Research in Plant Sciences, 2013, Vol. 1, No. 2, 20-23
Available online at http://pubs.sciepub.com/plant/1/2/3
© Science and Education Publishing
DOI:10.12691/plant-1-2-3

The Effects of Heat Stress on Reactive Oxygen Species Production and Chlorophyll Concentration in *Arabidopsis Thaliana*

Erica Kipp*, Michael Boyle

Department of Biology and Health Sciences, Pace University, New York, USA
*Corresponding author: ekipp@pace.edu

Received June 19, 2013; Revised September 06, 2013; Accepted September 25, 2013

Abstract Environmental and ecological changes due to temperature increase, such as those associated with global climate change, have been well documented. Since temperature fluctuations are greater on a regional rather than global scale, research quantifying plant responses to heat stress has become a principal objective for environmental and ecological biologists. Aerobic organisms generate reactive oxygen species (ROS) in healthy cells at a controlled rate, however, at elevated temperatures, oxidative stress can create an imbalance between the production of ROS and the ability to detoxify and remove the reactive intermediates. ROS concentration was compared between heat-stressed and optimally grown *Arabidopsis thaliana*, ecotype *Col-0*. The control group was grown at 23°C/23°C day/night for a 12 hour photoperiod while the heat stressed group was grown at 29°C/23°C day/night for a 12 hour photoperiod. All plants were grown on Murashige and Skoog (M&S) Basal Salt agar plates enriched with 1N KOH. Measuring the concentration of Thiobarbituric Acid Reactive Substances (TBARS) such as malondialdehyde (MDA), a common ROS, is a well-established method for detecting and quantifying oxidative stress. Results using a colorimetric assay demonstrated that heat stressed plants had a 68.30% higher concentration of TBARS than optimally grown plants ($P = 0.0005$). And, since TBARS production is associated with lipid peroxidation and membrane integrity, it stands to reason that photosynthesis may also be affected. In order to measure chlorophyll concentration, chloroplasts were isolated and purified. Results of colorimetric assays indicated that chlorophyll was reduced by 41.4% in heat stressed plants $P = 0.0005$. Thus, heat stress results in an increase in oxidative stress, lipid peroxidation, a higher than optimal concentration of ROS in the plant tissue and may further impair the plant's ability to photosynthesize by reducing chlorophyll content.

Keywords: *heat stress, climate change, Arabidopsis thaliana, reactive oxygen species, chlorophyll*

Cite This Article: Erica Kipp, and Michael Boyle, "The Effects of Heat Stress on Reactive Oxygen Species Production and Chlorophyll Concentration in *Arabidopsis thaliana*." *Research in Plant Sciences* 1, no. 2 (2013): 20-23. doi: 10.12691/plant-1-2-3.

1. Introduction

Global climate change models predict a 2°C to 6°C temperature increase over the next Century [6,12]. This is driven in large measure by increasing atmospheric carbon dioxide, which is up by 31% since 1750 [15]. Concern over temperature increases and quantifying plant responses to heat stress has become of particular interest to a wide range of scientists and disciplines as evidence suggests that increased temperatures, such as those associated with global warming, will have widespread adverse effects on species diversity, food-web and ecosystem structuring, dominant vegetation, plant physiology and development, climate, and phenology [2,7,11,12]. Since temperature is one of the main factors affecting plant productivity [4,7,13,14], understanding how elevated temperature affects the plant's ability to grow and reproduce is critical. This is especially important from a financial standpoint as many economically valuable ornamental and agricultural plants will be affected. It has been determined, for example, that heat adversely affected pollen and anther development in *Lycopersicon esculentum* (Solanaceae), the tomato, which, in turn, contributed to a decreased fruit set [14]. *Phaseolus vulgaris* (Fabaceae), the common bean, showed that heat applied prior to anthesis resulted in pollen and anther development abnormalities [13]; heat was found to affect the reproductive capacity of *Linum usitatissimum* (Linaceae), flax, when applied after floral initiation [3] and heat reduced the development and reproductive success in *Arabidopsis thaliana* (Brassicaceae) [7].

While aerobic organisms generate ROS at a controlled rate, a common plant response to heat stress is the accelerated generation of ROS [8]. Reference [15] noted that plants produced antioxidant enzymes as part of enzymatic scavenging systems when ROS was produced at a controlled rate, however, when plants are subjected to adverse conditions, such as high temperature resulting in

an increase in ROS concentration, the scavenging system loses its overall function [9,15]. ROS can be destructive towards essential cellular components and structural elements [5]; and higher concentrations of ROS are associated with lipid peroxidation, making cellular membranes particularly susceptible to oxidative damage [9]. And since the thylakoid membrane is the site of the light-dependent reaction of photosynthesis, damage to this membrane may result in reduced photosynthetic activity. Reference [4] suggested that membrane damage results in leakage, which is considered an early event in a plant's response to heat stress. This leakage, however, may result in chlorophyll spilling out and degrading in the stroma. Reference [1] determined that heat in excess of 40°C affected antenna complexes and that just over 30°C, there was a decrease in overall Photosystem II photochemistry.

A TBARS-sensitive assay was used in order to quantify the amount of heat stress-induced ROS concentration and; the chloroplast isolation and purification protocol was employed in order to colorimetrically determine chlorophyll concentration in heat-stressed and optimally grown plants. Both protocols targeted ROS and chlorophyll concentrations in the leaves and stems of pre-reproductive plantlets, harvested after 14 days of heat or control temperature treatment.

2. Materials and Methods

2.1. Origin of Germplasm

Controlled laboratory experiments were conducted using *Arabidopsis thaliana* ecotype Col-0, from the *Arabidopsis* Biological Resource Center, Columbus, OH. ABRC was established in 1991 at The Ohio State University. ABRC seed stock was accessed via The *Arabidopsis* Information Resource (TAIR) web site (http://www.arabidopsis.org). Table 1 lists stock numbers and corresponding origin, latitude, elevation, and other relevant information for Col-0 ecotype. This table was compiled with information from The *Arabidopsis* Information Resource, 'localities and habitats of seed stock origin.'

Table 1. Summary of information for *A. thaliana* Col-0 ecotype used in this study

Stock Number	Name and Abbreviation	Country/ Location	Latitude Longitude (min/max)	Description
6673	Columbia Col-0	USA/ Columbia	N38/N39 W92/W93	Height 25-35cm, Serrated lf margins

2.1.1. Background on *Arabidopsis Thaliana*

A. thaliana is a plant in the Brassicaceae family and has quickly become a model organism across plant biology, molecular biology, and genetics. *A. thaliana* is a small herbaceous rosette annual that inhabits a wide range of ecological and geographical regions. At only about 25 cm at maturity, *A. thaliana* completes its life-cycle within a 6-8 week period. The flower is complete, perfect, and self-compatible; the stamens are longitudinally dehiscent and the resulting fruit type, typical of Brassicaceae, is the silique.

2.2. Plant Growth

Seeds were surface-sterilized in 1 mL 70% ethanol for 1 minute and 4 mL 20% bleach for 20 minutes, followed by 4 sterile water washes in a laminar flow hood. The seeds were placed in 4 mL sterile water and allowed to cold germinate at 4°C for 4 days. The germinated seeds, with evident hypocotyl-radical axis, were transferred within a sterile hood onto Murashige and Skoog (M&S) Basal Salt agar plates enriched with 1N KOH. The plates were sealed with 3M micropore surgical tape to prevent them from drying out over the course of the experiment and during heat treatments.

2.3. Heat Stress Treatment

In order to develop a study more applicable to field conditions, experimentally heat stressed *Arabidopsis* plants received a temperature 4°C to 6°C above its optimum temperature range, an increase which corresponds tightly to climate change model predictions. Control plants were grown under optimal conditions at 23°C both day and night with a 12-hour photoperiod and for a total of 14 days. Heat stressed plants were grown at 29°C during the day and 23°C at night with a 12-hour photoperiod and for a total of 14 days. Plants were grown in a Percival E-41HO growth chamber with controlled temperature, humidity and light. Literature indicates optimum humidity for *Arabidopsis* is between 25-75% [10], thus we maintained a humidity of 55% and no more than 1.250 $\mu M/m^2/s$ of light irradiance, measured at a distance of 6" between the plant samples and the growth chamber lamps.

2.4. Quantitative Determination of TBARS

Biological specimens contain a mixture of TBARS, including lipid hydroperoxides and aldehydes, which increase as a result of oxidative stress [17]. TBARS are low-molecular-weight end products that are formed during the decomposition of lipid peroxidation products and are expressed in terms of MDA or malondialdehyde concentrations [17]. The level of lipid peroxidation, which is equal to the amount of MDA, corresponds to the amount of heat-stress induced oxidative damage. ROS was quantified using the BioAssay Systems QuantiChrom Thiobarbituric Acid Reactive Substances **(**TBARS) Assay Kit (DTBA-100).

2.5. TBARS Experimental Protocol

Twenty mg of leaf material was collected for each sample and homogenized in 200 µL ice-cold phosphate buffered saline (PBS). Tissue was homogenized by sonication on ice for 20 seconds in a Fisher Scientific FS6 sonicator. Two hundred µL of ice-cold 10% trichloroacetic acid (TCA) was added to 100 µL of tissue lysate and incubated 5 minutes on ice. The mixture was then centrifuged for 5 minutes at 14.000 rpm in a ThermoScientific Sorvall Legend Micro 21R Centrifuge. Two hundred µL of each clear supernatant was transferred into a 1.5 mL micro-centrifuge tube.

2.5.1. Developing Standards

Standards were made by adding 450 µL of (d) H_2O to 50 µL of 15 mM MDA standard, for a final of 1.5 mM

MDA. Fifteen µL of 1.5 mM MDA was mixed with 735 µL (d) H_2O for a final of 30 µM MDA.

2.5.2. Final Sample Preparation

Two-hundred µL of standard and 200 µL of sample was transferred into separate 1.5 mL tubes. Two-hundred µL of TBA (thiobarbituric acid) Reagent was added. The tubes were vortexed in a Fisher Scientific digital vortex mixer and incubated at 100°C for 60 minutes in an Isotemp heating block.

2.6. Colorimetric Assay

Individual samples were placed into a cuvette in order to read the optical density at 535 nm on a Thermo Scientific GeneSys20 Spectrophotometer. The optical density is determined by the reaction between the TBA Reagent and any TBARS present in the plant sample. Thus in order to calculate the TBARS concentration of each sample, the blank optical density standard was subtracted from all sample values. The resulting quantities were divided by the slope, determined from a standard curve, and multiplied by the dilution factor, summarized in Figure 1 in results and discussion section.

2.7. Measurement of Chlorophyll Content

Chloroplasts were isolated and purified using the protocol described in [10] and adapted from Lamppa. Briefly, 500 mg of plant tissue was homogenized along with 200 mL of cold Xpl homogenization buffer, which is a mixture of sorbitol, HEPES (pH 7.5), EDTA, (d) H_2O and $MgCl_2$ to which BSA and sodium ascorbate are added. Chloroplasts were pelleted by centrifuging in a Sorvall RC5C Plus at 3.500 rpm for 8 minutes. Supernatant was decanted and the resulting pellet was re-suspended in Xpl homogenization buffer. The re-suspended pellet was then layered on top of a 12.5 mL Percoll gradient comprised of 9.5 mL 40% (v/v) Percoll on top of 3 mL of 80% (v/v) Percoll, on ice. The sample was then centrifuged at 7.500 rpm for another 8 minutes. Broken chloroplasts and 40% of the Percoll were removed, leaving intact chloroplasts that were then washed in 20 mL of Xpl homogenization buffer and centrifuged at 3.500 rpm for 5 minutes. Ten µL of purified chloroplasts were then added to 1 mL of 80% acetone and to 40 µL of 100% acetone. The absorption was read at λ_{645} and λ_{663}, respectively. The chlorophyll concentration (µg/mL) was calculated using the equation summarized in Figure 2 in results and discussion section.

2.8. Statistical Analysis

A T-test was used in order to compare the mean values of the control plants with the test plants to determine if the ROS concentration and the chlorophyll concentration varied significantly between the two treatments. For the TBARS protocol, n = 148 and for the chlorophyll isolation, n = 300.

3. Results and Discussion

3.1. Results

TBARS concentration for optimally grown and heat stressed plants was calculated using an OD of 535 nm and the following equation (1), where n refers to the sample dilution factor.

$$[TBARS] = \frac{Rsample - Rblank}{Slope\ \ \mu M^{-1}} \times n(\mu M\ \ MDA\ equivalents) \quad (1)$$

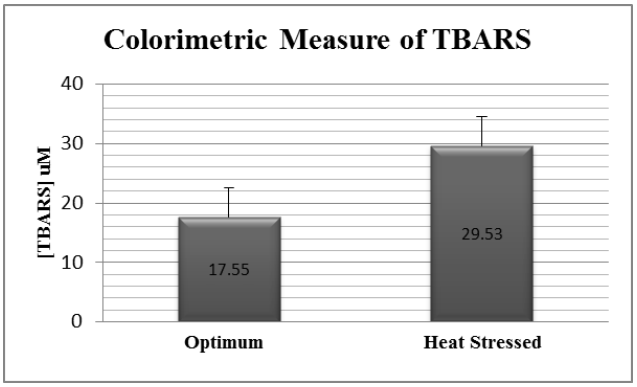

Figure 1. Mean TBARS concentration in µM for optimally grown plants versus heat stressed plants

Chlorophyll concentration for optimally grown and heat stressed plant samples was calculated using the following equation where A = Absorbance at 645 nm and 663 nm respectively.

$$[Chlorophyll\ \ \mu g/ml] = [A_{645} \times 202) + (A_{633} \times 80.2)] \times 10.5 \quad (2)$$

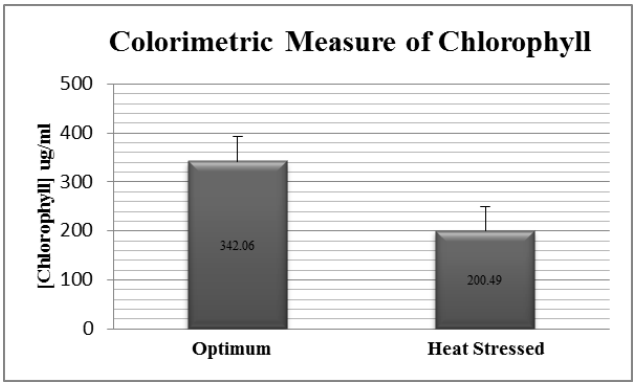

Figure 2. Mean chlorophyll concentration in µg/ml for optimally grown plants versus heat stressed plants

3.2. Discussion

Global climate change is believed to cause a variety of heat-related stress responses in plants, which may alter their ability to survive and reproduce. This study therefore quantified the concentrations of ROS and chlorophyll in *Arabidopsis thaliana Col-0* in order to correlate projected temperature increase with a potential impairment of plant function.

3.2.1. Conclusion of Results

We found a significant increase, 68.3%, of thiobarbituric acid reactive substances (TBARS) in heat stressed plants as compared to the control plants (29.54:17.55 µM). We also found a statistically significant decrease, 41.4%, in the amount of chlorophyll in heat stressed plants as compared to control plants (342.07:200.50 µM). Both sets of T-test data earned a

probability value of P = 0.0005. Thus, temperature is adversely affecting the plant's health.

An increase in heat is affecting chlorophyll concentrations in the plant and as a result, photosynthesis may be impaired. The results from this research indicate that there are significant consequences resulting from even modest increases in temperature between 4°C to 6°C and hint at some additional challenges plants may face in the near future.

3.2.2. Future Studies

Further research, however, is needed in order to determine if heat-stressed plants are overall producing higher levels of ROS or if the ROS concentration is high because the plant's ability to detoxify the ROS has been compromised. To that end, I have secured a Pace University, Dyson College 2012-2013 Undergraduate Student-Faculty Research Initiative grant and will collaborate with a colleague in the chemistry department to train an undergraduate biochemistry student. We will investigate the hypothesis that, as a result of ROS signaling, the mitogen-activated protein kinase (MAPK) pathways are induced to a greater extent in *Arabidopsis* plants exposed to elevated temperatures, as compared to plants grown under optimum conditions.

Acknowledgement

The research was made possible with a grant from Pace University, Dyson College 2011-2012 Undergraduate Student-Faculty Research Initiative and conducted within the research facility at Pace University, Department of Biology and Health Sciences. We would like to thank Dr. Richard Schlesinger for his help with the statistical analysis and we would like to thank Professor Spiro Sinanis for reading through the manuscript and providing valuable feedback.

Statement of Competing Interests

The authors have no competing interests.

References

[1] Briantais, J., Dacosta, J., Goulas, Y., Ducruet, J., and Moya I, "Heat stress induces in leaves an increase of the minimum level of chlorophyll fluorescence, F_o: A time-resolved analysis," *Photosynthesis Research*, 48 (1-2). 189-196. May 1996.

[2] Cao, M., and Woodward, F. "Dynamic responses of terrestrial ecosystem carbon cycling to global climate change," *Nature*, 393. 249-252. May 1998.

[3] Cross R.H., McKay S.A.B., McHughen A.G., and Bonham-Smith P.C, "Heat-stress effects on reproduction and seed set in *Linum usitatissium* L. (flax)," *Plant, Cell and Environment*, 26. 1013-1020. Jul.2003.

[4] Georgieva, K., "Some mechanisms of damage and acclimation of the photosynthetic apparatus due to high temperature," *Bulgarian Journal of Plant Physiology*, 25 (3-4). 89-99. Aug.1999.

[5] Gur, A., Demirel, U., Ozden, M., Kahraman, K., and Copur, O., "Diurnal gradual heat stress affects antioxidant enzymes, proline accumulation and some physiological components in cotton (*Gossypium hirsutum* L.)," *African Journal of Biotechnology*, 9 (7). 1008-1015. Jan. 2010.

[6] Houghton, J., Ding, Y., Griggs, D., Noguer, M., and van der Linden, P, *Climate change 2001: the scientific basis*, Cambridge University Press, Cambridge, UK, 2010.

[7] Kipp, E, "Heat stress effects on growth and development in three ecotypes of varying latitude of Arabidopsis," *Journal of Applied Ecology and Environmental Research*, 6 (4). 1-14. Oct. 2008.

[8] Kovtun, Y., Chiu, W., Tena, G., and Sheen, J., "Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants," *Proceedings of the National Academy of Sciences*, 97 (6). 2940-2945. March 2001.

[9] Liu, X., and Huang, B., "Heat stress injury in relation to membrane lipid peroxidation in creeping bentgrass," *Crop Science*, 40 (2).503-510. March 2001.

[10] Mach, J., *Arabidopsis: A laboratory manual*, *Preparation of Arabidopsis chloroplast*, Cold Spring Harbor Laboratory Press, Cold Springs, NY, 2002, , *217-219*.

[11] McCarty, J, "Ecological consequences of recent climate change," *Conservation Biology*, 15 (2).320-331. April 2001.

[12] Petchey, O., McPhearson, T., Casey, T., and Morin, P, "Environmental warming alters food-web structure and ecosystem function," Nature, 402.69-72. Sept.1999.

[13] Porch T.G. and Jahn M, "Effects of high-temperature stress on microsporogenesis in heat-sensitive and heat-tolerant genotypes of *Phaseolus vulgaris*," *Plant, Cell and Environment*, 24. 723-731. June 2001.

[14] Sato S., Peet M.M. and Thomas J.F, "Physiological factors limit fruit set of tomato (*Lycopersicon esculentum* Mill.) under chronic, mild heat stress," *Plant Cell and Environment*, 23. 719-726. July 2000.

[15] Sharkey, T., "Effects of moderate heat stress on photosynthesis: importance of thylakoid reactions, RUBISCO deactivation, reactive oxygen species, and thermo-tolerance provided by isoprene," *Plant, Cell and Environment*, 28. 269-277. March 2005.

[16] Walther G.R., Post E., Convey P., Menzel A., Parmesan C., Beebee T.J.C., Fromentin J.M., Hoegh-Guldberg O., and Bairlein F, "Ecological responses to recent climate change," *Nature*, 416. 389-395.March 2002.

[17] Yagi, K. "Simple procedure for specific assay of lipid hydroperoxides in serum or plasma," *Methods in Molecular Biology: Free Radical and Antioxidant Protocols*, 108. 107-110. January 1998.