World Academy of Science, Engineering and Technology
International Journal of Agricultural and Biosystems Engineering
Vol:6, No:11, 2012

Interspecific Variation in Heat Stress Tolerance and Oxidative Damage among 15 C_3 Species

Wagdi S. Soliman, and Shu-ichi Sugiyama

***Abstract*—**The C_3 plants are frequently suffering from exposure to high temperature stress which limits the growth and yield of these plants. This study seeks to clarify the physiological mechanisms of heat tolerance in relation to oxidative stress in C_3 species. Fifteen C_3 species were exposed to prolonged moderately high temperature stress 36/30°C for 40 days in a growth chamber. Chlorophyll fluorescence (Fv/Fm) showed great difference among species at 40 days of the stress. The species showed decreases in Fv/Fm and increases in malondialdehyde (MDA) content under stress condition as well as negative correlation between Fv/Fm and MDA ($r = -0.61^*$) at 40 days of the stress. Hydrogen peroxide (H_2O_2) content before and after stress in addition to its response under stress showed great differences among species. The results suggest that the difference in heat tolerance among C_3 species is closely associated with the ability to suppress oxidative damage but not with the content of reactive oxygen species (ROS) which is regulated by complex network.

***Keywords*—**C_3 species, Fv/Fm, heat stress, oxidative stress.

I. Introduction

THE plants from different habitats have different optimum growth temperature. The C_3 species adapt to temperate climates, while C_4 species can be tolerant to hot and drought conditions. The anticipated higher summer temperatures under climate warming are likely to cause serious damages to the growth and yield of C_3 crops [1-2]. Therefore, improving the tolerance of C_3 crops to heat stress is a major target for breeders [3-4]. However, the key traits that confer such tolerance in the field have not been clearly identified so far [5-7].

Plants exposed to temperature stress are suffering from the accumulation of reactive oxygen species (ROS) which cause oxidative stress. ROS is produced in leaves as a result of the imbalance between electron transfer rate and carboxylation capacity in photosynthetic process [8-10]. In previous study, we found that the sensitive cultivar of *Lolium perenne* to summer climates showed greater accumulation of hydrogen peroxide (H_2O_2) in leaves than that in the tolerant cultivars under prolonged moderately high temperature stress [11]. This result suggested that functional damage under summer high temperature is mainly caused by oxidative stress, which is derived from excess light energy generated under heat stress.

The ROS are generated by aerobic respiration in mitochondria, photosynthetic light reaction in chloroplasts, and photorespiration in peroxisomes [12-13]. The balance of ROS content regulates by its production and scavenging system which in turn is regulated by a redundant and complex biochemical network. Breakdown of gene expression in two major scavenging enzymes, namely ascorbate peroxidase (APX) and catalase, does not bring substantial changes in oxidative balance [14-15].

To understand the tolerance mechanism of plants to heat stress, it is important to make comparative studies both within species and among species which differ in their tolerance. So far, most studies that compared heat tolerance have been limited to comparison among a few numbers of cultivars [16-19] or a few numbers of species which are closely related [20-22]. Few studies have examined differentiation among large number of unrelated species under long-term heat stress. In this study, responses to heat stress were compared among fifteen C_3 grass species belonging to different genus with diverse genetic background with special reference to the relationship between heat tolerance and oxidative tolerance.

W. S. Soliman is with the Faculty of Agriculture, South Valley University, Qena 83523 Egypt (phone: +20-5211-835; fax: +20-5211-835; e-mail: wagdi79@yahoo.com).
S. Sugiyama is with the Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki 036-8560 Japan, (e-mail: sugi@cc.hirosaki-u.ac.jp).

II. Materials and Methods

A. Plant Materials

In this study, fifteen C_3 species were used including; *Agrostis alba* L., *Agrostis tenuis* Sibth., *Anthoxanthum odoratum* L., *Bromus inermis* Leyss., *Dactylis glomerata* L., *Festuca arundinacea* Schreb., *Festuca ovina* L., *Festuca pratensis* Huds., *Festuca rubra* L., *Lolium multiflorum* Lam., *Lolium perenne* L., *Phalaris arundinacea* L., *Phleum pratense* L., *Poa annua* L., *Poa pratensis* L.

B. Growth and Heat Stress Conditions

Seeds of the 15 species were germinated on wet filter paper in Petri dishes, and the seedlings were transplanted into pots – one seedling in each pot– 7.5 cm in diameter and 8 cm deep and filled with sandy loam containing 0.35 g of each of N, P_2O_5, and K_2O for every kilogram of soil. The plants were grown in a controlled growth chamber with day/night temperatures of 23/16 °C, a 16-h photoperiod (4:00 to 20:00 h) with photon flux of 250 µmol m^{-2} s^{-1}, and relative humidity of 70% round the clock. Forty days after transplanting, the plants were exposed to 30 ºC for 3 days for acclimation and then to 36/30 ºC (day/night) for 40 days. The plants were watered daily to avoid water stress. The experiment was set up in a randomized block layout incorporating four replications.

C. Chlorophyll Fluorescence Measurement

The minimum (F_0) and maximal (F_m) levels of fluorescence were measured in leaves adapted to dark for 20 min with a portable photosynthesis measuring system (LI-6400, Li-cor, Lincoln, Nebraska, USA). The maximal photochemical efficiency of photosystem II (PSII), the most heat-sensitive component in photosynthesis, was calculated as $F_v/F_m = (F_m - F_0)/F_m$. Measurements were made before the acclimation (control) and at 10-day intervals during the period of exposure to high temperatures.

D. Physiological Measurements

Membrane lipid peroxidation (MDA) and hydrogen peroxide (H_2O_2) content were recorded twice, before the acclimation and at 40 days of stress exposure. Membrane lipid peroxidation was determined by malondialdehyde (MDA) content using the thiobarbituric acid (TBA) method as described before [11, 23]. Fresh leaves (50 mg samples) were ground in 1.5 mL of 0.1% solution of trichloroacetic acid (TCA). The homogenate was centrifuged at 10 000 rpm at 3 ºC for 5 min, and 1 mL of the supernatant was mixed with 2 mL of 0.5% TBA in 20% TCA. After heating the mixture for 20 min in boiling water and cooling it quickly in an ice bath, the supernatant was used for spectrophotometric determination of MDA. Absorbance at 532 nm was recorded and corrected for non-specific absorbance at 600 nm. Concentrations of MDA were calculated on fresh weight (FW) basis by the following formula with an extinction coefficient of 155 $mmol^{-1} cm^{-1}$.

A modified version of the ferrous ammonium sulphate/ xylenol orange (eFOX) method was used to measure H_2O_2 content of leaves following the methods of [24-25]. Leaf extracts were prepared by grinding 50 mg leaf samples in 500 µL of 0.1 M potassium phosphate buffer (pH 6.5) containing 5 mM Na N_3 as an inhibitor of peroxidase activity. The extracts were centrifuged at 10 000 rpm at 5 ºC for 5 min. The supernatant (200 µL) was added to 5 mL of the assay solution containing 250 µM ferrous ammonium sulphate, 100 µM sorbitol, 100 µM xylenol orange, 1% ethanol, and 25 mM H_2SO_4, which had been deoxygenated with gaseous nitrogen to prevent artefact production in hydrogen peroxide during the reaction. The spectrophotometric assay was conducted by measuring the difference in absorbance between 550 nm and 800 nm after 15 min of the reaction. H_2O_2 content was calculated by a standard curve using a series of diluted solutions of commercial, high-grade 30% H_2O_2.

E. Statistical Analysis

Analysis of variance (ANOVA) was used to test the significance of differences among the species for each measurement. The statistical analysis was carried out using JMP (ver 4. SAS Institute, Cary, NC, USA).

III. Results

Chlorophyll fluorescence (Fv/Fm) showed no significant differences among 15 species before the exposure to heat stress with overall mean value of 0.779±0.001. Fv/Fm significantly decreased at 40 days of heat stress (0.636±0.032). The differences among species began to appear at 10 days of the stress and the differences became two-folds at 40 days of the stress (Fig. 1). The species were divided into three categories according to the degree of damage: (1) high tolerant species (seven species) which maintained more than 85% of Fv/Fm at 40 days of the stress, (2) medium tolerant species (six species) which maintained 75 ~ 85% of Fv/Fm and (3) sensitive species (two species) with less than 50% of Fv/Fm (Fig. 1).

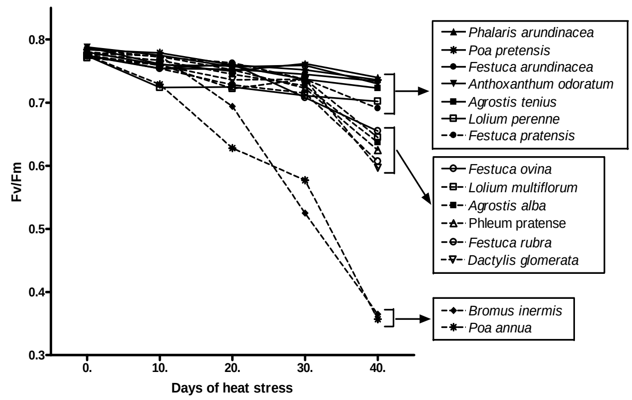

Fig. 1 Response pattern of chlorophyll fluorescence in 15 C_3 species at different durations (days) of continuous exposure to heat stress

Lipid peroxidation of membrane (malondialdehyde, MDA) and hydrogen peroxide (H_2O_2) showed highly significant differences among species before and after exposure to heat stress (Table I). MDA showed significantly negative correlation with Fv/Fm at 40 days of the stress (Fig. 2). The MDA content differed by eightfold before exposure to stress and by threefold after exposure to stress (Table I). *Bromus inermis* and *Festuca rubra* had the highest values of MDA content both before and after exposure to the stress. After exposure to the stress, MDA content increased significantly in all species except *Phalaris arundinacea* (Table II). H_2O_2 content showed the same response to MDA except for the significant decrease of H_2O_2 content in *Dactyles glomerata* and *Poa annua* (Table II). The H_2O_2 content differed by fifteen-folds and six-folds before and at 40 days of the stress, respectively (Table I). The highest values of H_2O_2 content both before and after exposure to stress were in *Festuca rubra* and *Festuca ovina*, respectively.

TABLE I
MINIMUM AND MAXIMUM VALUES AS WELL AS THE F VALUE OF VARIATION AMONG THE 15-SPECIES OF MALONDIALDEHYDE (MDA, µMOL G^{-1} FW) AND HYDROGEN PEROXIDE (H_2O_2, µMOL MG^{-1} FW)

	Control		40-day	
	Rang	F value	Rang	F value
MDA	6.3 ~ 52.5	50.2*	20.9 ~ 72.9	32.9*
H_2O_2	0.16 ~ 2.38	188.5*	0.51 ~ 3.12	110.7*

The value represents significance at probability level of $p > 0.001$

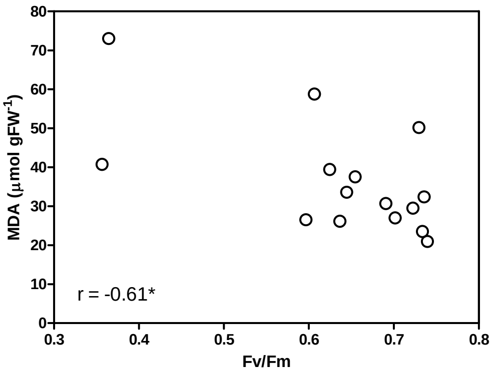

Fig. 2 The correlation between chlorophyll fluorescence (Fv/Fm) and malondialdehyde content (MDA, µmol g^{-1} FW) at 40 days of exposure to heat stress

TABLE II
THE RELATIVE CHANGES OF MALONDIALDEHYDE (MDA) AND HYDROGEN PEROXIDE (H_2O_2). THE RELATIVE CHANGES WERE CALCULATED AS PERCENTAGE OF THE VALUES AT 40 DAYS OF THE STRESS AGAINST CONTROL AND THE PROBABILITY OF SIGNIFICANT (*, **, *** AT 0.05, 0.01, AND 0.001, RESPECTIVELY) REPRESENT THE SIGNIFICANCE UNDER STRESS CONDITION COMPARE TO BEFORE STRESS

Species	MDA	H_2O_2
1. *Agrostis alba*	317.8***	268.6***
2. *Agrostis tenuis*	238.2***	132.1*
3. *Anthoxanthum odoratum*	407.9***	325.0***
4. *Bromus inermis*	138.9*	175.0**
5. *Dactylis glomerata*	417.7***	67.3**
6. *Festuca arundinacea*	135.2***	145.7**
7. *Festuca ovina*	156.1*	142.0**
8. *Festuca pratensis*	241.0***	157.1***
9. *Festuca rubra*	130.4**	131.1***
10. *Lolium multiflorum*	133.5***	303.8***
11. *Lolium perenne*	143.9***	241.2**
12. *Phalaris arundinacea*	124.2	116.2
13. *Phleum pratense*	475.2***	244.4***
14. *Poa annua*	284.3***	52.2***
15. *Poa pratensis*	339.6***	159.5*

*, **, ***, significant difference at 5, 1 and 0.1% levels, respectively

IV. DISCUSSION

Chlorophyll fluorescence (Fv/Fm) is used widely as an indicator of physiological damage to abiotic stress [26]. In this study, the decreases in Fv/Fm varied greatly among species, ranging from less than 10 % to more than 50 % at 40 days of exposure to heat stress. The decreases in Fv/Fm varied significantly even within the same genus (Fig. 1). This indicates that there are great differences among the C_3 species in tolerance to heat stress (Fig. 1). The decline of Fv/Fm represents that the reaction centre of PSII was damaged and inactivated by the stress [27].

Reactive oxygen species (ROS) plays the two opposite roles in processes of heat stress responses: a toxic molecule and a signal transduction molecule [28-30]. Levels of hydrogen peroxide (H_2O_2) vary greatly among species under natural conditions [24-25]. In this study, the species showed great differences in H_2O_2 content even under unstressed conditions. The great differences in H_2O_2 suggest that the species have different strategy to utilize H_2O_2 in regulating molecular and physiological networks. The significant increases in MDA content and decreases in Fv/Fm under stress condition as well as the significant correlation between them at 40 days of the stress (r = –0.61*) suggest that the difference in heat tolerance is closely associated with the ability to suppress oxidative stress. This is consistent with our previous studies within *Lolium perenne* cultivars [11, 23]. The differences in Fv/Fm and MDA after the stress were not associated with H_2O_2 content, this may be due to that the species used in this study had wide genetic background and roles of H_2O_2 in stress response cascade differed with each other as exemplified by the two species, *Poa annua* and *Dactylis glomerata*, which showed great sensitivity to stress and H_2O_2 content significantly decreased after the stress exposure (Fig. 1 and Table II).

Plants develop several defense mechanisms against toxic reactive oxygen molecules. These mechanisms include suppressing ROS production, scavenging the produced ROS and repairing the damage caused by ROS [31]. The results of this study suggest that the differentiation among species in heat stress tolerance is mainly associated with the ability to suppress the producing of ROS species. The great variation among species in H_2O_2 content even under unstressed condition is due to the wide genetic background among them. This wide genetic background led to difficulty of determining the role of antioxidants, not included, in heat stress tolerance among the species.

ACKNOWLEDGMENT

This paper is part from the Ph.D. Dissertation of WS Soliman. He received financial support from the Egyptian government in the form of a scholarship for his doctoral programme.

REFERENCES

[1] D. B. Lobell, and G. P. Asner, “Climate and management contributions to recent trends in U.S. agricultural yields,” *Science*, vol. 299, pp. 1032, 2003.
[2] D. B. Lobell, and C. B. Field, “Global scale climate–crop yield relationships and the impacts of recent warming,” *Environ. Res. Letters,* vol. 2, 014002 (7 pp), 2007.
[3] B. Barnabás, K. Jäger, and A. Fehér, “The effect of drought and heat stress on reproductive processes in cereals,” *Plant Cell Environ.,* vol. 31, pp. 11–38, 2008.
[4] M. A. Semenov, and N. G. Halford, “Identifying target traits and molecular mechanisms for wheat breeding under a changing climate,” *J. Exp. Bot.,* vol. 60, pp. 2791–2804, 2009.
[5] M. Tester, and A. Bacic, “Abiotic stress tolerance in grasses. From model plants to crop plants,” *Plant Physiol.,* vol. 137, pp. 791–793, 2005.
[6] Y. Zhang, M. A. R. Mian, and J. H. Bouton, “Recent molecular and genomic studies on stress tolerance of forage and turf grasses,” *Crop Sci.,* vol. 46, pp. 497–511, 2006.
[7] A. Wahid, S. Gelani, M. Ashraf, and M. R. Foolad, “Heat tolerance in plants: an overview,” *Environ. Exp. Bot.,* vol. 61, pp. 199–223, 2007.
[8] T. D. Sharkey, “Effects of moderate heat stress on photosynthesis: importance of thylakoid reactions, rubisco deactivation, reactive oxygen species, and thermotolerance provided by isoprene,” *Plant Cell Environ.,* vol. 28, pp. 269–277, 2005.
[9] V. Velikova, and F. Loreto, “On the relationship between isoprene emission and thermotolerance in Phragmites australis leaves exposed to high temperatures and during the recovery from a heat stress,” *Plant Cell Environ.,* vol. 28, pp. 318–327, 2005.

[10] V. Locato, C. Gadaleta, L. De Gara, and M. C. De Pinto, "Production of reactive species and modulation of antioxidant network in response to heat shock: a critical balance for cell fate," *Plant Cell Environ.,* vol. 31, pp. 1606–1619, 2008.

[11] W. S. Soliman, M. Fujimori, K. Tase, and S. Sugiyama, "Oxidative stress and physiological damage under prolonged heat stress in C3 grass *Lolium perenne*," *Grassland Sci.*, vol. 57, pp. 101–106, 2011.

[12] R. Mittler, S. Vanderauwera, M. Gollery, and F. Van Breusegem, "Reactive oxygen gene network of plants," *Trends Plant Sci.,* vol. 9, pp. 490–498, 2004.

[13] G. Miller, N. Suzuki, S. Ciftci-Yilmaz, and R. Mittler, "Reactive oxygen species homeostasis and signalling during drought and salinity stresses. *Plant Cell Environ.,* vol. 33, pp. 453–467, 2010.

[14] L. Rizhsky, E. Hallak-Herr, F. Van Breusegem, S. Rachmilevitch, J. E. Barr, S. Rodermel, D. Inzé, and R. Mittler, "Double antisense plants lacking ascorbate peroxidase and catalase are less sensitive to oxidative stress than single antisense plants lacking ascorbate peroxidase or catalase," *Plant J.,* vol. 32, pp. 329–342, 2002.

[15] G. Miller, N. Suzuki, L. Rizhsky, A. Hegie, S. Koussevitzky, and R. Mittler, "Double mutants deficient in cytosolic and thylakoid ascorbate peroxidase reveal a complex mode of interaction between reactive oxygen species, plant development, and response to abiotic stresses," *Plant Physiol.,* vol. 144, pp. 1777–1785, 2007.

[16] B. Huang, X. Liu, and Q. Xu, "Supraoptimal soil temperatures induced oxidative stress in leaves of creeping bentgrass cultivars differing in heat tolerance. *Crop Sci.,* vol. 41, pp. 430–435, 2001.

[17] J. Larkindale, and B. Huang, "Changes of lipid and saturation level in leaves and roots for heat-stressed and heat-acclimated creeping bentgrass (*Agrostis stolonifera*)," *Environ. Exp. Bot.,* vol. 51, 57–67, 2004.

[18] M. Almeselmani, P. S. Deshmukh, R. K. Sairam, S. R. Kushwaha, and T. P. Singh, "Protective role of antioxidant enzymes under high temperature stress," *Plant Sci.,* vol. 171, pp. 382–388, 2006.

[19] Y. He, and B. Huang, "Protein changes during heat stress in three kentucky bluegrass cultivars differing in heat tolerance," *Crop Sci.,* vol. 47, pp. 2513–2520, 2007.

[20] S. Xu, J. Li, X. Zhang, H. Wei, and L. Cui, "Effects of heat acclimation pretreatment on changes of membrane lipid peroxidation, antioxidant metabolites, and ultrastructure of chloroplast in two cool-season turfgrass species under heat stress," *Environ. Exp. Bot.,* vol. 56, pp. 274–285, 2006.

[21] Y. Xu, and B. Huang, "Differential protein expression for geothermal Agrostis scabra and turf-type *Agrostis stolonifera* differing in heat tolerance," *Environ. Exp. Bot.,* vol. 64, pp. 58–64, 2008.

[22] C. Xu, and B. Huang, "Differential proteomic response to heat stress in thermal Agrostis scabra and heat–sensitive *Agrostis stolonifera*," *Physiol. Plantarum,* vol. 139, pp. 192–204, 2010.

[23] W. S. Soliman, M. Fujimori, K. Tase, and S. Sugiyama, "Heat tolerance and suppression of oxidative stress in: comparative analysis of 25 cultivars of the C_3 grass *Lolium perenne*," *Environ. Exp. Bot.*, vol. 78, pp. 10–17, 2012.

[24] J. M. Cheeseman, "Hydrogen peroxidase concentrations in leaves under natural conditions," *J. Exp. Bot.,* vol. 57, pp. 2435–2444, 2006.

[25] G. Queval, J. Hager, B. Gakière, and G. Noctor, "Why are literature data for H2O2 contents so variable? A discuusion of potential difficulties in the quantitative assay of leaf extracts," *J. Exp. Bot.,* vol. 59, pp. 135–146, 2008.

[26] K. Maxwell, and G. N. Johnson, "Chlorophyll fluorescence- a practical guide," *J. Exp. Bot.,* vol. 51, pp. 659–668, 2000.

[27] S. P. Long, S. Humphries, and P. G. Falkowski, "Photoinhibition of photosynthesis in nature," *Ann. Rev. Plant Physiol. Plant Mol. Biol.,* vol. 45, pp. 633–662, 1994.

[28] C. H. Foyer, and G. Noctor, "Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context," *Plant Cell Environ.,* vol. 28, pp. 1056–1071, 2005.

[29] N. Suzuki, and R. Mittler, "Reactive oxygen species and temperature stresses: A delicate balance between signaling and destruction. *Physiol. Plantarum,* vol. 126, pp. 45–51, 2006.

[30] P. Jaspers, and J. Kangasjärvi, "Reactive oxygen species in abiotic stress signaling," *Physiol. Plantarum,* vol. 138, pp. 405–413, 2010.

[31] K. Asada, "The water-water cycle in chloroplasts: Scavenging of active oxygens and dissipation of excess photons," *Ann. Rev. Plant Physiol. Plant Mol. Biol.,* vol. 50, pp. 601–639, 1999.