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Effect of High Temperature on Different Genotypes of Citrus

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Global warming as a result of greenhouse gases can have a detrimental effect on crop productivity. High temperature can be deleterious to the growth and development of citrus trees. In the present investigation, 'Marsh' grapefruit and 'Hamlin' sweet orange trees were exposed to a temperature 38 °C (100 °F) to explore the mechanism of high temperature tolerance in these two genotypes. Trees were grown in 15 × 8 cm plastic pots containing Fafard Mix 4P. Mineral nutrition was provided periodically using Peters' professional fertilizer. Trees were kept at 700 µmole·m^{-2}·s^{-1} *PAR* with a 12-h light and 12-h dark photoperiod in environmental growth chambers and acclimated for 90 days at 23 to 27 °C (73 to 80 °F) before applying high temperature treatments. The experiment was conducted as a randomized complete-block design and mean values per plant were determined and subjected to analysis of variance. Plants were exposed to 38 °C (100 °F) for 10 days. Heat-induced oxidative stress was more pronounced in 'Hamlin' leaves than in grapefruit leaves in terms of H_2O_2 production. However, concentrations of H_2O_2 were same in both the genotypes in control conditions. Grapefruit leaves displayed a better antioxidant defense system under high temperature regimes than 'Hamlin' leaves. It seems 'Hamlin' sweet orange leaves will be more susceptible to rising global temperatures than grapefruit leaves.

Citrus, an economically important crop, is grown worldwide under climatic conditions that include hot humid equatorial and warm humid subtropical climates (Spiegel-Roy and Goldschmidt, 1996). Citrus growth, development, and distribution is severely affected by low temperatures but usually not by higher temperatures (Raveh et al., 2003; Spiegel-Roy and Goldschmidt, 1996). However, during changing environmental conditions and future projections of global climate change, high temperatures are likely to affect crop growth and productivity (Lobell and Field, 2007; Solomon et al., 2007). The optimum temperature requirement for citrus growth is 25 to 30 °C (Spiegel-Roy and Goldschmidt, 1996) but under warm-subtropical climate during summer leaf temperature can rise up to 39 to 41 °C (Syvertsen and Lloyd, 1994), which can adversely affect the rate of net photosynthesis and promote fruit abscission (Raveh et al., 2003).

During heat stress, limiting CO_2 fixation and stomatal closure generates reactive oxygen species (ROS) through the chloroplastic electron transport chain (Guo et al., 2006). ROS in excess are toxic for plant cells and consist of superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^·$; Foyer et al., 1997). In addition, high temperature-induced photorespiration also enhanced the levels of H_2O_2 in leaves (Guo et al., 2006).

Throughout the course of evolution, plants have developed enzymatic and non-enzymatic antioxidant defense mechanisms to control the rate of ROS formation (Fu and Huang, 2001). Enzymatic machinery constitutes superoxide dismutases (SOD) that dismutate O_2^- radical to form H_2O_2, which is further cleaved into water and oxygen by the action of catalase (CAT) and ascorbate peroxidase (APOD; Jeffery, 2002; Mittler, 2002). Catalases are specifically located in peroxisomes while APOD is located in multiple organelles in plant cells (Mittler, 2002).

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The metabolic shift in antioxidant machinery during heat exposures is a part of adaptive response to cope with deleterious effect of ROS (Xu et al., 2006). Transgenic plants with over-expression of antioxidant enzymes showed greater resistance against oxidative stress (Mittler, 2002). The strength of antioxidant defense in plant species determines their thermo-tolerance (Xu et al., 2006). Therefore, the main objective of this work was to determine the intrinsic antioxidant abilities of 'Hamlin' sweet orange and grapefruit to test their fitness in high temperature regimes.

Materials and Methods

Plant material and culture. Young 'Hamlin' sweet orange [*C. sinensis* (L.) Osbeck] and 'Marsh' grapefruit (*Citrus paradisi* Macfad.) trees on Swingle citrumelo rootstock were purchased from a commercial nursery (Harris Citrus, Lithia, FL) and maintained in 15 × 8 cm plastic pots containing Fafard Mix 4P (Southern Agricultural Insecticides Inc., Palmetto, FL). Mineral nutrition was provided periodically using Peters' professional fertilizer (The Scott Co., Marysville, OH). Trees were grown in 700 µmol·m^{-2}·s^{-1} photosynthetically active radiation (*PAR*) with a 12-h light and 12-h dark photoperiod in environmental growth chambers and acclimated for 90 d at relative humidity (RH) of 65% to 75% and optimum temperature ranges from 23 °C (73 °F) to 27 °C (80.6 °F) before applying the high temperature (HT) treatments.

Treatments. Control trees were maintained at optimal conditions (temperature, 23 to 27°C; RH 65% to 75%) and marked as grapefruit control (GFC) and 'Hamlin' control (HAMC). High temperature-treated plants were exposed to 38 °C (100.4 °F) for 10 d at 65% to 75% RH and marked as grapefruit high temperature (GFHT) and 'Hamlin' high temperature (HAMHT). All trees were watered every day to avoid water stress. Matured leaves (fully expanded) were used for all the biochemical estimations.

H_2O_2 concentration. Leaf samples (0.5 g) were homogenized in 0.1 % trichloroacetic acid (TCA) and centrifuged at 12,000 ×

g for 15 min at 4 °C. The supernatant (0.3 mL) was mixed with 1.3 mL potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide (KI) solution and incubated for 5 min before measuring oxidation product at A_{390}. H_2O_2 concentration was calculated from a standard curve prepared from known concentrations of H_2O_2 and expressed as μmol·g^{-1} FW (Velikova et al., 2000).

Leaf preparation for enzyme assays. Leaf samples (0.5 g) were homogenized in 100 mM potassium phosphate buffer pH 7.0 containing 0.5 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 2 % PVP in a pre-chilled pestle and mortar. The extraction buffer also contained 5 mM ascorbate and homogenate was centrifuged at 4 °C for 30 min at 15,000 g. Since maintenance of consistent catalase electrophoretic mobility required the presence of dithiothreitol (DTT; Anderson et al., 1995), an aliquot of each sample was made to 10 mM DTT to be used for catalase spectrophotometric assays. For enzymatic analysis, the supernatant was gel filtered over Sephadex G-25 (PD-10 column, GE Healthcare Bio-Science AB, Uppsala, Sweden) equilibrated with 50 mM potassium phosphate buffer pH 7.0. All enzyme activities were measured in a final volume of 3 mL using various aliquots of the supernatants (10–25 μL for SOD; 70–125 μL for CAT; 50 μL APOD).

Superoxide dismutase. SOD activity was determined by the method of Dhindsa et al. (1981). A preliminary study revealed that overnight dialysis of crude extract using cellulose ester dialysis tubing (MWCO 8000–10000 Dalton) showed no interference by small molecular weight impurities. Similar results were also reported in different plant species such as pea, oat, and corn (Giannopolitis and Ries, 1977). The assay solution (total volume 3.0 mL) contained 201 mM methionine, 1.72 mM NBT, an appropriate aliquot of (10–25 μL) enzyme extract, 50 mM potassium phosphate buffer (pH 7.8), and 0.12 mM riboflavin. The riboflavin was added last. The tubes were shaken and placed 30 cm below a light source consisting of two 75-W fluorescent bulbs. The reaction was started by switching on the light for 10 min. The tubes were covered with black cloth immediately after switching off the light. Non-irradiated reaction mixtures containing the enzyme extract, which did not develop color, were used as controls. Absorbance of the reaction mixture was measured at 560 nm. Blanks lacked enzyme in the reaction mixture and developed maximum color. One unit of SOD activity was defined as the enzyme activity that caused a 50% inhibition of the initial rate of the reaction in the absence of enzyme (Dhindsa et al., 1981). Activity was expressed in units of min^{-1}·mg^{-1} protein.

Catalase (CAT). CAT activity was assayed in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2, and leaf extract. The decomposition of H_2O_2 was followed at 240 nm (extinction coefficient = 39.4 mM·cm^{-1}; Cakmak and Marschner, 1992).

Ascorbate peroxidase (APOD). APOD activity was assayed in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 1.0 mM H_2O_2, 0.25 mM ascorbic acid and leaf extract. The activity was determined by the rate of ascorbate oxidation at 290 nm (extinction coefficient = 2.8 mM cm^{-1}; Nakano and Asada, 1981). Protein content was determined by the method of Bradford (1976) using BSA as the standard.

Statistical analysis. The experiment was conducted as a randomized complete-block design with five blocks, and one plant of each treatment per block in a growth chamber. Five plants were used for each treatment. On each sampling date, three leaves per plant were removed and all assays were conducted on each leaf, which represented five replicates (plants) and each replicate with three sub-samples (leaves). Three independent experiments were conducted. Mean values per plant were determined and subjected to analysis of variance (SAS Institute, Cary, NC) with means separated using a protected least significant difference (LSD) at $P < 0.05$. The standard error (SE) of the mean was also calculated.

Results and Discussion

High temperature can adversely affects plant growth and development (Mittler et al., 2012) and at the biochemical level, high temperature is known to inhibit ATP synthesis, chloroplastic electron transport chain and also to inactivate rubisco (Ribulose-1,5-bisphosphate carboxylase oxygenase) activation (Haldimann and Feller, 2004; Huve et al., 2011). Given conditions of limited CO_2 fixation, excessive heat absorption caused transfer of electron to molecular oxygen, which can lead to the formation of toxic O_2^- radicals (Asada, 1999).

Superoxide dismutases are the primary defense against of O_2^- radicals in plant systems during various abiotic stresses (Tsang et al., 1991). Superoxide dismutase activity increased in leaves of both citrus genotypes after high temperature exposure (Table 1). However, SOD activity was higher in grapefruit in comparison to 'Hamlin' leaves throughout the period of heat stress. This rise in SOD activity was 3.3-fold, 4.1-fold, and 2.1-fold at 4, 8, and 10 d after high temperature exposure (DAE), respectively, in grapefruit while only a 2.2-fold, 1.5-fold, and 1.5-fold rise was observed in 'Hamlin' at 4, 8, and 10 DAE, respectively. These observations showed that grapefruit leaves were better protected from the deleterious effect of O_2^- than 'Hamlin' leaves.

The byproduct of SOD activity is H_2O_2. In spite of higher SOD activity in grapefruit, the levels of H_2O_2 were lower in grapefruit leaves than in 'Hamlin' leaves (Table 2). Levels of H_2O_2 remained

Table 1. Effect of high temperature (HT; 38 °C) on SOD activity (units mg^{-1} protein min^{-1}) in leaves of grapefruit (GF) and 'Hamlin' (HAM) leaves. The standard error (±SE) of each mean (n = 15) was calculated and asterisks indicate mean values that are significantly different from the 27 °C controls (means separated using LSD at $P < 0.05$).

	Treatments			
DAE[z]	GFC (27 °C)	GFHT (38 °C)	HAMC (27 °C)	HAMHT (38 °C)
0	0.75 (± 0.10)	0.78 (± 0.12)	1.01 (± 0.13)	1.02 (± 0.11)
4	0.60 (± 0.11)	1.99 (± 0.13)*	0.98 (± 0.14)	2.18 (± 0.11)*
8	0.57 (± 0.20)	2.37 (± 0.14)*	1.56 (± 0.14)	2.38 (± 0.13)*
10	0.88 (± 0.12)	1.85 (± 0.13)*	0.90 (± 0.13)	1.39 (± 0.14)*

[z]Days after high temperature exposure.

Table 2. Effect of high temperature (HT; 38 °C) on H_2O_2 (μmol·g^{-1} fw) concentration in leaves of grapefruit (GF) and 'Hamlin' (HAM) leaves. The standard error (±SE) of each mean (n = 15) was calculated and asterisks indicate mean values that are significantly different from the 27 °C controls (means separated using LSD at $P < 0.05$).

	Treatments			
DAE[z]	GFC (27 °C)	GFHT (38 °C)	HAMC (27 °C)	HAMHT (38 °C)
0	2.87 (± 0.10)	2.61 (± 0.20)	3.01 (± 0.11)	2.94 (± 0.20)
4	3.38 (± 0.12)	2.85 (± 0.11)*	3.26 (± 0.13)	5.31 (± 0.12)*
8	2.26 (± 0.11)	2.41 (± 0.14)*	2.56 (± 0.13)	6.22 (± 0.11)*
10	3.42 (± 0.13)	2.49 (± 0.12)*	3.15 (± 0.14)	5.95 (± 0.16)*

[z]Days after high temperature exposure.

higher in 'Hamlin' with the highest H_2O_2 (6.2 µmol·g^{-1} fw) accumulation observed at 8 DAE. The lower SOD activity and higher H_2O_2 accumulation in 'Hamlin' leaves might cause the generation of $OH^{\cdot}$ radicals by the Fenton reaction (Pospicil, 2009). Hydroxyl radicals are the most toxic ROS and directly attack membrane lipids and cause lipid peroxidation and finally cell death (Halliwell, 1991). Therefore, survival of plant cells exposed to high temperature stress requires an efficient H_2O_2 removing system.

We have evaluated the activities of H_2O_2 catabolizing enzymes to further understand the mechanism of oxidative stress tolerance in citrus genotypes. Catalase and ascorbate peroxidase are the major H_2O_2 degrading enzymes in plants (Mittler, 2002). CAT removes the bulk of H_2O_2 in peroxisomes, and APOD fine tunes its levels in different cellular compartments (Mittler, 2002; Willekens et al., 1997).

Catalase activity was enhanced by high temperature and remained consistently higher in grapefruit than in 'Hamlin' leaves while this activity showed a significant rise only at 4 DAE in 'Hamlin' and then declined by 1.6-fold at 10 DAE (Table 3). The highest CAT activity (0.088 µmol H_2O_2 mg^{-1} protein·min^{-1}) was observed at 4 DAE in grapefruit and showed a rise of 3.1-fold compared to controls, which reflected the early CAT mediated removal of H_2O_2 when in the high temperature regime. However, only a 1.3-fold rise in CAT activity was observed in 'Hamlin' at 4 DAE which thereafter declined. 'Hamlin' had a low degradation of H_2O_2 in terms of CAT activity. Similar results were found for APOD activity in 'Hamlin' (Table 4). Ascorbate peroxidase activity increased (2.1-fold) up to 4 DAE and then declined. The APOD activity never declined in grapefruit and showed 2.0-fold, 3.4-fold, and 3.0-fold increase at 4, 8, and 10 DAE, repectively. Hydrogen peroxide metabolism was efficient in grapefruit leaves from the beginning of high temperature stress. The functioning of higher SOD, CAT, and APOD activities probably maintained the low levels of H_2O_2 in grapefruit leaves and thus avoided the potential oxidative damage. In comparison, the complete failure of SOD, CAT, and APOD association 4 DAE in 'Hamlin' leaves appeared to be at least a contributing cause of its susceptibility to high temperature stress.

Table 3. Effect of high temperature (HT; 38 °C) on catalase activity (µmol H_2O_2 mg^{-1} protein min^{-1}) in leaves of grapefruit (GF) and 'Hamlin' (HAM) leaves. The standard error (±SE) of each mean (n = 15) was calculated and asterisks indicate mean values that are significantly different from the 27 °C controls (means separated using LSD at $P < 0.05$).

	Treatments			
DAE[z]	GFC (27 °C)	GFHT (38 °C)	HAMC (27 °C)	HAMHT (38 °C)
0	0.053 (±0.001)	0.051 (±0.002)	0.037 (±0.001)	0.035 (±0.001)
4	0.028 (±0.002)	0.088 (±0.003)*	0.028 (±0.002)	0.038 (±0.001)*
8	0.028 (±0.002)	0.071 (±0.001)*	0.037 (±0.001)	0.037 (±0.002)
10	0.047 (±0.001)	0.063 (±0.002)*	0.055 (±0.002)	0.035 (±0.001)*

[z]Days after high temperature exposure.

Table 4. Effect of high temperature (HT; 38 °C) on APOD activity (µmol asc. mg^{-1} protein min^{-1}) in leaves of grapefruit (GF) and 'Hamlin' (HAM) leaves. The standard error (±SE) of each mean (n = 15) was calculated and asterisks indicate mean values that are significantly different from the 27 °C controls (means separated using LSD at $P < 0.05$).

	Treatments			
DAE[z]	GFC (27 °C)	GFHT (38 °C)	HAMC (27 °C)	HAMHT (38 °C)
0	0.16 (± 0.002)	0.17 (± 0.002)	0.13 (± 0.002)	0.15 (± 0.001)
4	0.11 (± 0.001)	0.23 (± 0.001)*	0.11 (± 0.003)	0.24 (± 0.003)*
8	0.10 (± 0.003)	0.34 (± 0.004)*	0.11 (± 0.004)	0.05 (± 0.004)*
10	0.13 (± 0.002)	0.39 (± 0.003)*	0.08 (± 0.002)	0.07 (± 0.001)

[z]Days after high temperature exposure.

Literature Cited

Anderson, M.D., T.K. Prasad, and C.R. Steward. 1995. Changes in isozyme profiles of catalase, peroxidase, and glutathione reductase during acclimation to chilling in mesocotyls of maize seedlings. Plant Physiol. 109:1247–1257.

Asada, K. 1999. The water–water cycle in chloroplast: Scavenging of active oxygen and dissipation of excess photons. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50:601–639.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72:248–254.

Cakmak, I. and H. Marschner. 1992. Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves. Plant Physiol. 98:1222–1227.

Dhindsa, R.A., P. Plumb-Dhindsa, and T.A. Thorpe. 1981. Leaf senescence: Correlated with increased permeability and lipid peroxidation and decreased levels of superoxide dismutase and catalase. J. Expt. Bot. 32:93–101.

Foyer, C.H., H. Lopes-Degado, J.F. Dat, and I.M. Scott. 1997. Hydrogen peroxide and glutathione associated mechanism of acclamatory stress tolerance and signaling. Plant Physiol. 100:241–254.

Fu, J. and B. Huang. 2001. Involvement of antioxidants and lipid peroxidation in the adaptation of two cool season grasses to localized drought stress. Environ. Exp. Bot. 45:105–114.

Giannopolitis, C.N. and S.K. Ries. 1977. Superoxide dismutases. I. Occurrence in higher plants. Plant Physiol. 59:309–314.

Guo, Y-P., H-F. Zhou, and L-C. Zhang. 2006. Photosynthetic characteristics and protective mechanisms against photo-oxidation during high temperature stress in two citrus species. Scientia Hort. 108:260–267.

Haldimann, P. and U. Feller. 2004. Inhibition of photosynthesis by high temperature in Oak (*Quercus pubescens* L.) leaves grown under natural conditions closely correlates with a reversible heat-dependent reduction of the activation state of ribulose-1,5-bisphosphate carboxylase/oxygenase. Plant Cell Environ. 27:1169–1183.

Halliwell, B. 1991. Oxygen radicals: Their formation in plant tissues and their role in herbicide damage. In: N.R. Baker and M.P. Percival (eds.). Herbicides. Elsevier Science.

Huve, K., I. Bichele, B. Rasulov, and U. Niinemets. 2011. When it is too hot for photosynthesis: Heat-induced instability of photosynthesis in relation to respiratory burst, cell permeability changes and H_2O_2 formation. Plant Cell Environ. 34:113–126.

Jeffrey, A.A. 2002. Catalase activity, hydrogen peroxide content and the thermotolerance of pepper leaves. Scientia Hort. 95:277–284.

Lobell, B.B. and C.B. Field. 2007. Global scale climate–crop yield relationship and the impact of recent warming. Environ. Res. Lett. 2:014002 (7 p.).

Mittler, R. 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7:405–410.

Mittler, R., A. Finka, and P. Goloubinoff. 2012. How do plants feel the heat? Trends Plant Sci. 37:118–125.

Nakano, Y. and K. Asada. 1981. Hydrogen peroxide is scavenged by ascorbate specific peroxidases in spinach chloroplast. Plant Cell Physiol. 22:887–892.

Pospisil, P. 2009. Production of reactive oxygen species by photosystem II. Biochim. Biophys. Acta 1787:1151–1160.

Raveh, H., S. Cohen, T. Raz, D. Yakir, and E.E. Goldschmidt. 2003. Increased growth of young citrus trees under reduced radiation load

in a semi-arid climate. J. Expt. Bot. 54:365–373.
Solomon, S., D. Qin, M. Manning, M. Marquis, K. Averyt, M.M.B. Tignor, M.H. Leroy, and Z. Chen. 2007. Climate Change 2007: The physical science basis. Contribution of working group I to the fourth assessment report of the intergovernmental panel on climate change. Cambridge University Press.
Spigel-Roy, P. and E.E. Goldschmidt. 1996. Biology of horticultural crops: Biology of citrus. Cambridge University Press.
Syvertsen, J.P. and J.J. Lloyd. 1994. Citrus. In: B. Schaffer and P.C. Andersen (ed.). Handbook of environmental physiology of fruit crops. Vol. II. Subtropical and tropical crops. CRC Press, Boca Raton, FL.
Tsang, E.W.T., C. Bowler, D. Herouart, W. Van Camp, R. Villaroel, C. Genetello, M. Van Montagu, and D. Inze. 1991. Differential regulation of superoxide dismutase in plants exposed to environmental stress. Plant Cell 8:783–792.
Velikova, V., I. Yordanov, and A. Edreva. 2000. Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective roles of exogenous polyamines. Plant Sci. 151:59–66.
Willekens, H., S. Chamnongpol, M. Davey, M. Schraudner, C. Langebartels, M. Van Montagu, D. Inze, and W. Van Camp. 1997. Catalase is a sink for H_2O_2 and is indispensable for stress defense in C_3 plants. EMBO J. 16:4806–4816.
Xu, S., J. Li, X. Zhang, H. Wei, and L. Cui. 2006. Effect of heat acclimation pretreatment on changes of membrane lipid peroxidation, antioxidant metabolites, and ultra-structure of chloroplast in two cool-season species under heat stress. Environ. Expt. Bot. 56:274–285.