* 2007, Hao *et al.* 2012, Huang *et al.* 2012). Nonetheless, extended exposure of roots to high temperatures can reduce carbon exchange rates, increase respiration, and alter saccharide allocation (Rachmilivitch *et al.* 2015) as well as photosynthetic performance (Monje *et al.* 2007), which negatively influence the growth of aboveground tissues and/or the whole plant metabolism (Benlloch-González *et al.* 2017). Subsequently, root zone temperature is an important factor in leaf stress perception and responses (Suzuki *et al.* 2008, 2011). Besides, such excessive temperature can elicit the overloading of reactive oxygen species (ROS) (Hasanuzzaman *et al.* 2013). To cope with the heat-induced oxidative damage, plants have developed several antioxidant pathways, including detoxifying enzymes, such as superoxide dismutase (SOD), catalase (CAT), peroxidases (PRX), and glutathione reductase (GR), and non-enzymatic antioxidants such as tocopherols, ascorbic acid, glutathione (GSH), and carotenoids (Gill and Tuteja 2010, Mishra *et al.* 2015).

The response of plants depends on the severity and duration of stress, plant species, and tissue types as well as the interactions of multiple stresses (Aghamolki *et al.* 2014). The developmental stage at which plants are exposed to heat stress may affect the extent of damages experienced by the crop (Wahid *et al.* 2007). High-temperature injury increases dramatically within the flowering time which can result in a failure of fruit set due to the defect in pollen developmental processes and pollination inducing flower sterility (Morrison *et al.* 2016, Müller *et al.* 2016). In this study, the tomato was used as a model plant. Micro-Tom cultivar is characterized by its compact size (10 cm tall) which is preferentially convenient with our experimental design in controlled conditions, short life cycle (~70 d) and its ability to grow at high density (Campos *et al.* 2010).

The present research investigates the implications of high root temperatures at the flowering phase on the content of chlorophylls, H_2O_2, and malondialdehyde, and the scavenging activity of antioxidants which can affect the response of tomato plants to short term heat stress. It is important to enhance our understanding of adaptation

strategies of tomatoes under warmer temperatures events that are occurring globally and that can critically affect plant behaviour, especially during flowering which is the outcome of final yield.

Materials and methods

Plants, growth conditions, and stress treatments: Seeds of tomato (*Solanum lycopersicum* L. cv. Micro-Tom) were washed thoroughly in running water, sterilized by sodium hypochlorite (0.1 %, m/v) and washed in distilled water. Thereafter, seeds were germinated in the dark at 25 °C ± 1 °C on glass Petri dishes containing two layers of filter paper. To avoid the interaction of roots with any other surrounding environmental factor, root medium and shoot air temperature were controlled independently and plants were grown in a liquid hydroponic system, to better characterize the direct effect of high temperatures on roots and to ensure that observed changes were not due to any alteration in the nutrient supply or any other factor.

Five days old seedlings were transferred to plastic containers of 15 dm^3 each containing full strength Hoagland's nutrient solution (Hoagland and Arnon 1950) and kept in a growth chamber under controlled conditions (a relative air humidity of 60 ± 10 %, day/night temperatures of 25/20 °C, a 16-h photoperiod, and a photosynthetic photon flux density of 200 µmol m^{-2} s^{-1}). The nutrient solution was replaced every third day to avoid the depletion of nutrients. At the stage of the 7 - 8 leaves, plant roots were subjected to heat stress at two different temperatures of 40 and 45 °C for 4 d. Plants maintained at a day/night temperature of 25 °C served as a control. Shoots of plants were continuously exposed to an air temperature of 25 °C ± 2 °C. The root heating device consisted of two glass aquariums equipped resistances to heat the growth medium and immerged pumps to aerate and maintain a uniform temperature distribution in the aquarium. The control of the root zone temperature within the desired range was achieved using thermostats (*Huber CC-208B*, *Peter Huber Kältemaschinenbau AG*, Offenburg, Germany) to maintain constant water growth-medium temperatures during the treatment period. The temperature was measured accurately with an immerged thermometer. Solution aquariums are appropriate for short-term high-temperature treatments performed in the dark to prevent phototoxicity and to better exploit the thermal conductivity of the water compared to the air or the soil.

Complete harvesting of all mature leaves and roots of six plants from each heat treatment group was done 24, 72, and 96 h during heat treatments. The leaves and roots were weighed and frozen in liquid nitrogen and immediately transferred to -80 °C to prevent enzyme degradation.

Measurement of photosynthetic pigments: Approximately 0.1 g of fresh leaves initially ground in liquid nitrogen was placed in pre-weighed tubes and homogenized in 1.5 cm^3 of chilled 80 % (v/v) acetone. Tubes were vortexed and incubated in the dark in an ice bath with occasional shaking until the complete discolouration of the leaf powder. After centrifugation at 15 000 *g* and 4 °C for 5 min, chlorophyll content was determined by absorbance measurements at 645 and 663 nm and carotenoids at 470 nm using a spectrophotometer (*Lightwave II*, *Biochrom*, Cambridge, UK). The content of photosynthetic pigments was estimated using the equation of Arnon (1949).

Determination of hydrogen peroxide: Hydrogen peroxide content was determined as described by Murshed *et al.* (2013) with some modifications. Frozen leaf tissue (0.25 g) was homogenized in an ice bath with 1 cm^3 of 0.1 % (m/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12 000 *g* and 4 °C for 15 min. Aliquots of 100 mm^3 from each tube were placed in 96-well plates, and 50 mm^3 of 10 mM potassium phosphate buffer (pH 7.0) and 100 mm^3 of 1 M potassium iodide were added to each well. The plate was briefly shaken, and after incubation for 30 min at room temperature, the absorbance readings were taken at 390 nm with a microplate reader (*PowerWave XS*, *Biotek*, Winooski, USA). The content of H_2O_2 was determined using a calibration curve generated using a series of standard dilutions of commercial H_2O_2.

Lipid peroxidation assay: The thiobarbituric acid reactive substances (TBARS) assay is the most used test to determine lipid peroxidation by measuring the content of malondialdehyde (MDA), as the final product of the lipid peroxidation, using the method described by Dhindsa *et al.* (1981). A 0.25 g leaf sample was homogenized in 1 cm^3 of 0.1 % (m/v) trichloroacetic acid (TCA) solution. The homogenate was centrifuged at 12 000 *g* for 15 min, and 0.5 cm^3 of the supernatant was added to 1 cm^3 of 0.5 % (m/v) thiobarbituric acid (TBA) in 20 % TCA. The mixture was incubated in boiling water for 30 min, and then quickly cooled in an ice bath. The tubes were briefly vortexed and 200 mm^3 aliquots were taken from each tube and transferred in triplicates to 96-well plates, and the absorbance was measured at 532 nm with a microplate reader (*PowerWave XS*). The value for a non-specific absorption at 600 nm was subtracted. The amount of MDA-TBA complex (red pigment) was calculated using the coefficient of absorbance of 155 mM^{-1} cm^{-1}.

Determination of ascorbate: The extraction and quantification of total ascorbate (AsA), reduced AsA, and dehydroascorbate (DHA) in leaf extracts followed the method of Kampfenkel *et al.* (1995). A 0.25 *g* sample of frozen leaf powder was homogenized in 1 cm^3 of cold 6 % (m/v) TCA. The homogenate was centrifuged at 16 000 *g* and 4 °C for 15 min. The supernatant was used for total AsA and reduced AsA determination. For measurements of total AsA, 10 mm^3 of extract were added to 10 mm^3 of 10 mM DL-dithiothreitol (DTT) and 20 mm^3 of 0.2 mM phosphate buffer (pH 7.4). After incubation for 15 min at 42°C, 10 mm^3 of 0.5 % (m/v) N-ethylmaleimide (NEM) was added with an additional incubation of 1 min at room temperature to remove the excess of DTT. This was followed by adding 150 mm^3 of a reagent prepared just before use by mixing 50 mm^3 of 10 % TCA, 40 mm^3

of 42 % (v/v) of H_3PO_4, 40 mm^3 of 4 % (m/v) 2,2-bipyridyl dissolved in ethanol (70 %) and 20 mm^3 of 3 % (m/v) ferric chloride. After further incubation for 40 min at 42 °C, the absorbance was measured at 525 nm with a microplate reader (*PowerWave XS*) using a calibration curve generated using standard solutions of commercial L-ascorbic acid. For reduced AsA determination, the same reaction was used, but 0.2 M phosphate buffer (pH 7.4) was used in place of DTT and NEM. The amount of DHA was estimated from the difference between total ascorbate and reduced AsA.

Determination of antioxidant enzyme activities in leaf and root extracts: Frozen tissues (from leaves or roots) were homogenized in a 1:2 ratio (m/v) phosphate buffer containing 1 % (m/v) of polyvinylpolypyrrolidone, 2 mM of DTT and 0.5 mM of *Pefabloc* (a protease inhibitor). Extracts were centrifuged at 15 000 *g* and 4 °C for 10 min and supernatants were used immediately for the analysis of enzyme activities. Protein content was quantified according to the method of Bradford (1976), using a calibration curve constructed with bovine serum albumin as the standard.

All enzyme activities were measured in 200 mm^3 volume by monitoring enzyme reactions at 25 °C, using a microplate reader (*Power Wave XS*). All samples and blanks were analyzed in triplicate.

CAT activity was measured using a method adapted from that of Aebi (1984) with a slight modification, in a reaction mixture containing 0.1 M phosphate buffer (pH 7.0), 60 mM H_2O_2, and 10 mm^3 of plant extract supernatant. The enzyme activity was determined by monitoring the disappearance of H_2O_2 at 240 nm for 5 min and calculated using the coefficient of absorbance of 43.6 M^{-1} cm^{-1}.

Guaiacol peroxidase (GPX) activity was determined according to Cakmak (1994) with some modifications. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.7 mM H_2O_2, 35 mM guaiacol, and 15 mm^3 of supernatant. The enzyme activity was estimated by following the increase in absorbance due to the oxidation of guaiacol in the presence of H_2O_2 at 470 nm for 1 min using the coefficient of absorbance of 4 500 M^{-1} cm^{-1}.

Ascorbate peroxidase (APX) activity was determined following a method adapted from Nakano and Asada (1981), by measuring the decrease of absorbance at 290 nm for 5 min and calculated using the coefficient of absorbance of 2800 M^{-1} cm^{-1}. GR activity was assayed as described by Murshed *et al*. (2008), after analyzing the decrease in absorbance at 340 nm due to the oxidation of NADPH using the coefficient of absorbance of 6 220 M^{-1} cm^{-1}.

Statistical analyses: The experiment was carried out with six plants per treatment, representing 36 plants for each experimental group (25, 40, and 45 °C) in three independent biological experiments of plant growth and stress treatment. Collected data were analyzed using a one-way *ANOVA* following an initial check of *ANOVA* assumptions using the statistical analysis system (*CoStat*, *CoHort Software*, Birmingham, UK), followed by Duncan's new multiple range tests. Significant differences between treatments were identified at the 0.05 probability level. The data shown are means ± SE of three biological replicates.

Results

Heat stress treatment triggered various morphological changes in tomato leaves consisting of necrotic and chlorotic lesions for plants exposed to 40 and 45 °C, noticeable after 72 h of heat treatment, whereas control plants cultivated at 25 °C did not show any leaf damage symptoms (Fig. 1). Necrotic lesions were observed especially in the peripheral areas of the leaves and increased with increasing temperature and duration of heat stress. Leaf lesions were accompanied by necrotic damage in the roots which took a brown colour at 40 °C and 45 °C (Fig. 2). Moreover, plants exposed to high growth-medium temperatures showed a significantly decreased chlorophyll *a* and *b* content at both 40 and 45 °C as compared to control plants, more visible by the end of heat stress (Fig. 3*A*,*B*). In fact, the chlorophyll *a* and *b* content were reduced compared to control plants by about 45 and 31 % at 40 °C, and by 61 and 48 % at 45 °C, respectively, as determined 96 h after the initiation of heat stress. Consequently, the total chlorophyll content decreased below control values during the entire period of heat stress (Fig. 3*C*). This decline was highest at the end of the stress period with a 38 % decrease for plants treated at 40 °C, and 54 % for those at 45 °C. Higher content of total carotenoids was found at a root temperature of 40 °C with a 24 and 43 % increase after 24 h and 72 h, respectively, and remained constant during the last 24 h of the experiment compared to the control (Fig. 3*D*). Furthermore, plants subjected to 45 °C showed a remarkable increase of carotenoids content starting from the first 24 to 96 h, the increase was 1.5-, 2.2-, and 2,3 fold, respectively, compared to 25 °C. In contrast, the chlorophyll *a*/*b* ratio steadily decreased under both high temperatures as compared to 25 °C, with the lowest values recorded 72 h after the stress treatment (Fig. 3*E*). Significant differences between stressed and non-stressed plants were seen also in chlorophyll/carotenoids ratio throughout the different intervals of heat treatment, where the chlorophyll/carotenoids ratio in stressed plants decreased to 59, 53, and 55 % of the control at 40 °C, and even to 50, 38, and 31 % of the control at 45 °C after 24, 72, and 96 h of heat treatment, respectively (Fig. 3*F*).

Under normal growth conditions at 25 °C, tomato plants during the time intervals of 24, 72 and 96 h had similar H_2O_2 content (Fig. 4*A*). Heat stress at 45°C resulted in a continuous increase of H_2O_2 content to about 110, 126 an 130% of the control plants at 24, 72, and 96 h, respectively (Fig. 4*A*). On the other hand, increased root temperature to 40 °C led to a significant increase of H_2O_2 only during the first 24 h followed by a significant decline from 72 to 96 h of heat stress to 78 or 60 % of the control, respectively.

In accordance with the observed increase of H_2O_2 content, the increased MDA content indicated a higher

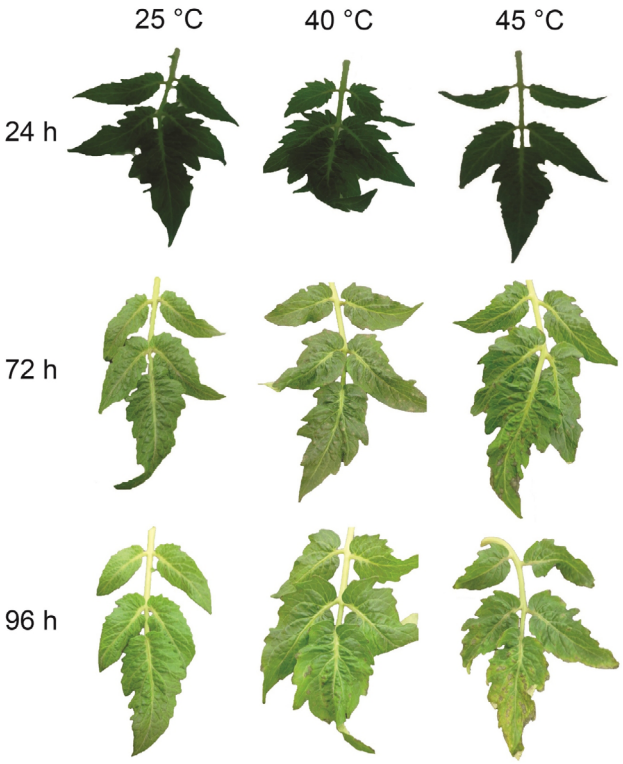

Fig. 1. Representative images of leaves of tomato (*Solanum lycopersicum* L. cv. Micro-Tom) plants exposed to high root temperatures during 24, 72, and 96 h.

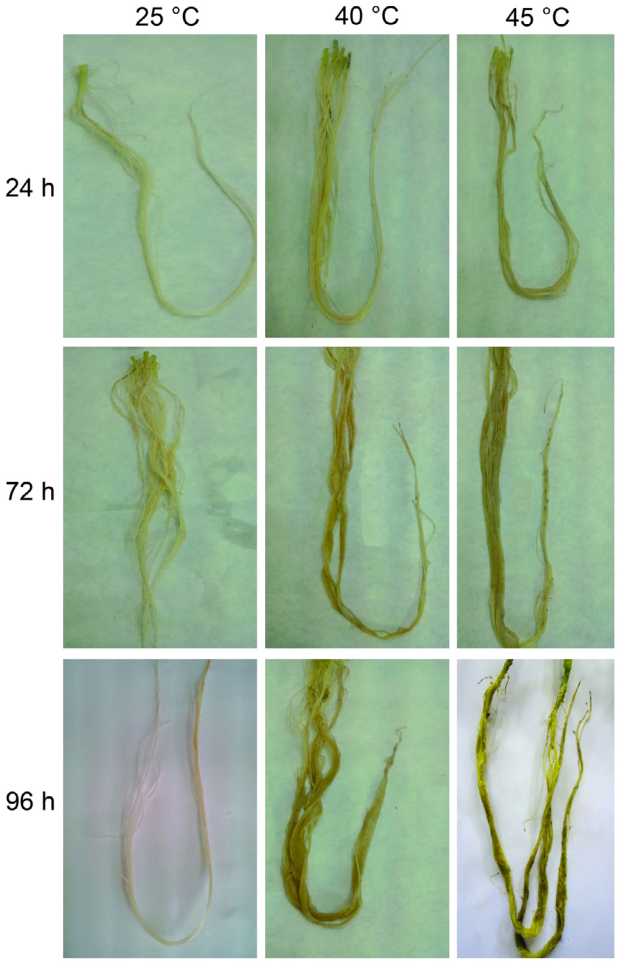

Fig. 2**.** Representative images of roots of tomato cv. Micro-Tom plants exposed to high root temperatures during 24, 72, and 96 h.

lipid peroxidation in plants exposed to 45 °C, it increased approximately 3-fold at 96 h of the heat stress (Fig. 4*B*). Interestingly, despite the observation that H_2O_2 content decreased at 40 °C after 72 and 96 h of treatment, the amount of MDA was approximately equal to that of control followed by a significant 3-fold rise at the end of heat treatment.

We analysed the influence of applied heat stress on the leaf content of the antioxidant compounds that participate in the glutathione-ascorbate cycle (Fig. 5). The content of total and reduced AsA showed a significant increase at 45 °C during the entire heat stress period by about 14 and 21 % after 24 h and by 20 and 30 % at the end of stress, respectively, as compared to that at 25 °C. However, there was no significant difference in the content of total AsA at 40 °C whereas the content of reduced AsA increased significantly, especially after 72 h of heat stress.

In the leaves, exposure to high growth-medium temperatures resulted in a significant enhancement of CAT activity at 72 and 96 h after heat treatment with maximum values at 45 °C as compared to the control (Fig. 6*A*). Peroxidases are another group of enzymes considered to be involved in the removal of high concentrations of H_2O_2. The largest increase was seen 72 h after heat stress where APX activity increased 3.4-fold at 45 °C in comparison with 25 °C. However, after 96 h of heat treatment at 45 °C, APX activity showed a significant reduction below the control values by 20 % whereas, for plants maintained at 40 °C, the activity rises 2.2-fold compared to control plants (Fig. 6*B*). In parallel with CAT activity and with increasing time after the onset of heat stress, GPX activity indicated a systematic increase at 40 °C and much greater at 45 °C, especially after 96 h of heat stress. GPX activity increased 8.7- and 9.6-times at 40 °C and 12.2- and 22.2-times at 45 °C after 72 and 96 h of heat stress, respectively, as compared to 25 °C (Fig. 6*C*). GR activity increased compared to the control at 40 °C since the 24 h of treatment and remained quite high till the end of the experiment. The most marked enhancement for this enzyme was registered at 45 °C from 72 up to 96 h of heat stress, with an increase by 12.2-fold and 10.1-fold to that at 25 °C (Fig. 6*D*).

In the roots, under normal growth conditions, no changes in CAT activity were detected for control plants while at the high growth-medium temperature of 45 °C, CAT activity increased significantly during the entire heat stress period (Fig. 7*A*). At 40 °C, the activity decreased after 72 h and then increased after 96 h of heat stress as compared to 25 °C. Besides, APX activity showed a similar trend as CAT activity at 40 °C with the largest values after 24 h followed by a decline after 72 h of the experiment (Fig. 7*B*). However, at the temperature of 45 °C, APX activity indicated a steady decrease below the

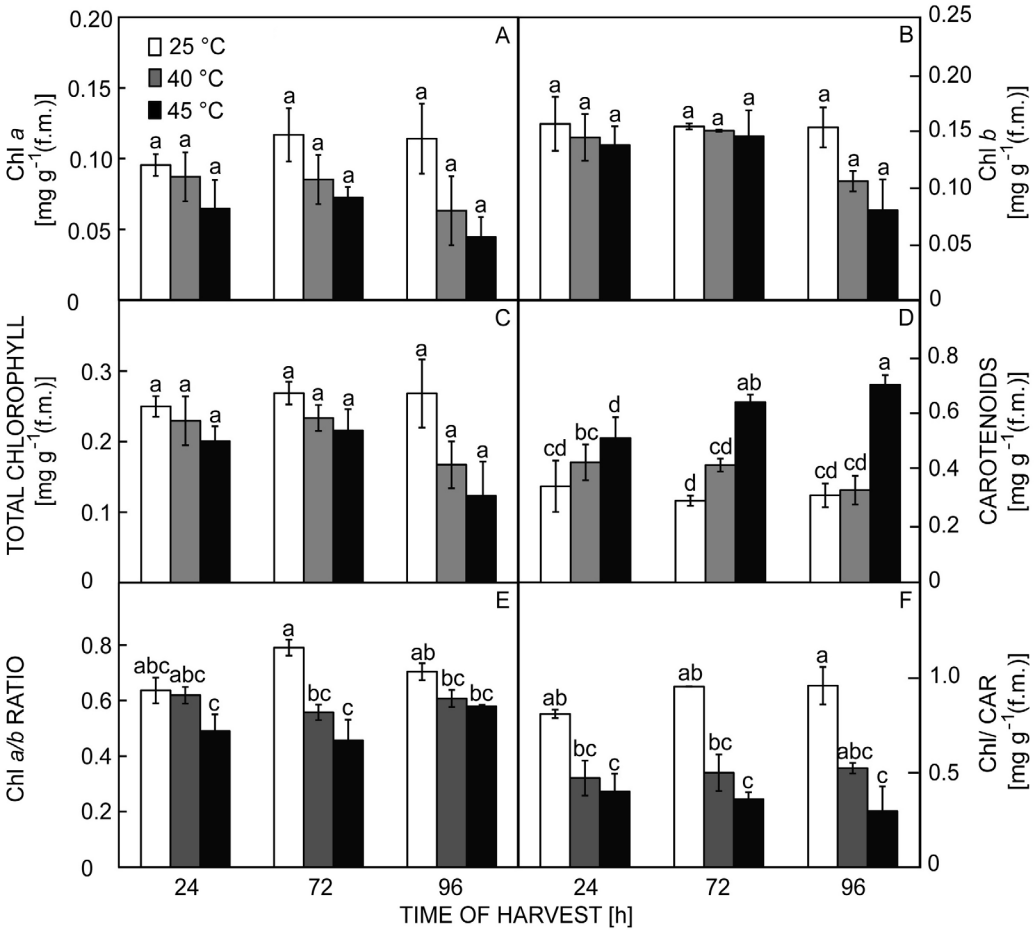

Fig. 3. Effect of high growth-medium temperatures on chlorophyll *a* (Chl *a*; *A*), chlorophyll *b* (Chl *b*, *B*), total chlorophyll content (*C*), total content of carotenoids (*D*), chlorophyll *a*/*b* ratio (*E*), and chlorophyll/carotenoids ratio (*F*) in leaves of *Solanum lycopersicum* L. cv. Micro-Tom. Means ± SEs, $n = 3$; significantly different values are denoted by different letters.

control during the entire experiment. Furthermore, GPX activity reached the maximum at 24 h of stress duration by 2.1- and 6-fold at 40 and 45 °C, respectively, and remained approximately equal to control values during the rest of the heat treatment (Fig. 7*C*). GR activity showed no significant differences between non-stressed and stressed plants during the first 24 h of heat treatment whereas it increased significantly under both high temperatures with 1.6-fold and 1.8-fold after 72 h, and with 1.9-fold and 2.6-fold after 96 h of heat stress at 40 °C and 45 °C, respectively, compared to control plants at 25 °C (Fig. 7*D*).

Discussion

For tomatoes, the optimal growth conditions include a temperature range of 25 - 35 °C during the daytime and 20 °C during the night (Camejo *et al*. 2006). The elevated mean daily temperature at the leaf or the root level in many agricultural regions harmfully affects different developmental stages of such crops (Mathur *et al*. 2018) and contributes up to 70 % losses in tomato yield (Sato *et al*. 2004). High temperatures negatively affect all aspects of the photosynthesis apparatus which is known to be highly sensitive to heat stress (Mathur *et al*. 2014). The present results showed significant damage to both the leaf and roots of stressed plants (Fig. 1, 2) and a remarkable decrease in chlorophyll *a*, chlorophyll *b* and total chlorophyll content to about 50 % of control values at 45 °C after 96 h of heat treatment. The decrease in chlorophyll content under high-temperature may be due to an impaired chlorophyll synthesis or its accelerated degradation or a combination of both (Mathur *et al*. 2014). The content of chlorophyll *a* was more affected than chlorophyll *b* content (Fig. 3*A*,*B*) resulting in a lower chlorophyll *a*/*b* ratio (Fig. 3*E*). A similar result was found by Ding *et al*. (2016) who observed that in cucumber plants exposed to a high root temperature of 35 °C for 4 d down-regulation of Rubisco and the enzymes of RuBP regeneration limit the photosynthetic rate. Another study focused on the effect of high temperatures on the leaves described that photosynthesis is depressed in most plants when leaf temperature exceeds 38 °C (Edwards and Walker 1983) due to limitations in either electron transport or Rubisco activase capacity (Sage and Kubien 2007). In tomatoes subjected to moderate heating (30/35 °C night/day) or severe heating (37/42 °C night/day), the photosynthetic rate is diminished in response to a high temperature of

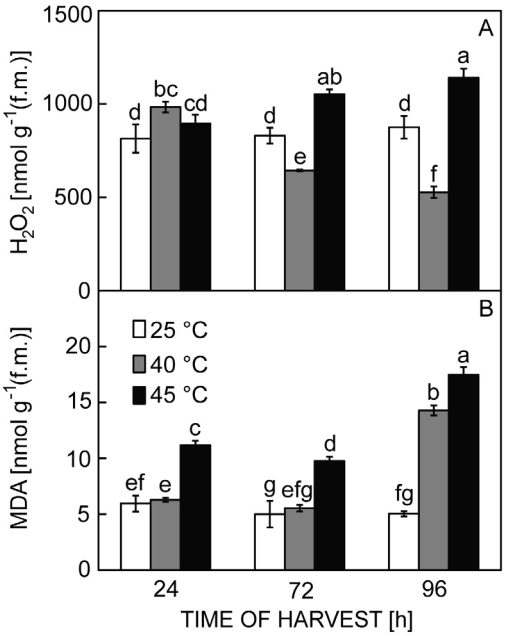

Fig. 4. Effect of high growth-medium temperatures on hydrogen peroxide (H_2O_2; *A*) and malondialdehyde (MDA; *B*) content in leaves of *Solanum lycopersicum* L. cv. Micro-Tom plants exposed to high root temperatures over a time interval of 96 h. Means ± SE, $n = 4$; significantly different values are denoted by different letters.

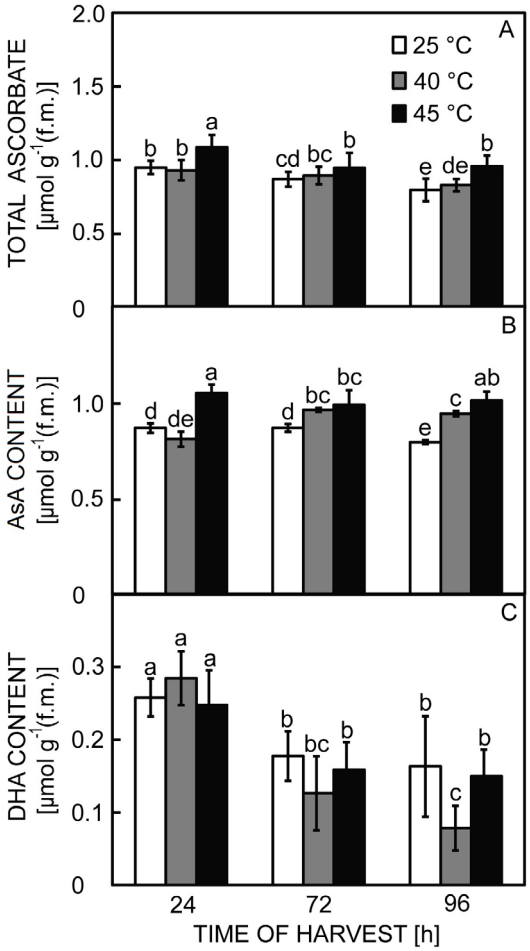

Fig. 5. Effect of high growth-medium temperatures on leaf content of total ascorbic acid (AsA; *A*), reduced AsA (*B*), and dehydroascorbic acid (DHA; *C*). Means ± SE, $n = 3$; significantly different values are denoted by different letters.

42 °C, while root membrane damage increases at both 35 and 42 °C (Giri *et al.* 2017). The impact of heat on root growth is larger than on shoot growth as indicated by a decrease in root-to-shoot ratio, especially for severely stressed plants.

Nada *et al.* (2003) reported that exposing roots to high temperatures of 38 °C for up to 10 d, with the aerial part remaining at an optimal temperature for growth (25 °C), causes a decline in photosynthesis and gas exchange in cucumber leaves. Besides, they suggested that increased leaf ABA content after heat stress reduces stomatal conductance and the activation state of Rubisco leading to impaired photosynthesis. Similarly, in tomato plants exposed to heat (root treatment of 55 °C for 4 d), photosynthetic rate and stomatal conductance are reduced as compared to plants grown at 25 °C (Willkinson and Davies 2002, Guenthner 2019). This is contrary to the findings of Cocetta *et al.* (2018) who indicated that short-term exposure of roots of *Diplotaxis tenuifolia* to heat stress at 40 °C has a little effect on the chlorophyll content that is slightly higher after 48 h compared to control and content of glucose remains unchanged in both leaves and roots. These findings might be related to the specific capacity of rocket plants to adapt their primary metabolism during short-term heat stress, in contrast to profound modifications of the secondary metabolism of glucosinolates.

Further, low chlorophyll content in high temperature-stressed plants is suggested to be associated with increased ROS production and so oxidative stress in plants (Chalanika De Silva and Asaeda 2017). The mechanisms by which high root temperature reduces growth and photosynthesis are not well understood. Reactive oxygen species (ROS) accumulation resulting from high root temperatures can damage cell membranes and impact metabolic activity in shoots. Studies have suggested that increased ROS accumulation stimulates photodamage to photosystem PS II by inhibiting its repair (Allakhverdiev *et al.* 2008). At the chloroplast level, high temperatures induced structural modifications of thylakoids and changes of the grana stacking or swelling (Zhang *et al.* 2014); causing a decline in photochemical reactions of thylakoid pigment-protein complexes (Su *et al.* 2014, Chen *et al.* 2016), which might be one of the important reasons for notably decreased photosynthesis under heat stress (Shaheen *et al.* 2015). These results revealed that photosynthesis is very sensitive to changes in the root environment and that the susceptibility of leaves to stress seemed to be modified by the degree of root temperature and its duration.

Unlike chlorophyll, the content of carotenoids in tomatoes subjected to 45 °C showed a significant increase proportionally with the duration of heat stress for plants subjected to 40 °C (Fig. 3*D*). The accumulation of

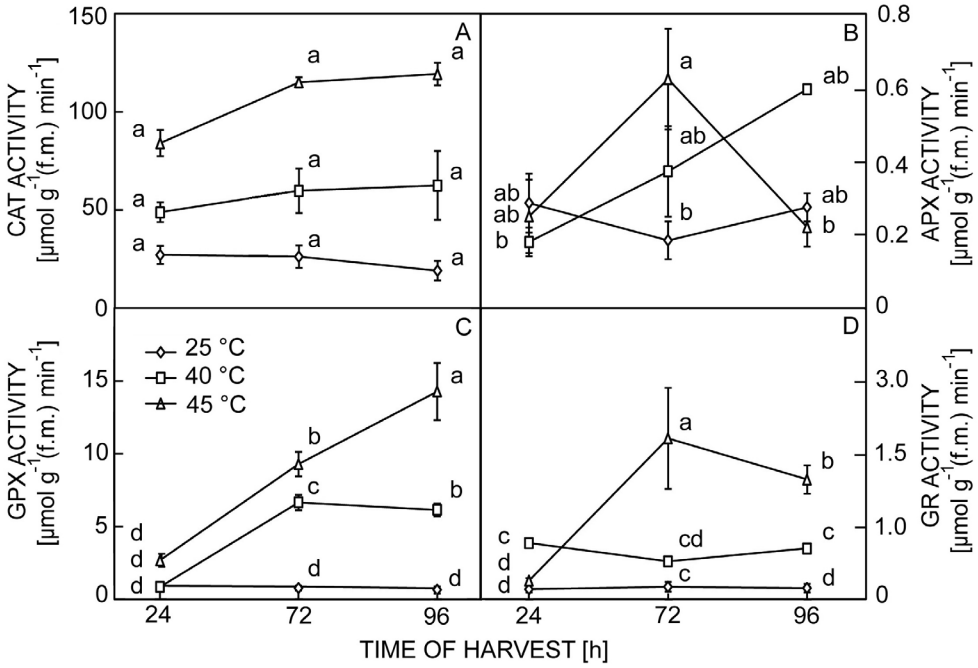

Fig. 6. Impact of high growth-medium temperatures on catalase (CAT; *A*), ascorbate peroxidase (APX; *B*), guaiacol peroxidase (GPX; *C*), and glutathione reductase (GR, *D*) activities in leaves of *S. lycopersicum* L. cv. Micro-Tom. Means ± SE, $n = 3$; significantly different values are denoted by different letters.

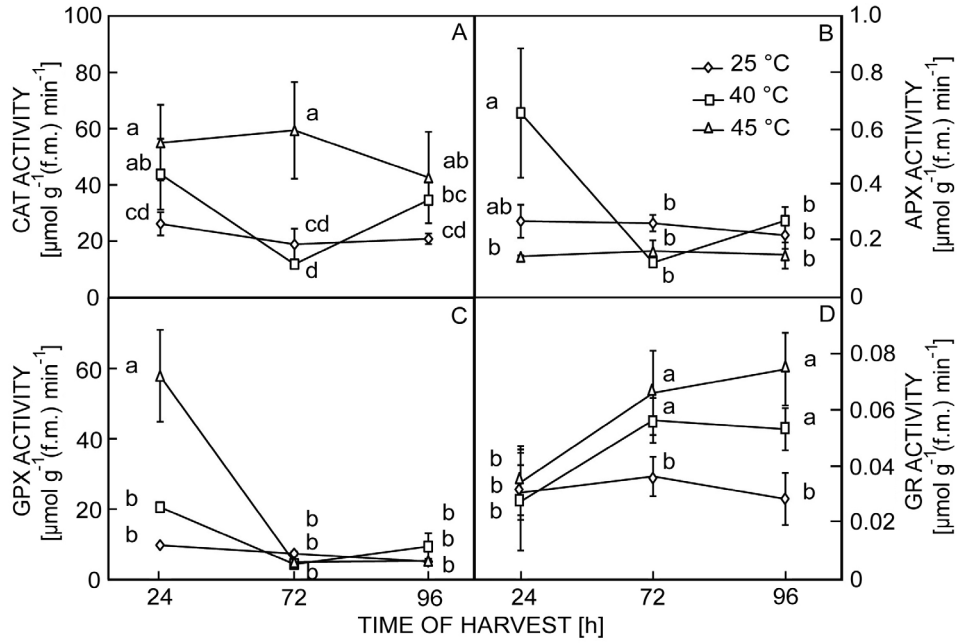

Fig. 7. Impact of high growth-medium temperatures on catalase (CAT; *A*), ascorbate peroxidase (APX; *B*), guaiacol peroxidase (GPX; *C*), and glutathione reductase (GR; *D*) activities in roots of *S. lycopersicum* L. cv. Micro-Tom. Means ± SE, $n = 3$; significantly different values are denoted by different letters.

carotenoids as non-enzymatic antioxidants (Mir-Aafaq *et al.* 2013) reduces the oxidative and peroxidative injury to membrane lipids by ROS ensuring the protection of cellular structures in different plant species (Wahid *et al.* 2007). For sustained photosynthesis, carotenoids are involved in the quenching of singlet oxygen ($^1O_2$), lipid peroxyl radicals as well as excited triplet chlorophyll which generates $^1O_2$ by a transfer of excitation energy to oxygen (Kadkhodaie *et al.* 2014). Camejo and Torres (2001) observed that total carotenoid content does not change in tomato cv. Amalia sensitive to heat stress, whereas it increases in the thermotolerant cv. Nagcarlang when exposed to heat treatment of 45 °C for 2 and 3 h, indicating that maintaining a higher or invariable content

of total carotenoids during stress conditions has a great implication in the plant tolerance to heat stress. Thereby, an over-accumulation of ROS in plants under high temperatures is often the major cause of degradation of chlorophyll, as photosynthetic reactions represent a well-established source of cellular oxidants, especially under stress conditions (Chalanika De Silva and Asaeda 2017). In this regard, Ding *et al*. (2016) reported that high root zone treatment of 35 °C for 4 d significantly reduces the content of carotenoids in cucumber plants, indicating oxidative damage in plant tissues (Wahid *et al*. 2007), contrarily to our findings.

It is well established that free radical-induced membrane damage accompanied by higher lipid peroxidation is detrimental to plant tissues in response to environmental stresses (Ruelland and Zachowski 2010). In the current study, we observed a direct relationship between heat stress and oxidative stress evaluated in terms of H_2O_2 and MDA content in leaf extracts. Generally, heat stress triggers the generation of H_2O_2 leading to so-called oxidative stress (Djanaguiraman *et al.* 2010). Based on our data, high H_2O_2 content caused oxidative damage in tomato leaves with an increase in heat stress at 24 h of the treatment at 45 °C. Consistently, increased ROS production is recorded in turf leaves exposed to high soil temperatures (Huang *et al.* 2001). In bentgrass roots and leaves, ROS accumulation corresponds to the reduced photosynthetic rate associated with premature leaf tissues senescence at a root temperature of 35 °C (Liu and Huang 2004).

Conversely, at 40 °C, the production of leaf H_2O_2 in stressed plants was prevented (Fig. 4*A*), which may be dependent upon an enhanced antioxidant potential in the tissues and the reppression of ROS formation during heat stress by inhibiting the electron transfer processes in chloroplasts or mitochondria (Mittler 2002, Suzuki *et al.* 2011). In addition, the lowest content of H_2O_2 might be related in part to the highest accumulation of carotenoids acting as free radicals scavengers under high root temperatures. Similarly, the results provided by Silva *et al*. (2017) indicated that when *J. curcas* roots were imposed to 42 °C regardless of shoot temperature, the content of leaf H_2O_2 is barely affected compared to control plants.

Under stress conditions, the extent of ROS production can exceed the antioxidant defence capability of plants, causing damage to cell components (Van Breusegem and Dat 2006). Lipid peroxidation has long been used as a criterion to assess heat injury to plant cell membranes (Savicka and Škute 2010). In the present experiment, we found that tomato leaf MDA content was increased significantly under a high root temperature of 45 °C during the entire heat period compared to control (Fig. 4*B*), reflecting a serious membrane injury by lipid peroxidation.

The destabilization of cell membranes by exposure to high root temperatures is partially a product of ROS accumulation due to increased respiration (Van Breusegem and Dat 2006), in agreement with previous reports for cucumber (Ding *et al*. 2016). However, at 40 °C till 72 h of the heat stress, tomato plants sustained low membrane damage suggesting that constant rates of MDA production compared to control plants could be used as a biochemical marker for heat tolerance. Changing to a more rigid membrane state would modify membrane transport properties to protect cells against the damage by acute stress stimuli (Horvárth *et al*. 1998). Besides, as a signalling molecule H_2O_2 regulates cell growth and activates acclimation responses under unfavourable conditions (Grennan 2008, Gill and Tuteja 2010). Therefore, high H_2O_2 content detected upon 24 h under 40 °C may explain the constant MDA content during the 72-h time interval of stress treatment, whereas it was not the major cause of the membrane injury occurring 96 h after the onset of heat stress. We suppose that other ROS such as superoxide or hydroxyl radical would be rather implicated (Jeffrey 2002).

Antioxidant enzymes function as part of the defence system is extremely important against deleterious ROS accumulation in plant cells (Siddiqui *et al.* 2013). The current study indicates that CAT, GPX, and APX activities in tomato leaves showed no significant variations as compared to control plants during the first 24 h at 40 °C but later increased (Fig. 6*A-C*). Reduced activities of CAT and peroxidases in parallel with early H_2O_2 generation have also been reported in other plants under heat shock (Jiang and Huang 2001, Liu *et al*. 2012). Moreover, CAT, APX, and GPX showed a greater increase after 72 h of heat stress leading to low content of H_2O_2 which might alleviate the damage to cell membranes (Harsh *et al.* 2016). Indeed, tolerance to high temperature is associated with an increase in antioxidant enzyme activities (Sairam *et al*. 2000). Other enzymes play a vital role in H_2O_2 detoxification by accelerating the regeneration of ascorbate and glutathione throughout the ascorbate-glutathione cycle including glutathione reductase (GR) which exhibited an enhanced activity at 40 °C. Here, we observed that an increase in root temperature to 40 °C elicited an efficient ROS-scavenging system that would dissipate excess energy and thereby limit critical damage to the photosynthetic tissues (Tan *et al.* 2011). Supporting these findings, Jiang and Huang (2001) claimed that CAT, G-POD, APX, and GR activities are stimulated in leaf samples of cucumber plants subjected to high root zone temperature treatment of 35 °C.

Regarding tomato plants heat-stressed at 45 °C, it is evident that leaf CAT, GPX, and GR activities significantly increased especially after 72 and 96 h compared to plants treated at 40 °C. In agreement with this result, Kumar *et al.* (2012) recorded a higher expression of antioxidant enzymes in rice plants when imposed to a day/night temperature of 40 and 35 °C. No variations are observed for the antioxidants in *Jatropha curcas* leaves under root/shoot treatment of 42/27 °C as compared to control plants sustained at root/shoot temperatures of 27/27 °C for 12 h (Silva *et al*. 2017). Thus, the enhanced activities of ROS scavenging enzymes may represent a positive input that is triggered when plants are exposed to long-term heat stress (Paul *et al*. 2016). In this experiment, the highest activities were registered for CAT, GPX, and GR under 45 °C in parallel with enhanced H_2O_2 content during the heat stress period. In plant cells, CAT has a high capacity to scavenge H_2O_2 by breaking it down directly into water and

oxygen but with a lower affinity compared to peroxidases (Mittler 2002). Generally, high temperatures lead to increased antioxidative enzymes activities up to a certain temperature limit after which they decline (Zafar *et al.* 2018). This limit temperature differs between the tolerant and susceptible crop cultivars (Hameed *et al.* 2012). In accordance with this report, a high root temperature of 45 °C induced a trend of variable changes in APX activity in tomato leaves. This was probably due to an APX inactivation with prolonged periods of heat stress for 96 h. Chakraborty and Pradhan (2011) reported that catalase (CAT) and ascorbate peroxidase (APX) in *Lens culinaris* shows an initial increase before declining at 50 °C, while PRX and glutathione reductase (GR) activities decrease at all temperatures ranging from 20 to 50 °C. Furthermore, the declining trend of APX since 72 h is adverse with the production of H_2O_2 and TBARS demonstrating that the inactivation of antioxidant enzymes under extreme long-term heat stress reduces the removal of ROS and promotes their accumulation.

In this regard, Mano *et al.* (2001) suggested that antioxidative enzymes are early targets of various environmental stresses, but each enzyme is inactivated in a stress-specific manner. Alternatively, Dash and Mohanty (2001), reported that the enzymatic efficiency in metabolizing H_2O_2 is differential due to temperature-dependent alterations of CAT and APX in heat-sensitive wheat cultivars. From these findings, we infer that tomato was able to induce a coordinate function of antioxidant enzymes represented by CAT, GPX, and GR involved in ROS degradation when roots were subjected to a high temperature of 45 °C. Also, the exposure durations resulting in oxidative damage in leaf tissues declined as root temperature increased. Scavenging ROS by antioxidant enzymes is of great importance to maintain the stability of the photosynthetic apparatus in various plants under heat stress (Tanaka and Makino 2009).

Along with the high activities of antioxidant enzymes in tomato leaves, the content of total and reduced AsA substantially increased at 40 °C and was more noticeable at 45 °C (Fig. 5). Ascorbate, as one of the most abundant redox compounds, is located in the cytosol, vacuole, chloroplasts, and apoplastic space in leaf cells (Polle *et al.* 1990). AsA has a potent antioxidant capacity in keeping ROS under control in plants through the ascorbate-glutathione cycle (Orsavová *et al.* 2019). Hence, the maintenance of the cellular AsA pool in a reduced state is associated with defence responses (Léchaudel *et al.* 2013). Generally, an increase in the foliar AsA content is suggested as a short-term acclimation to resist heat stress (Badiani *et al.* 1997). Moreover, elevation in the AsA pool is observed to stimulate APX and GR activity (Das and Roychoudhury 2014). Therefore, efficient enzymatic degradation of H_2O_2 and AsA regeneration allows electron flow through photosystems controlling the redox state of photosynthesis which could protect the chloroplastic components against oxidation by H_2O_2. As known, AsA can strongly affect the expression of genes encoding photosynthetic components (Kiddle *et al.* 2003). These results are largely in agreement with studies involving heat acclimated turf grass (Xu *et al.* 2006).

In contrast, the antioxidant enzymes in the roots show different behaviour compared to the leaves. A considerable and a significant increase in CAT, APX, and GPX activities at a root temperature of 40 °C was observed after 24 h with a later decrease till the end of the experiment, whereas GR activity was enhanced during all heat stress periods (Fig. 7*A-D*) In cucurbit plants, boosting root temperature from 24 to 34 °C induces increased activities of APX, CAT, and GPX (Zhang *et al.* 2007). Inactivation of the detoxification enzymes due to high temperatures is observed in wheat (Savicka and Škute 2010). In this context, Mittler *et al.* (2006) suggested that higher ROS content under elevated temperatures possibly causes cellular injury due to lower antioxidant activity in the stressed tissues.

On the other hand, our data showed that extreme root temperature of 45 °C preferentially triggered CAT and GR activities in roots during the entire heat stress experiment, alike APX and GPX activities which had the same response as in plants exposed to 40 °C. It can be speculated that with further increase in root temperature, the activity of the enzymatic system in plant roots was affected suggesting that the occurrence of oxidative damage was more pronounced in roots than in leaves as they are in direct contact with high temperatures. The significant increase of antioxidant enzymes activities might reveal a stronger tolerance of roots to heat treatment of 45 °C mediated by better protection against increased ROS accumulation. However, Silva *et al.* (2017) showed that root CAT, SOD, and APX activities are inhibited under root/shoot treatment of 42/27 °C for 12 h in *Jatropha curcas*, leading to higher oxidative damage in the roots.

In leaves, ROS are produced mainly in mitochondria, chloroplasts and peroxisome, whereas in root tissues they are generated basically in mitochondria (Navrot *et al.* 2007). Specifically, during prolonged heat stress, the mitochondrial H_2O_2 and O_2^- production increases with increased root respiration in different plant species (Savicka and Škute 2010). Thus, root respiratory activity and production of reactive oxygen species play a main role in root temperature sensitivity (Rachmilevitch *et al.* 2006). A similar result was found by Silva *et al* (2017) who reported that exposure of *Jatropha curcas* roots to 42 °C for 12 h induces higher ROS content and membrane peroxidation than in shoots grown at 27 °C. Consequently, it is vital for plants to adjust the enzymatic machinery and activate specific ROS scavenging enzymes to counteract oxidative stress (Mishra *et al.* 2015).

Interestingly, our data showed that temperature changes in roots affected tomato leaves even under non-limiting air temperatures. The root system may be affected by high temperatures in accordance with our findings as root browning occurred after 24 h at a root temperature of 45 °C, which has profound influences on plant growth (St Clair and Lynch 2010). It has been reported that root zone temperature is an essential factor in plant growth from germination and emergence through vegetative growth to floral initiation and reproductive growth (Adebooye *et al.* 2009). Moreover, root responses to increased temperature

can be species-specific, as different species have different optimum temperatures for root growth (Gray and Brady 2016). Indeed, roots act as an early detection system against unfavourable soil conditions and can adjust the physiological and molecular responses of the whole plant to changes within the soil profile (Aidoo *et al.* 2016). Furthermore, high root temperatures can trigger signals inducing downregulation of the shoot growth (Benlloch-Gonzalez *et al.* 2017), leading to reduced photosynthesis (Wise *et al.* 2004) and leaf senescence (Wang *et al.* 2003), which at critical stages of plant development affect the final yield (Sato *et al.* 2006). Wang *et al*. (2003) indicated that shoot injury of the creeping bentgrass is associated with an induction of oxidative stress. The enhancement of antioxidant metabolism is also anticipated in leaves when roots face stressful conditions (Silva *et al*. 2017). It should be noted that the specific antioxidant scavenging compounds or enzymes contributing to stress defence vary across plant species, cultivars, age, organs, and in response to stress type, severity, and duration (Gill and Tuteja 2010). The earlier findings showed possible targets of heat stress and the importance of signal transduction between roots and leaves to balance stress effects, ultimately leading to the activation of ROS metabolisms and the expression of antioxidant enzymes.

Conclusions

Our results uncover the importance of root growth-media temperatures in controlling plant growth and contribute to a deeper understanding of plant leaf responses to raised root temperatures regardless of shoot temperature. Presented data revealed that a high root temperature of 45 °C was more deleterious to photosynthesis than 40 °C, leading to higher oxidative damage in leaf tissues compared to roots treated at a temperature of 40 °C. Nonetheless, tomato plants under a root temperature of 40 °C exhibited an efficient enzymatic and non-enzymatic antioxidant system avoiding the photodamage and ensuring the protection of photosynthetic tissues under heat stress. In summary, a rise in root temperature changed the antioxidant metabolism in leaves with different scavenging components between the two plant organs due to their subcellular locations in different tissues. The biochemical changes observed show variable responses and degree of tolerance depending on the temperature and the exposure time, suggesting that species with greater root resilience have a competitive advantage to face stressful conditions. Field studies from the perspective of the regulatory and signalling network, sugar translocation process and the root architecture would further contribute to assessing tomato cultivars adapted to warmer environments.

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