The effects of high temperature stress on the enzymatic antioxidant system in *Zea mays*

By

Kovin Ashley Chetty

Submitted in partial fulfillment of the requirements for the degree of Philosphiae Doctor in the Department of Biotechnology, Faculty of Natural Sciences.

Supervisor: Professor Ndiko Ludidi

August 2017

University of the Western Cape

The effects of high temperature stress on the enzymatic antioxidant system in *Zea mays*

Keywords

Zea mays

Heat Stress

Antioxidant

Reactive Oxygen Species

Native-PAGE

Abstract

High temperature stress is synonymous with the attenuation of plant growth, metabolism and eventually death resulting in major loss of crop productivity worldwide. Part of the metabolic perturbations associated with heat stress leads to the excessive formation of reactive oxygen species (ROS), which have highly deleterious effects on cellular homeostasis. Naturally, through millions of years of evolution and adjustment, plants have developed antioxidant enzymes that neutralize harmful ROS species offering a protective role in the annulment of oxidative damage in response to high temperature. The aim of this study was to measure the activity of several antioxidant enzymes in response to heat stress in *Zea mays*.

To achieve this, maize plants at the V2 developmental stage were grown in separate growth chambers that exposed the plants to two separate day/night temperature cycles. Control plants were kept at 25˚C whilst, heat-stressed plants were exposed to a 42ºC/25ºC cycle for 12 hours each day for 5 days. Thereafter the activities of Superoxide dismutase (SOD), catalase (CAT), glutathione Peroxidase (GPX), ascorbate Peroxidase (APX), glutathione reductase (GR) and dehydroascorbate Reductase (DHAR) were measured via native-polyacrylamide gel electrophoresis and spectrophotometric means. In addition, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) content were also measured in leaves.

Our results show that heat stress resulted in the upregulation in the activities in all enzymes chosen for investigation: Total SOD activity was increased to about 458.9 Units.g^{-1} fresh weight in the 25˚C treated plants compared to 650 Units g^{-1} fresh weight ($P < 0.01$) in heat stressed leaves which was supported by a ~1.2-fold increase in Cu/ZnSOD as revealed by Native-Page. Total hydrogen peroxide content increased from 2 nanomoles per gram fresh weight to 4.02 nanomoles per gram fresh weight ($P < 0.01$) whilst MDA content increased non – significantly by ~1.2-fold when compared to the non- heated controls. Heat stress resulted in the up-regulation in the activity of 2 CAT isoforms: CAT1, which was not active in the 25ºC plants, was induced ~ 1.5-fold ($P < 0.001$) in response to heat stress at 42 ºC; which also caused a ~2.5-fold increase ($P < 0.0001$) in the CAT2 isoform. Spectrophotometric determination of the total CAT activity revealed that heat stress resulted in a 13%

increase ($P < 0.001$) in total enzymatic activity when compared to controls. Regarding APX activity, two isoforms were active in both plant groups: APX1 activity was increased by ~2.3-fold ($P < 0.01$) whereas APX2 activity was also induced by ~ 2.3-fold ($P < 0.001$). Total APX activity at 290nm was 13% higher ($P < 0.05$) in heat stressed samples when compared to room temperature controls. Heat stress also resulted in the upregulation of two GPX isoforms: firstly, GPX1 was induced ~1.4-fold ($P < 0.001$) whereas GPX2 activity was intensified ~1.5-fold ($P < 0.001$) when compared to their room temperature counterparts. Heat treatment resulted in stimulation in total GPX activity from 5.2 to 6.2 ($P < 0.3$) millimoles of NADPH oxidized per gram fresh weight. Total GR activity increased by 11% ($P < 0.2$) as indicated by the reduction of DTNB to TNB at 412 nm and the upregulation of 4 isoforms. GR1 by ~1.5-fold ($P < 0.01$), GR2 and GR3 were more active by ~1.4- ($P <0.01$) and 2-fold respectively ($P< 0.001$) when compared to the 25˚C controls whilst GR4 was induced by ~1.4-fold ($P< 0.001$). Total DHAR activity as indicated by ascorbate formation at 265 nm showed a 25% upregulation while by Native-PAGE , one isoform of DHAR activity was found to be increased in responses to heat stress which presented with a ~1.2-fold ($P <0.47$) exacerbation when compared to 25ºC controls. These results show that the increase in activities of antioxidant enzymes as a result of high temperature stress form part of thermotolerance mechanisms in *Zea mays*. Furthermore, several of the Native-Page data reveal the upregulation of isoforms that are of significance in that this study and present with new findings regarding specific antioxidant isoforms in response to heat stress

Table of Contents

Acknowledgements

I would like to offer my gratitude to Professor Ndiko Ludidi for allowing me to conduct this work in his lab and for his assistance throughout this duration of this study.

I would also like to acknowledge the Plant Biotechnology Research Group for their help, support as well as Arun Gokul of the Environmental Biology Research Group for his assistance.

I am also grateful to the University of Western Cape and the National Research Foundation for funding the work entailed in this thesis.

Declaration

I declare that *The Effects of Heat Stress on the Antioxidant Machinery in Zea mays* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full name..... Date..... Signed.....

List of Figures

List of Abbreviations

ABA	Abscisic acid
APX	Ascorbate peroxidase
AsA	Ascorbate
AT	Aminotriazol
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CaM	Calmodulin
$CaSO_4$	Calcium sulphate
CAT	Catalase
CMT	Cell membrane thermostability
$CoSO_4$	Cobalt sulphate
Cu	Copper
$CuSO_4$	Copper sulphate
Cu/ZnSOD	Copper-zinc superoxide dismutase
Cyt	Cytosol
DHA	Dehydroascorbate
DHAR	Dehydroascorbate reductase

DNA	Deoxyribonucleic acid
DTNB	5,5-dithio-bis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylenediaminotetraacetate
FAD	Flavin adenine nucleotide
$FeCl_3$	Iron III chloride
Fe-NaEDTA	Iron-sodium ethylenediaminotetraacetate
FeSOD	Iron superoxide dismutase
Fm	Maximum fluorescence
Fo	Base fluorescence
FWT	Fresh weight
Fv	Variable fluorescence
GB	Glycinebetaine
GLDH	L-galactone-1,4-lactone dehydrogenase
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GST	Glutathione-S-transferase
H_2O_2	Hydrogen peroxide

H_3BO_4	Boric acid
HCl	Hydrochloric acid
HSF	Heat shock factor
HSP	Heat shock protein
IPCC	Intergovernmental panel on climate change
kDa	Kilodalton
K_2HPO_4	Di-potassium hydrogen phosphate
$K_3[Fe(CN)_6]$	Potassium ferricyanide
KPO_4	Potassium phosphate
K_2SO_4	Potassium sulphate
KCN	Potassium cyanide
KNO_3	Potassium nitrate
MDA	Malondialdehyde
MDAsA	Monodehydroascorbate
MES	2-(N-morphlino) ethanesulphonic acid
$MgSO_4$	Magnesium sulphate
$MnSO_4$	Manganese sulphate
MnSOD	Manganese superoxide dismutase
mRNA	Messenger ribonucleic acid
MTT	Thiazolyl blue tetrazolium bromide

MV	Methylviologen
N_2	Nitrogen
NBT	Nitrotetrazolium blue chloride
NaClO	Sodium hypochlorite
NADPH	Nicotinamide adenine dinucleotide phosphate
NH_4NO_3	Ammonium nitrate
Na_2MOO_4	Sodium molybdate
NaN_3	Sodium azide
O_2	Oxygen
$^1O_2$	Singlet oxygen
$O_2\bullet^-$	Superoxide anion
OH•	Hydroxyl radical
OEC	Oxygen evolution complexes
PAGE	Polyacrylamide gel electrophoresis
PCD	Programmed cell death
PCR	Polymerase chain reaction
PHGPX	Phospholipid hydroperoxide
PMS	Phenazine methosulphate
Pn	Nett photosynthesis
PS	Photosystem

PRx	Peroxiredoxin
PVPP	Polyvinylpolypyrrolidone
R	Reproductive
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
RuBisCo	ribulose-1,5-biphosphate carboxylase/oxygenase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TNB	2-nitro-5-thiobenzoate
TRX	Thioredoxin
V	Vegetative
VE	Vegetative emergence
Zm	*Zea mays*
$ZnSO_4$	Zinc sulphate

Chapter One

Background, Aims and Justification of the Study

1.1. Background

Global warming, for which there is enough scientific evidence to support the phenomenon, has instilled in most humans a proactive adjustment of our resources, attitudes, farming methods and an overall discerning disposition to survive abiotic stresses and their highly complex and often interrelated onslaughts. Heat, drought, cold and salinity are the four major abiotic stresses that work in tandem to enforce acute cellular damage in many plant species and particularly in food-staple crops (Bita & Gerats, 2013). With regards to heat stress for which this study orbits, plants are exposed to temperature fluctuations on a daily basis however during the earths most choleric season; hot summers (40 °C to 55°C) trigger dysfunction of molecular and biochemical events that impair plant development, metabolism and productivity (Hasanuzzaman, Nahar, Alam, Roychowdhury, & Fujita, 2013). Heat stress is thought to uncouple enzymes and metabolic pathways that allow for the formation of surplus and damaging reactive oxygen species (ROS), which have the availability to react with surrounding pigments, lipids, proteins and DNA or with any biochemical moiety within close vicinity (Camejo et al., 2006; Soliman, Fujimori, Tase, & Sugiyama, 2011). Oxidative attack is also synonymous with the dysfunction of the electron transport chains found within the chloroplast and mitochondria whereby altered carbon metabolism results in reduced energy production diminished stature of plants, reproductive development, and crop yield (Xu, Li, Zhang, Wei, & Cui, 2006).

Curbing the harmful effects of ROS-mediated damage are the enzymatic and the non-enzymatic antioxidants; that bring balance to redox stress to all eukaryotic cells. Due to their ability to bring cellular homeostasis and oxidative balance under normal and under compromising scenarios, they are routinely analyzed for their activities and

expression levels in the laboratory setting. Many of these enzymes are have also been genetically altered using biotechnological and breeding mechanisms to operate more efficiently in crop cultivars thereby allowing plants to survive better under conditions of stress (Almeselmani, Deshmukh, Sairam, Kushwaha, & Singh, 2006; Nagesh Babu & Devaraj, 2008; Tang et al., 2006; W. Wang, Vinocur, & Altman, 2003).

1.2. Aims

High temperature stress in plants has been shown to impair plant growth, development in addition to numerous physiological and biochemical processes which often result in decreases in yield. One of the greatest harms posed by heat stress in the generation of excess reactive oxygen species (ROS) that upsets cellular redox balance and homeostasis and results in the cellular damage and cell death (Hasanuzzaman, et al., 2013). A noted response by plants under heat stress is the change in antioxidant activities and synthesis which restore cellular redox balance in an attempt to promote cell survival (Bita & Gerats, 2013). This study focuses on six major antioxidant enzymes that scavenge ROS; a) Superoxide dismutase b) catalase, c) Ascorbate peroxidase, d) Glutathione peroxidase, e) Glutathione reductase and f) Dehydroascorbate reductase. This study was designed to measure the respective activities of these six enzymes using spectrophotometric assays and non-denaturing or native polyacrylamide gel electrophoresis with post electrophoretic staining with reagents and cofactors that initiate enzyme activity in response to heat stress in *Zea mays*. The chosen experimental design is based around the hypothesis on previous studies (Djanaguiraman, Prasad, & Al-Khatib, 2011; Kumar, Gupta, & Nayyar, 2012; Savicka & Škute, 2010) that have measured these six antioxidant enzymes in response to heat stress only via spectrophotometric means. In addition several other studies have measured isoform antioxidant activities in response to heat stress using Native-PAGE however these outputs only focused on specific enzymes. This study aims to conclusively measure all six enzymes using both techniques i.e. spectrophotometric assays and isoform pattern emergence revealed

through Native-PAGE. We expect to subordinate findings made by previous authors as heat is thought to enhance enzyme activity however spectrophotometric data will be cross-referenced to antioxidant enzyme isoform pattern.

1.3. Justification of the Study

Climate change poses a serious threat to all life on earth. The Intergovernmental Panel on Climate Change (IPCC) released their fourth assessment in 2007, where it was predicted that the ambient air temperature to increase by 0.2ºC per decade (Bernstein et al., 2008). This prognostication denotes higher temperatures of 1.8-4.0ºC by the year 2100 which has raised alarm bells and a heightened state of anxiety regarding the future with respect to food security (Change, 2007). This increase in temperature has also been predicted to affect crop yields negatively where an increase of 3-4ºC may result in a drop of crop yield of wheat by 15-35% in Asia and Africa (Ortiz et al., 2008). The mean crop yield growth rate of global maize as revealed by the IPCC, has decreased from ~2% in 1960 to 1.8% in 2000 (Change, 2007).

Closer to home, the South African Highveld region is the most crucial area of crop production in the country where 70% of all cereals and 90% of the country's maize is cultivated there. The western half of the region produces a variable yield of maize each season whereas the eastern half gets more rainfall and there remains high a retention of crop output. Thus the western half is highly susceptible to climate changes especially heat or thermal stress. It has been shown by Lobell et al., 2011, that there exists a non-linear relationship between the increase of ambient temperature and crop yield. They have revealed that if crops spend each degree spent above 30ºC the final maize output is diminished by 1% under rain-fed conditions and by 1.7% under drought conditions including the South African Highveld. These statistics coupled with the fact that the global population rises by a billion people every 10 years creates a scenario whereby the need for more tolerant crops is justified and pressing development through research-based strategies to improve crop tolerance and food security is compulsory.

Chapter Two

Literature Review

2.1 Synopsis

This review of literature is dedicated to provide pertinent background information for a sound understanding of the work entailed in this thesis. It aims mostly to ensure that enough scientific evidence is subordinated against a backdrop of knowledge that is essential to understand the effects of thermal or heat stress on the enzymatic antioxidant machinery in *Zea mays.*

This chapter is divided into the following topics:

- A brief overview of the developmental stages of *Zea mays.*
- The general morphological, biochemical and molecular effects of heat stress in plants.
- A review of ROS and the enzymes that detoxify them.
- An in-depth review of the six antioxidant enzyme chosen for investigation for this study and their respective biochemical changes in response to heat stress in *Zea mays* and various other plants.

2.2 *Zea Mays*

2.2.1 Overview

Zea mays, commonly known as 'corn' or 'maize' is one of the three most cultivated cereal crops grown in the world. It is considered a grass and thus belongs to the Poaceae family (Gibson & Benson, 2002). In 2010, 844.4 million metric tons of dried maize was produced commercially which was harvested from 161.9 million hectares of land. Maize is grown in moderate and tropical countries worldwide however the U.S produces more than 33%

of the worlds dried corn; other countries that produce notable amounts are Brazil, China, Mexico and Argentina (Compass, 2011; R. L. Paliwal, Granados, Lafitte, Violic, & Marathée, 2000).

Corn is a staple human food worldwide where is dried and ground into flour or maize-meal. The oil obtained from corn kernels is used in cooking but more importantly maize is a global source of starch which is usually used as an ingredient in both its native and chemically modified form in cooking and industry (White, 2000). It is also given to livestock where the stem, leaves and kernel provide high energy and feed value (Farnham, Benson, Pearce, White, & Johnson, 2003). More recently it has been used as a biofuel in the form of biodiesel or ethanol, which is derived from the alcoholic fermentation process. Interestingly, this ethanol is added to gasoline in various quantities. In the last decade, owing to increased environmental concerns, global corn production was boosted by 42% to substantiate its involvement as an biofuel (Boyer & Hannah, 2000; Compass, 2011).

2.2.2. Maize Morphology

Modern-day maize was derived from *Tripsacum dactyloides*, which is a sod-forming bunch grass endemic to Mexico and the United States more than 7000 years ago (Piperno & Flannery, 2001). It spread to the rest of the world after Europeans discovered the Americas during the 15th century (R. Paliwal, 2000). A typical maize plant, which is a grass and therefore a monocot, can grow up to 4m tall. The six main morphological structures of the maize plant are:

- The **coleoptile** is a modified leaf structure that environs the plumule (rolled up leaves) during germination. It provides protection of the plumule and facilitates pushing of the plumule through the pericarp (kernel covering) and finally through the soil surface.

- The **leaf** is made up the of the blade, midrib and the leaf collar; leaves are usually broad and made of up of single plumes which develop on either side of each node, the term denoting this type of leaf arrangement is distichous (Esau, 1977). Each of the leaves sheath its surrounding stalk and forms and expanded blade which connects the sheath by a blade joint called a collar. The typical plant can possess up to 30 leaves

with disparity regarding the leaf size, number and positioning (Evert, 2006). High leaf numbers are seen in tropical maize cultivars than the temperate ones.

- The stem or **stalk** is made of nodes and internodes and provide rigidity and support for leaves to trap sunlight.

- The **root** system of maize is made up of lateral seminal roots, nodal roots and brace roots that emerge from the kernel itself. Seminal roots provide anchorage for seedlings; when the nodal roots are well developed (Vegetative stage 3, V3). The brace roots are said to form much later above the soil surface.

- The central cob or **ear** has a cylindrical array of flowers possessing silks (elongated style) and ovaries which if fertilized produces kernels. Well-developed ears are capable of producing up to 1000 kernels.

- The tassel is the male flowering appendage that produces pollen (usually between 2-5milligrains) that fertilizes the ear.

2.2.3 Maize Developmental Stages

Maize growth stages can be divided into two measures: vegetative growth and reproductive growth. Vegetative growth involves defining a growth stage that is dependent on the number or leaf collars present. Hence, a plant with five visible leaf collars would be denoted V5 (Refer to Figure 1). As the maize plants grows new leaves the earlier leaves senesce and fall of due to aging and expansion of the stem. The final vegetative stage is denoted VT which occurs when all branches of the tassel have emerged.

The first reproductive stage or R1 in maize occurs when silks have emerged at the tip of the ear husk. The following reproductive stages involve the development of kernels on the ear husk; after this stage husk removal is advised for stage identification (Refer to Table 1).

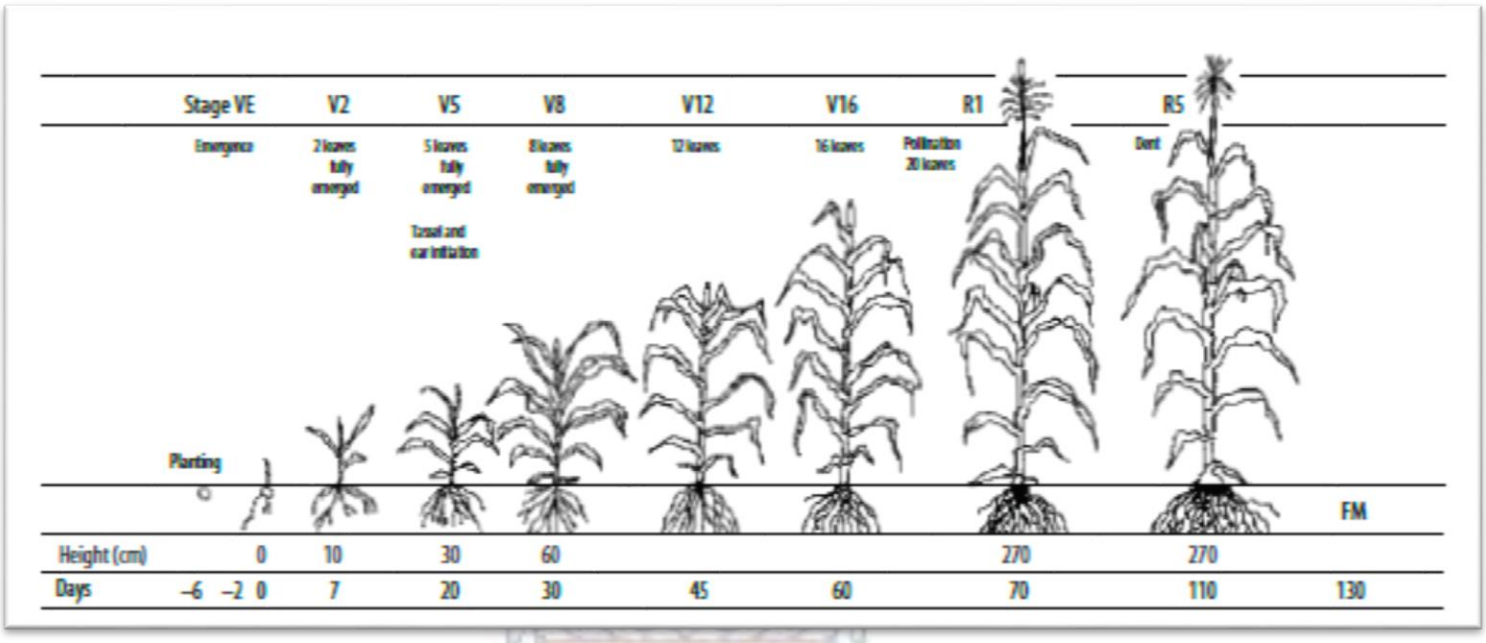


Figure 1: Progressive Maize Development Depicting the Vegetative and Reproductive Stages Relative to Plant Height and Onset of Planting and Emergence (Coless, 1992).

Table 1: Typical Developmental Stages of Maize Development and descriptions of the progressive Vegetative (V) and Reproductive (R) stages of *Zea mays* and their associated responses to moisture stress.

Developmental	Description of Stages
Vegetative Emergence (VE)	Following planting, the kernel absorbs 30% of dry weight as water to ensure enzyme activity and growth. Emergence of the radicle ensues under favorable conditions through the seed coat within 2 -3 days. Plumule and leaf development begin to elongate and form respectively as part of the developing seedling. The radicle is followed by the seminal root system that provide anchorage and nutrient uptake. This root system sustains the kernel for 2-3 weeks until the permanent root system develops. Once the coleoptile emerges and experiences sunlight (6-10 days after planting) it splits to reveal two true leaves; around 7 days after emergence, the next new leaves grow upwards inside the first two leaves and unfurl one leaf every 3 days (Colless, 1992; Hoeft, Aldrich, Nafziger, & Johnson, 2000).
V1-V2	Occurs 7 days after plant emergence where 1 to 2 leaves are apparent and the primary root system is still developing (Colless, 1992).
V3-V5	Begins 14 days after emergence, the seedling root system no longer grows and nodal roots now begin to form an integral part of the root system. Leaf and ear shoot are initiated at V5, which is when the ear shoot number is determined. Using light microscopy a small tassel begins to develop which occurs at the V5 stage when the plant is around 20cm tall. The ear or female flowering structure is initiated which consists of a cob with flowers arranged in a cylindrical pattern. These flowers possess an ovary which becomes a kernel if fertilized and silks, which are elongated styles. An ear is said to be well developed if there are 700 to 1000 potential kernels (Jones, Kiniry, & Dyke, 1986).
V6-V7	The plant is at the V6 stage after 21 days after post-emergence; the root system is well developed and spans 45cm deep and 60cm wide. Elongation of the third whorl is apparent and rapid stem elongation begins. Kernel number is also determined at V7; new ovules (kernels) begin to develop at the base of the ear (Ritchie, Hanway, & Benson, 1992).

V8-V9	The maize plant is said to be at V8 approximately 4 weeks after emergence where the 4th whorl of nodal roots are elongating. Ear shoots are also present where they develop at ever node above ground except the terminal 6-8 nodes (Kaiser, 1997).
V10-V11	At this stage (5 weeks after planting) the plant now accumulate dry matter and nutrients at a fast rate (Colless, 1992).
V12-V13	Occurs a week after V13; race roots begin to develop from the 5th node above the soil. Potential number of kernels and their rows are established. Ovule formation is complete as the meristematic dome is no longer functional (Jones, et al., 1986).
V12-V13	The plant is now entering the late vegetative stages (8 weeks after emergence) in which moisture is of utmost importance as water stress at this stage greatly diminishes grain yield. At V17, the tips of the upper ear shoots become visible as well as the tip of the tassel (Heisey & Edmeades, 1999).
V18-VT	The plant is now reaching full size; silks from ovules from the base of the ear begin to elongate which are then followed be silks from terminal ovules. At the stage the ear is developing rapidly and is only 7 days away from viable silking (Bolaños & Edmeades, 1996).

R1	Two main processes occur at this stage: Pollen shedding and Silking. Pollen grains develop in the anthers of the tassel which split open to release. 100 pollen grains/cm^{-2}/day is the amount required to successfully pollenate a growing maize field. One to three days after pollen shed has been initiated, the first silks emerge from closed husks which will be viable for pollination with enough time before tassel pollen shedding stops. Interestingly, silk elongation occurs mostly at night and appear in the morning ready to accept pollen. If viable pollen grains fall on these silks, tiny hair-like protrusions called trichromes traps the pollen to aid pollination. Within a space of 12-28hours a pollen tube grows to the length of the silk and releases a nucleus into the ovule. Fertilization of the egg and polar nucleoli occurs which then develops in to the endosperm of the new maize kernel (Colless, 1992).
R2	Called the 'Blister Stage' where after fertilization, developing kernels resemble watery blisters. The cob is now at its full growth length. In a fortnight, the kernels grow profusely and metabolism of the plant shifts to filling of the kernels(Hoeft, et al., 2000).
R4	The 'Dough Stage' or R4 in maize development is synonymous with a high conversion of simple sugars to starch in the form of dextrin which have the consistency of dough (Kaiser, 1997).
R5	Hardening of crown of the kernel occurs which forms a 'dent' or definite band also called the milk line which demarcates between the milky substance and dry starch (Jones, et al., 1986)
R6	Eight weeks post-pollination, the kernel is now mature. At the tip of the kernel is a specialized tissue which conduits sugar and other substances. At maturity ensues this layer collapses and turns black which is an outward sign of physiological maturity (Ritchie, et al., 1992)

2.3. Plant Responses to Thermal Stress

2.3.1. Overview

Abiotic stresses, if not at all, rarely inflict damage independently; plants undergoing thermal stress will often experience drought and salinity stresses simultaneously as is the nature of things. It is important however, and possible to measure the biological consequences of each stress separately as is performed under laboratory and controlled settings. As revealed by such experiments, the degree of damage that plants are vulnerable to is largely dependent on the developmental stage at which the stress is enforced. As this study focussed on the effects that heat stress has on the antioxidant machinery in *Zea mays*, the physiological mechanisms will only be mentioned briefly, leaving more focus and resolution for a review of relevant literature that includes studies that have revolved around antioxidant enzymes and their biological responses under heat stress in *Zea mays* and other genera of plants.

2.3.2 Definition of Heat Stress, Heat Tolerance and Heat Stress Threshold

Heat stress occurs when the ambient temperature rises to a level above the threshold level whereby permanent damage to plant development and growth is observed. Generally in plants, a transitory rise in ambient temperature usually 10-15ºC is considered either heat shock or stress. As with most abiotic stresses, the degree of stress that the plant experiences is a complex arrangement of variables that include intensity, duration and the rate of the temperature flux (Wahid, Gelani, Ashraf, & Foolad, 2007). Heat tolerance is the ability of the plant to overcome high temperature onslaughts and grow and produce good economic yield. There is much debate between professionals regarding temperature fluxes; some researchers promote that nocturnal temperatures provide the most limitation whilst other groups have reported that the diurnal/nocturnal swing of temperature do not affect plants independently. They believe that it is more suitable to judge plants responses to high stress according to the day mean temperature with the day temperature having a subordinate role (Willits & Peet, 1998).

Concerning the heat stress threshold, which is defined at the daily mean temperature at which a measurable reduction in growth occurs. As one would expect, varying plant species have their established upper and lower threshold limits at which plant growth and developments stops. A comprehensive study by Sanchez et al., 2014, which addresses the threshold temperatures in maize reported that -1.8ºC to be the Tmin lethal and 46ºC as the Tmax lethal (Sánchez, Rasmussen, & Porter, 2014). This group reviewed that maize pollination is highly sensitive to high temperatures; which increased non-germinated pollen by 51%. In similar studies by Herrero & Johnson 1980, maize exposed to 38ºC completely failed to germinate whereas in semi-arid conditions Carberry et al., 1989, reported that optimal temperatures for maize growth was 30.5°C with Tmin at 7.7°C and Tmax at a value of 37.3°C (Carberry, Muchow, & McCown, 1989; Herrero & Johnson, 1980; Schoper, Lambert, & Vasilas, 1987).

2.3.3. The Morpho-Anatomical and Physiological Effects of Heat Stress in Plants

Typical morphological injuries to plants under high temperatures include sunburn of typical structures such as leaves and stems, leaf abscission and senescence, root/shoot growth inhibition, fruit damage and reduced crop yield (Guilioni, Wery, & Tardieu, 1997; Ismail & Hall, 1999; Vollenweider & Günthardt-Goerg, 2005). Chronic heat stress has been shown to modify the patterns of development in plants, decreasing germination rates, impeding vegetative emergence and establishment of seedlings. In maize, coleoptile growth is reduced at 40ºC and completely halted at 45ºC furthermore, significant reductions in relative growth rates, shoot dry mass and net assimilation rate was observed in response to high temperature stress was in sugar cane (Ashraf & Hafeez, 2004; Wahid, et al., 2007).

Anatomical changes associated with heat stress mirror those to drought stress where reduced cell dimensions, stomatal closure, thicker trichrome tissue with enlarged xylem vessels in both shoot and roots are observed. In the salt tolerant dwarf shrub, reduced loss via transpiration which was attributed to significant changes in chloroplastic thylakoidal organization was observed in response to heat stress. Typical features also seen in the cellular level include diminished grana stacking and swelling; in grape, lamellae in the stroma also become swollen, vacuolar contents are seen in aggregates and mitochondria are empty which results in reduced

photosynthesis and cellular respiration (Adams, Cockshull, & Cave, 2001; MD Karim, Fracheboud, & Stamp, 1999; Sayed, 1996; J. H. ZHANG, HUANG, LIU, & PAN, 2005).

With regards to physiological changes, water status relations are the most susceptible and concerning factors under conditions of heat stress. Conditions of high temperature is known to concurrently result in decreased water availability. As expected, heat stress causes limitations in leaf water potential and root-water conductance; this was seen in sugar cane where water potential in leaves underwent changes even through soil water availability and relative humidity remained plausible (Wahid, et al., 2007). In summary, higher diurnal temperatures results in a greater degree of transpiration owing to decreased water potentials which affect and normal functioning of physiological processes (Tsukaguchi, Kawamitsu, Takeda, Suzuki, & Egawa, 2003).

With regards to adaptive responses under conditions of heat stress or decreased water potentials, plant accumulate osmolytes which are organic in nature and have low molecular weight. These include proline, polyols, sugars, ammonium- and sulphur containing compounds (Rao, Raghavendra, & Reddy, 2006). In maize the quaternary amphoteric amine known as glycinebetaine (GB) is synthesized in large quantities under conditions of high temperature. The accumulation of this osmolyte and the amino acid proline is thought to restore cell redox potentials however the generation of these compounds under stressful conditions has gleaned mixed theories with a portion of researchers indicating that GB and proline accumulation are a direct result of stress and therefore is not an adaptive mechanism. Other theorists claim that these compounds offer an osmoprotectant role improving growth and adjusting leaf water potentials across many species of plants; even in those that cannot produce GB which is found in cases where the compound was applied exogenously (Ashraf & Foolad, 2007; Kishor et al., 2005; Quan, Shang, Zhang, Zhao, & Zhang, 2004).

At the sub-cellular chloroplastic level, photosynthesis is one of the most susceptible physiological process to thermal stress; plants with C3 metabolism display sensitivity to heat stress than C4 plants (X. Yang et al., 2006). The thylakoidal lamellae are the key sites of injury in response to heat stress where swelling and loss of grana stacks are observed (Marchand, Mertens, Kockelbergh, Beyens, & Nijs, 2005; J. Wang, Cui, Wang, & Li, 2009).

Photosystem II (PSII) is very thermolabile; heat stress has been shown to completely eradicate activity of this system (Bukhov, Wiese, Neimanis, & Heber, 1999). Usually, thermotolerance of any plant species is closely associated with photosynthetic parameters such as chlorophyll fluorescence, which is the ratio of variable fluorescence (*Fv)* divided by the maximum fluorescence (*Fm*) and the base fluorescence (*Fo*) (Yamada, Hidaka, & Fukamachi, 1996). As long as the upper limits of the heat stress threshold is not surpassed, increased leaf temperature and higher levels of photon flux density lead to adaptive mechanisms that optimize photosynthesis are noticed and revolve around the aforementioned parameters at PS II (Crafts-Brandner & Salvucci, 2002). This however is also predetermined by water status in the leaves, stomatal conductance under moderate heat and CO_2 concentration (Greer & Weedon, 2012).

Other thermotolerance parametric quotients that reflect are the ratios between chlorophyll a: chlorophyll b and chlorophyll : carotenoids; in tomato and sugarcane both these ratios are decreased in response heat stress as well as chlorophyll degradation as a result of the formation of reactive oxygen species and subsequent lipid peroxidation (Camejo et al., 2005; Wahid & Ghazanfar, 2006). Oxygen generation at the PS II reaction center is also measured frequently as a measure of heat tolerance as high temperatures are believed to dissociate oxygen evolution complexes (OECs) resulting is improper electron flow; as shown in *Triticum aestivum*, heat stress was shown to inflict damage at PS II which resulted in complete loss of oxygen evolution (De Ronde, Cress, Krüger, Strasser, & Van Staden, 2004; Sharkova, 2001).

Gaseous exchange during heat stress is only major determinant in thermotolerance in plants as moderate temperatures result in stomatal closure and therefore decreased CO_2 assimilation. This also deactivates ribulose-1, 5-biphosphate carboxylase/oxygenase (RuBisCo), an important enzyme in carbon fixation, and affects net photosynthesis (Pn). Crafts-Brandner and Salvucci, 2002, comprehensively provided data regarding the sensitivity of photosynthesis in maize in response to heat stress and reported that Pn was inhibited by 95% in response to a temperature of 45° even when atmospheric CO_2 was exacerbated 3-fold. This shows that net photosynthesis is not-dependent on stomatal conductance at higher temperatures and interestingly suggests that global warming may not affect Pn (Crafts-Brandner & Salvucci, 2002).

Cell membranes are also structural features that are susceptible to heat stress as increases in total kinetic energy and molecule movement across lipid membranes is thought to alter their fluidity by mechanisms that include protein denaturation and increase in unsaturated fatty acids. Furthermore, high temperatures also cause conformational changes in the high order structures of membrane bound proteins thus increasing factors such as membrane permeability with subsequent electrolyte leakage (Savchenko, Klyuchareva, Abramchik, & Serdyuchenko, 2002). These parameters have been summarized and studied as an index called the cell membrane thermostability (CMT) in many plant species. Although the CMT which is a measure of electrolyte leakage and therefore membrane permeability and stability, the degree of leakage is also dependent on other factors such as plant age, developmental stage, season of growth and other factors. In maize, Karim et al., 1999, showed that mature leaves were more damaged by heat stress that younger leaves owing to a higher percentage of saturated fatty acids in developed leaves. They reported that the increase in saturated fatty acids changes the melting temperature of the membranes thereby limiting the heat tolerance of maize (MA Karim, Fracheboud, & Stamp, 1997; MD Karim, et al., 1999).

2.4. Reactive Oxygen Species Homeostasis in Plant Cells

2.4.1. Overview

Oxygen is believed to have appeared in the Earth's atmosphere as a product of photosynthesis and is deemed to 'cut both ways' as aerobic metabolism in eukaryotes results in the continual production of various forms of reactive oxygen species (ROS). They can be toxic at high concentrations but luckily, they are detoxified by innate enzymatic and non-enzymatic mechanisms in a homeostatic fashion that is believed to be the result of millions of years of evolution. In cells, there exists an oxidative equilibrium that is homeostatic in nature. Any jolt of this fine balance, especially those that tip the scales in favor excess ROS production results in a state called oxidative stress. It has been well documented that under condition of oxidative stress, excess ROS production dramatically

resulting in free radical attack to proteins, DNA and lipids (Apel & Hirt, 2004). Damaged molecules can be recovered, repaired or replaced by *de novo* synthesis however under conditions of extreme stress, a sequence of events occur that can eventually result in cell death (Mano, 2002; Reddy, Chaitanya, & Vivekanandan, 2004). In plants however, the production of ROS has an undercurrent theme of duality; as there is also purposeful production of ROS which occurs in response to environmental parameters such as pathogen attack, hormone signaling, polar growth and gravitropism (Apel & Hirt, 2004; Mori & Schroeder, 2004; Torres, Jones, & Dangl, 2006). This conscious motivation by plants to produce ROS reveals a multidimensional, layered, complex interplay of cellular integration that intertwines ROS production that is environmentally enforced and that of the plants owns will (Gill & Tuteja, 2010).

Singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), the superoxide anion ($O_2^{\cdot -}$) and the hydroxyl radical ($OH^{\cdot}$) are the highly reactive forms of oxygen that are capable oxidative damage to lipids, protein and DNA (Apel & Hirt, 2004). Singlet oxygen ($^1O_2$) is the most unusual of all the ROS as its formation is not a consequence of electron transfer but rather is produced as a result of photo-activation. It is therefore a key ROS that is measured and analyzed under conditions of photo-oxidative stress in plants (Triantaphylidès & Havaux, 2009; Triantaphylidès et al., 2008). Superoxide dismutase (SOD) neutralizes the $O_2^{\cdot -}$ which leads to the formation of H_2O_2 which is then detoxified by catalase (CAT), ascorbate peroxidase (APX) and glutathione peroxidase (GPX). The enzymes glutathione reductase (GR) and dehydroascorbate reductase (DHAR) are also antioxidant in nature replenishing pools of glutathione (GSH) and ascorbate (Asc) which are electron donors that are used by GPX and APX respectively. This study focuses on the activities of these six enzymes and this section aims to review these antioxidants and their ability to detoxify their respective harmful ROS substrates.

2.4.2. The Superoxide Radical ($O_2^{\cdot -}$) and Superoxide Dismutase (SOD)

Molecular oxygen (O_2) is considered unreactive unless acted upon by biological catalysts; in mitochondria, the seat of oxidative metabolism in cells, cytochrome oxidase adds four electrons to O_2 which results in the formation of water. However, one and two electron additions are also tangible and result in the formation of the superoxide

radical anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) respectively. In chloroplast thylakoids, the reaction centers photosystem I (PSI) and photosystem II (PSII) are also the primary source of the $O_2^{\cdot -}$ (Kozi Asada, 2006). The superoxide radical is considered to be a moderately reactive ROS with an approximate half-life of 2-4 μs and is usually the first ROS produced by cells (Gill & Tuteja, 2010). Phospholipid bilayers are impermeable to $O_2^{\cdot -}$ as revealed by Takahashi and Asada, 1983, in experiments that used phospholipid vesicles from *Glycine max* that contained riboflavin. Superoxide radicals were generated by exposing the vesicles and therefore the flavin mononucleotide to light which spontaneously produced the radical. The impermeability of $O_2^{\cdot -}$ to spinach thylakoidal membranes was determined by the inability of trapped superoxide dismutase (SOD) inside vesicles to neutralize exterior $O_2^{\cdot -}$; which only occurred when Triton X-100 was introduced (Takahashi & Asada, 1988). This is expected as the $O_2^{\cdot -}$ is a charged molecule.

Detoxifying $O_2^{\cdot -}$ are three groups of SODs that are categorized according to the metal co-factor that is used and present within the enzyme; these are the iron, manganese and copper-zinc SODs (Fe-SOD, Mn-SOD, and Cu-Zn SODs) which are located in different cellular compartments. Sequence similarity of these SODs have been undertaken and results have suggested that Mn and Fe are the more archaic versions and may have originated from an ancestral enzyme whereas Cu-Zn SODs bear little resemblance to their older counterparts and is thought to have evolved as a separate entity (Kanematsu & Asada, 1990; M. W. Smith & Doolittle, 1992).

In chloroplasts, Fe-SOD is the major variant found to detoxify $O_2^{\cdot -}$ as shown by Salin, 1981 via experiments using polyclonal antibodies against Fe-SOD in *Brassica campestrus* (Mustard (Salin & Bridges, 1981). Interestingly, Fe-SOD is not found in animals suggesting that the enzyme first became apparent in the plastid genome and eventually located into the nucleus; this is corroborated by its presence in photosynthetic bacteria and its absence in non-photosynthetic species (Bowler, Montagu, & Inze, 1992).

There appears to be two clear categories of Fe-SOD: the first enzyme is a 40 kDa homodimer that is comprised of two 20 kDa subunits that are identical in nature and retains 1-2 gram atoms of Fe in the active site. The second

variant is commonly found in higher order plants; it is ~90 kDa with 4 identical sububits and contains 2-4 gram atoms of Fe in the active center (Kusunose, ICHIHARA, NODA, & KUSUNOSE, 1976; Yost & Fridovich, 1973).

Manganese SODs (Mn-SODs) is thought to derive ancestrally from a Fe-SOD and are present in mitochondria and peroxisomes. They possess only one atom of Mn per subunit which is essential for catalytic activity. Fe-SODs and Mn-SOD share striking similarity in their structure however studies have shown that there has been enough evolution divergence as they cannot restore each other's activity (Fridovich, 1986). At the active sites of Mn-SODs, there is an abundance of positive amino acids, and as mentioned earlier, $O_2^{\cdot -}$ is negatively charged thereby allowing easy attachment through their electrical attributes and efficient direct donation of electrons to the radical thereby forming H_2O_2 reaction with a proton (Kozi Asada, 1994; Bowler et al., 1991).

Structurally, Mn-SODs are either homodimeric or homotetrameric with only one atom of Mn with a valency of +3 in each subunit. Neither potassium cyanide (KCN) nor H_2O_2 are able to inactivate the activity of Mn-SODs which is why these chemicals are used to inhibit Fe and Cu-ZnSODs when analyzing their respective activities in laboratory settings. Also thought to be the major mitochondrial based SOD, it appears that peroxisomes also holds a Mn-SOD as revealed by del Rio et al., 1992, in experiments that immunolocalized Mn-SODs in both the mitochondrion and peroxisome in *Citrullus lanatus* (watermelon). More relevantly, Zhu and Scandalios, 1993, reported 4 genes that encode Mn-SODs in maize however sequence analysis data later revealed that all four Mn-SODs were mitochondrially-bound and that there is translocational peptide sequence that suggests its presence in the peroxisome.

The historical generation of Cu-SODs is attributed to the replenishment of oxygen in the atmosphere which rendered Fe (with a valency of +2) unavailable; it is deemed that the oxidation of Cu^{+} to Cu^{2+} which also rendered the latter more soluble, allowed for organisms to absorb copper in their active sites of SODs. This is the attributing factor as to why Fe-SODs are highly related to Mn-SODs and how both of them differ greatly to Cu-ZnSOD which is apparent in their structures and their cellular distribution (Bannister, Bannister, Barra, Bond, & Bossa, 1991).

Copper-Zn-SODs are found throughout the cell and belong to two major groups:

The first group consists of the cytoplasmic or periplasmic variants that are homodimeric in structure. The second group is found in the chloroplast and the extracellular matrices which are homotetrameric with each subunit functioning independently as revealed by Fridovich, 1986. (Bordo, Djinovic, & Bolognesi, 1994; Fridovich, 1986). This soluble variant which is found in the stoma, is also similar to a cytosolic form in which it displays a 68% sequence homology to. Antibodies directed to this SOD have revealed that the enzyme is not uniformly distributed within the chloroplast but rather is closely aggregated to the stromal flank of the thylakoidal membrane in close vicinity to PS I (Ogawa, Kanematsu, Takabe, & Asada, 1995). Further immunolocalization studies by Ogawa et al., 1996, reported that Cu-ZnSODs were also present in the apoplasts in which it was deemed to aid in lignification and the nucleus, which Ogawa et al., 1996 suggesting its presence as a protector of fatal oxidative damage to DNA by the $O_2^{\cdot -}$ (Ogawa, Kanematsu, & Asada, 1996)$^{\cdot}$. Scandalios and coworkers, 1987, also reported a Cu-ZnSOD in the peroxisome which contributes to 18% of the total SOD activity in *Citrullus vulgaris* leaves (Sandalio & Del Río, 1987).

To determine differential gene expression of SODs in response to temperature stress, Tsang et al., 1991, exposed *Nicotania plumbaginifolia* plants to a 5 hour heat shock and thereafter a 15 hour chilling period and reported a ~10-fold induction in Cu/ZnSOD$_{cyt}$ mRNA abundance which remained elevated for 10 hours post. This study deduced that this SOD isoenzyme is the only one affected by high temperature that is independent to photon flux densities and that the cytosol is the major site of $O_2^{\cdot -}$ formation under conditions of high temperature (Tsang et al., 1991).

2.4.3. Hydrogen peroxide (H_2O_2) and Catalase (CAT)

Hydrogen peroxide has the longest half-life when compared to the rest of the ROS species ~ 1ms (Bhattacharjee, 2005) and as mentioned before, produced as a product in the dismutation of the $O_2^{\cdot -}$ as catalyzed by SOD. It is not a free radical *per se* as however it is a powerful oxidizing agent and has the ability to cause damage to

surrounding biomolecules (Apel & Hirt, 2004). Many metabolic proteins such as those found in the Calvin cycle such as glyceraldehyde-3-phosphate dehydrogenase, phosphoribulokinase, fructose biphosphatase and sedoheptulose biphosphatase where H_2O_2 reacts with important enzyme thiols thereby rendering them inactive (K Asada, 1987; Charles & Halliwell, 1981; Ellyard & Gibbs, 1969) In maize, H_2O_2 steady-state concentrations range from 1-2 mM (Jiang & Zhang, 2001) which can also be generation by enzymes in the apoplasts in response to pathogens in a mechanism called 'oxidative burst' (Bolwell, 1999). In addition to being a ROS, H_2O_2 readily traverses through membranes and therefore, has the potential to inflict damage far from the site of formation (Bienert, Schjoerring, & Jahn, 2006); it also mediates a whole range of biological processes these will be highlighted briefly:

Programmed cell death (PCD) is thought to be driven by H_2O_2 although the mechanism by which this occurs has not been fully elucidated. Nevertheless studies have shown that H_2O_2 influences the permeability of the mitochondrial transition pore; in a study performed using soybean cultures, that were challenged with an avirulent bacteria, Delledonne et al., 2001, showed that H_2O_2 concentrations were the main factor in initiating PCD (Delledonne, Zeier, Marocco, & Lamb, 2001). Furthermore, exposing *Arabidopsis* cultures to H_2O_2, Tiwari et al., 2002, reported that mitochondrial generation of H_2O_2 also increased which caused dysfunction of the organelle and PCD (Tiwari, Belenghi, & Levine, 2002).

Hydrogen peroxide has also been implicated in auxin-based root gravitropism. Gravity has been shown by Ju et al., 2001, to generate H_2O_2 asymmetrically in *Zea mays* roots; more specifically in the convex endodermis. This group also revealed that by applying H_2O_2 or antioxidants such as *N*-acetylcysteine, ascorbate and Trolox (Vitamin E), root gravitropism could be modulated. Interestingly, treatments with catalase had no effect on root verticality which led the authors to describe the inability of the enzyme to permeate the root epidermis (Joo, Bae, & Lee, 2001).

A more concretely elucidated mechanism is the relationship between H_2O_2 and abscisic acid (ABA) in stomatal closure. It has been well documented that ABA elicits the formation of H_2O_2 via nicotinamide adenine

dinucleotide phosphate (NADPH) oxidase in guard cells which facilitates closure of stomata (McAinsh, Clayton, Mansfield, & Hetherington, 1996). At millimolar concentrations H_2O_2 also opens Ca^{2+} channels at the plasmalemma which increases cytosolic calcium, activating a host of transcription factors thus appointing H_2O_2 as an important and potent signaling molecule (Pei et al., 2000). There have been numerous studies reporting the role of H_2O_2 as a signaling molecule in plants; in eukaryotes calcium perturbations affect protein phosphorylation through the binding calmodulin (CaM), a calcium binding protein, signaling which have been reported to initiate the activity and expression of catalases (CATs) (Pei, et al., 2000).

In plants, Catalases (CATs) and peroxidases are the two enzymatic families that remove H_2O_2 (Willekens, Inzé, Van Montagu, & Van Camp, 1995). Catalases catalyze the dismutation of H_2O_2 to water and molecular oxygen without the help of a reducing cofactor. Peroxidases on the other hand do require a reducing substrate and are classified accordingly. Glutathione peroxidase (GPX) which contains selenium removes H_2O_2 with the help of glutathione (GSH) and is present in the mitochondria and the cytosol (Drotar, Phelps, & Fall, 1985; Sabeh, Wright, & Norton, 1992). Crucial to the removal of H_2O_2 are three isoenzymes of ascorbate peroxidases (APX) that use ascorbate as a reducing cofactor in the cytosol, stroma and thylakoids of the chloroplast (Creissen, Edwards, & Mullineaux, 1994). Post scavenging, oxidized glutathione (G-S-S-G) and dehydroascorbate (DHA) are replenished by glutathione reductase (GR) and DHA reductase (DHAR) which is reviewed in the next section (K Asada, 1987).

As the name suggests, peroxisomes are major sources of H_2O_2; where a multitude of oxidases such as acyl-CoA oxidase, glycolate oxidase and urate oxidase exist and all contribute to the formation of H_2O_2 by removing two electrons from cellular substrates utilizing a flavodoxin intermediate and transferring the two to molecular oxygen thus forming the peroxide.(Kindl & Lazarow, 1982). Catalases are therefore abundant in peroxisomes in the glyoxylate cycle whereby fatty acids are metabolized and converted to succinic acid; the aforementioned acyl-CoA oxidase generates two molecules H_2O_2 directly for every molecule of succinate which is vital for seedling establishment (Beevers, 1982).

CATs generally consist of four 500 residue subunits (tetramers) each containing an extensive hydrophobic core however thus far the purification of a plant have eluded protein chemists as the large hydrophobic exterior of the tetramers in addition to the incidence of surface cysteine have rendered the enzyme difficult to obtain. (Díaz, Loewen, Fita, & Carpena, 2012).

In *Zea mays*, there exists three unlinked nuclear *CAT* genes: *Cat1, Cat2 and Cat3* which is the variant is found in the mitochondria, that aside, all plant catalases have been localized to the peroxisome and is biochemically unique when compared to other species of CATs. All of the CATs in *Zea mays* are comprised of four 60 kDa subunits and bears structural homology to CATs found in other phyla. Interestingly only CAT3 and CAT1 are functional in etiolated leaves and the coleoptile in the seedling (SCANDALIOS, 1974). In mature leaves, CAT2 is found in the peroxisomes of bundle sheath cells whilst CAT1 and 3 are found in the mesophyll (Tsaftaris & Scandalios, 1986). CAT3 has a 70-fold higher catalytic activity than the chloroplastic CAT-2 variant and is therefore more resistant to aminotriazol (AT) sodium azide (NaN_3) or cyanide (Havir & McHale, 1989). This suggests that CAT-3 plays a specific metabolic role from the other CAT isoforms. Furthermore, CAT1 and 2 can interact *in vitro* displaying heterotetramerism and *in vivo* when co-expressed in scutella of germinating embryos however CAT3 only displays homotetramerism even in locations of CAT 1 and 2 tissue specific areas (Chandlee & Scandalios, 1984; Scandalios, Tong, & Roupakias, 1980).

In most organisms, heat shock results in a fervent response in gene expression that results in a group of proteins called heat shock proteins (HSPs). Matters and Scandalios, 1986, exposed to maize mutants; the high CAT activity mutant (R6-67) and a wild type maize W64A to chronic 40°C high temperature stress. The results of this study showed that in scutella, CAT activity in W64A grown at 40°C was only a fraction less than in seedlings grown at 25°C. The R6-67 mutant grown at 40°C exhibited a lower CAT activity when compared to those grown at 25°C which was attributed to decreased levels of CAT-2 protein. As the mutant normally presents with a high CAT-2 activity, this group suggested high temperature stress resulted in the deactivation of CAT-2 transcription and translation and higher protein degradation rates. Furthermore CAT-1 mRNA and protein abundance remained static when compared to CAT-2 however in W64A scutella post day 1 germination, which was exposed to

temperatures of 35 and 40°C , both CAT-1 and CAT-2 proteins mRNA abundance increased and remained at steady state levels. In addition it was observed that CAT-1 mRNA and protein is more stable that CAT-2 at the higher temperature (Matters & Scandalios, 1986). In response to a 5 hour 37 °C treatment followed by a 22°C recession, Willekens et al., 1994, reported a decrease in *Cat 1 and Cat2* transcripts and no change in *Cat3* mRNA abundance in *N. plumbaginifolia* (Willekens et al., 1994).

2.4.4. Ascorbate Peroxidase (APX)

Ascorbate peroxidase (APX) makes use of two molecules of ascorbate (AsA) as a highly specific electron donor to also detoxify (reduce) harmful H_2O_2 to water and molecular oxygen. As a result, AsA is oxidized to 2 equivalent molecules of monodehydroascorbate (MDAsA) radical which is relatively unstable and therefore spontaneously disproportionates into AsA and dehydroascorbate at a rate of $10^5 M^{-1}s^{-1}$ (Noctor & Foyer, 1998; Shigeoka et al., 2002; Wells & Xu, 1994). In higher plants, studied have revealed at least four discrete cellular locations in which APX isoenzymes are located: In the chloroplast, there exists a free-unbound stromal ascorbate peroxidase (sAPX) and a thylakoidal membrane bound variant designated tAPX. There is also a cytosolic isoenzyme-cAPX-, and a peroxisome/glyoxysomes or microbody which is also membrane bound (Chen & Asada, 1989; Miyake, Cao, & Asada, 1993; K. Yamaguchi, Mori, & Nishimura, 1995). Two research groups also discovered a fifth mitochondrial APX (mitAPX) that was either assayed spectrophotometrically in *Pisum sativum* (Jimenez, Hernandez, del Rio, & Sevilla, 1997) leaves or isolated and characterized in potato tubers (De Leonardis, Dipierro, & Dipierro, 2000).

Ascorbate peroxidases like CATs, contain haeme and exhibits a high specificity for AsA. One of the most determining factors in APX catalysis is availability of its electron donor AsA which is especially vital for the mitAPX and the chloroplastic isoenzymes (De Leonardis, et al., 2000). Kinetic studies have shown that when concentrations of Asa fall below 20 µM, APX activity dramatically reduces; in the case of chlAPX and mitAPX the IC_{50} times are 30s and 60 mins or more respectively (Miyake, et al., 1993; Shigeoka, Nakano, & Kitaoka, 1980). The chloroplastic isoenzyme tAPX and sAPX both present as a monomer with a molecular mass of ~30

kDa (Nakano & Asada, 1987) while cAPX is homodimer of 57,5 kDa (Mittler & Zilinskas, 1991). Thylakoidal-APX is slightly heavier than sAPX by 4.5 kDa which is attributed to peptide sequences that are involved in membrane embedding (Chen & Asada, 1989; Ishikawa, Sakai, Yoshimura, Takeda, & Shigeoka, 1996; Miyake, et al., 1993). Both the microsomal- and mitochondria APXs are alike in that they present with a molecular weight of 31 kDa (De Leonardis, et al., 2000; Ishikawa et al., 1998; K. Yamaguchi, et al., 1995).

With regards to heat stress, Song et al., 2005, subjected cucumber plants to heat stress at the four-leaf stage to 40°C for days and measured all four isoenzymes of APX by spectrophotometric assays. They reported a total increase in catalytic activity 3 isoenzymes: cAPX, sAPX and mitoAPX showed significant increases in activity whilst tAPX only increased slightly but not significantly. Furthermore, northern blot analysis of sAPX showed an increase in mRNA transcript in response to high temperature stress (Song et al., 2005).

Shi et al., 2001, cloned a peroxisomal ascorbate peroxidase from *Hordeum vulgare* (barley) denoted *HvAPX1* and overexpressed it *Arabidopsis* which was controlled by the RNA 35S promoter of the cauliflower mosaic virus. This group then subjected these mutant plants, which were hydroponically grown, to 37°C as a heat stress treatment. The result of these experiments showed that in response to heat stress, the expression of HvAP1 is induced as revealed by RNA-blot analysis especially after 24hours where relative transcript levels were ~2-fold when compared to control plants that were grown at 25°C and that overexpressing *HvAPX1* in *Arabidopsis* provides thermotolerance to plants by scavenging H_2O_2 (W. Shi, Muramoto, Ueda, & Takabe, 2001).

In similar experiments, Tang et al., 2006, developed transgenic potato plants in which both Cu-ZnSOD and APX were expressed in chloroplasts and driven by the *SWPA2* promoter which is induced by oxidative stress in the form of methylviologen treatment (MV). These plants were then grown under conditions of high temperature (42°C) for 20 hours; plants grown at 25°C served as controls. Reverse transcriptase-polymerase chain reaction (RT-PCR) performed in both control and heat stressed plants for the expression levels for Cu-ZnSOD and APX showed that these enzymes were induced in response to MV when grown at 42°C (Tang, et al., 2006).

2.4.5. Glutathione Peroxidase (GPX)

Peroxidases are non-haeme containing enzymes that are found located in diverse subcellular compartments. They are divided into two categories namely the peroxiredoxins (PRXs) and the glutathione peroxidases (GPXs). The PRxs function in thiol-dependent redox reactions protecting enzymes from oxidation and subsequent oxidation (Chae, Kim, Kim, & Rhee, 1993; Kim, Kim, & Rhee, 1989; Lim, Cha, Kim, & Kim, 1994). They are also relevant in the detoxification of H_2O_2, phospholipid hydroperoxides and peroxynitrite to either water, alcohol or nitrite respectively (Bryk, Griffin, & Nathan, 2000; Hillas, del Alba, Oyarzabal, Wilks, & de Montellano, 2000; Nogoceke, Gommel, Kieß, Kalisz, & Flohé, 1997). Studies focusing on the reaction catalysis mechanisms have revealed that PRxs contain a peroxidatic cysteine which is oxidized by sulfenic acid, hydroperoxides; it is converted also shown to be converted to its native form by thioredoxin (TRX), glutaredoxin or GSH which all act on various isoenzymes of the PRx family (Gelhaye, Rouhier, Navrot, & Jacquot, 2005).Phylogenetic analysis of the GPX family of genes have resulted in categorizing them into three cohorts: The first group consists GPX1 and 2 which are selenium-containing and is localized to both the mitochondria and chloroplast. Group 2 consists of GPX3 and 5 which are found in the cytosol and mitochondrion and lastly the last group which is GPX4 that is specific for phospholipid hydroperoxide (PHGPX).

In *Populus trichocarpa* Navrot et al., 2006, reviewed six GPX genes using prediction and subcellular-targeting bioinformatics tools: PtrcGpx1 was found to be chloroplastic and had a predicted molecular mass of 18.2 kDa, PtrcGpx2 which was present in extracellular fluids and probably, a secreted GPX, had weighed 18.8 kDa. The cytosolic PtrcGpx3.1 is cytosolic and has a molecular weight of 18.6 kDa whilst PtrcGpx3.2 is either mitochondrial or chloroplastic or both and has a molecular weight of 18.5kDa. PtrcGpx4 is cytosolic and also has an estimated molecular mass of 18.9 kDa and lastly PtrcGpx5 is secreted and has a weight of 19.2 kDa, slightly heavier than other GPXs in poplar western balsam (Navrot et al., 2006). Other studies focusing on plant GPX-like activities, Sabeh et al., 1993, showed that in parenchymous leaf-gel of *Aloe vera* that the native enzyme had an apparent molecular mass of 62 kDa which was composed of 4 monomers of 16 kDa when subjected to denaturing polyacrylamide electrophoresis. In *Citrus sinensis*, exposed to salt stress, Ben Hayyim et al., 1993, a

GPX-like protein with a molecular mass of 22 kDa was shown to be induced by 0.2M NaCl (Ben-Hayyim et al., 1993; Sabeh, et al., 1992).

2.4.7 Glutathione Reductase (GR)

Glutathione reductases are NADPH-dependent oxidoreductases that are crucial in maintaining intracellular GSH pools by reducing G-S-S-G in chloroplasts, mitochondria, peroxisome and the cytosol (Edwards, Rawsthorne, & Mullineaux, 1990; Romero-Puertas et al., 2006). Although the enzyme appears to be found widespread intracellularly, 80% of total cellular GR activity was found to occur in chloroplasts (Kumar, et al., 2012). The reductase is known to coalesce with flavin adenine dinucleotide (FAD) to form a homodimer that is roughly 100-150 kDa when one FA is attached i.e. as a monomer. Glutathione reductase does also exhibits tetramerism in the absence of thiols however it is GSH presence that maintains the homodimer form (Yousuf, Hakeem, Chandna, & Ahmad, 2012). In *Zea mays*, Mahan and Burke, 1987, revealed using affinity chromatography that native GR had a relative molecular weight of 190 ±30 kDa which consisted of four polypeptides with a molecular weight of 32, 34, 63 and 65 kDa as a result of SDS-PAGE (Mahan & Burke, 1987).

Concerning catalysis, GR possesses a vital disulphide group moiety that uses one mole of NADPH to reduce one mole of G-S-S-G to GSH. Romero-Puertas et al., 2006, exposed pea plantlets to high temperature conditions of 30°C for 1hour, 35°C and finally 38°C for 4 hours and reported a 0.4 fold decrease in chloroplastic *GR1* gene expression and no change in cytosolic *GR2* expression as shown by semi-quantitative RT-PCR; however total GR activity was induced by 18-24% when measured spectrophotometrically thus suggesting post-translational methods of enzyme activation (Romero-Puertas, et al., 2006). Pastori et al., 1996, assayed GR gene activity in both the bundle sheath and mesophyll cells of *Zea mays* plants which were grown at the 20°C, 18°C and 15°C; their data showed that GR catalytic activity and mRNA expression increased only in the 15°C treatment and that by probing plant tissue with GR-specific antibodies, mesophyll cells were the main site of GR protein aggregation whilst mRNA transcripts as revealed by northern blotting of GR was found to be present in both bundle sheath cells and mesophyll cells (Pastori, Mullineaux, & Foyer, 2000).

2.4.8. Dehydroascorbate Reductase (DHAR)

Dehydroascorbate reductase (DHAR) was first characterized by Foyer and Halliwell, 1997 in spinach chloroplasts and in *Pisum sativum* by Yamaguchi and Joslyn, 1952. The enzyme present in spinach chloroplast is 23 kDa and uses 2 molecules of GSH to reduce DHA to Asa; its apparent K_m value for GSH is 2.5 mM and for DHA is 0.07 mM (C. H. Foyer & Halliwell, 1977; M. Yamaguchi & Joslyn, 1952).

There has been much debate amongst researchers regarding the cellular distribution of DHAR as there has been claims that *in vivo* the measurements of DHA in chloroplastic extracts are negligible as its accumulation is a result of side reactions of proteins involved in thiol metabolism. Morell et al., 1997, provided evidence that DHA and DHAR are 'phantom' indicators of oxidative stress by subjecting leaf and chloroplast extracts to native gel electrophoresis with specific post electrophoretic staining for DHAR and western blot analysis. Their results concluded that proteins observed as DHAR in native page gels are actually in fact thioredoxins and trypsin inhibitors as revealed by western blotting. Their conclusions were based on the ability of DHA to directly inactivate chloroplastic thioredoxins as at concentration of 50 µM. It appears that the stroma has been found to contain millimolar concentrations of DHA which then would oxidize thioredoxins-dependent redox system and have major implications for chloroplast and cell viability as a whole. They provided data that represented a dramatic decrease in NADP-malate dehydrogenase (a member of the thioredoxin system) which supported their notions on the effects of DHA accumulation in chloroplasts however what this group did not consider is the effects of any abiotic stresses on the levels of these proteins that possessed DHAR activities thus showing recruitment and involvement of enzymes which would make them necessary and therefore not 'phantom' reflectors of oxidative stress (Morell, Follmann, De Tullio, & Häberlein, 1997). It is vitally important that DHA be converted to AsA but as Foyer and Mullineaux , 1998, argue that thioredoxin just as GSH and NAPDH, can also initiate the reduction DHA back to AsA due to their presence of DHAR activity as revealed by activity staining and western

blot analysis. In summary DHAR can exist and is not oblivious to conditions of oxidative stress which renders its measurement viable and necessary (C. H. Foyer & Mullineaux, 1998).

2.4.8. Ascorbate (AsA) & Glutathione (GSH)

Plants can synthesize AsA in both non-and photosynthetic tissues that when measured can accumulate to millimolar concentrations; in leaves AsA is more abundant that chlorophyll expressing the AsA pool, which comprises ten percent of the soluble carbohydrate (C. Foyer, Rowell, & Walker, 1983). It is therefore seen as the plants chief antioxidant and possesses the ability to detoxify hydroxyl radicals, $O_2^{\cdot -}$, and $^1O_2$ non-enzymatically and enzymatically as an electron donor by APX as a part of the Ascorbate-Glutathione Cycle (Buettner & Jurkiewicz, 1996) Ascorbate can be synthesized from glucose in the 'inversion pathway' as the D-glucose molecule is inverted to its enantiomeric stereoisomer and its carbon skeleton converted to L-galactono-γ-lactone and eventually L-ascorbic acid. The 'non-inversion' pathway of AsA synthesis occurs when D-glucose is converted to D-glucosone and then L-ascorbic acid (LOEWUS, 2012). Although the site of AsA synthesis is unknown the presence of L-galactono-1, 4-lactone dehydrogenase (GLDH), the key enzyme in involved in ascorbate synthesis is found both in the cytosol and the mitochondria as revealed by Oba et al., 1995 (LOEWUS, 2012; Ôba, Ishikawa, Nishikawa, Mizuno, & Yamamoto, 1995). Ascorbate is not synthesized in chloroplasts however it is found at high concentrations in this organelle (20-50 mM); this is achieved via facilitated diffusion across the chloroplast envelope. Interestingly, the thylakoidal membranes have no known ascorbate transport mechanism (C. Foyer, et al., 1983; C. H. Foyer & Lelandais, 1996).

Glutathione (GSH) is a tripeptide containing glutamate, cysteine and glycine that is involved in sulphur metabolism and defense (Noctor & Foyer, 1998). At the level of the roots, GSH is involved in sulphur uptake and is used as a cofactor in the removal and detoxification of xenobiotics by GSH-S-transferases (GSTs) throughout the whole plant (Herschbach & Rennenberg, 1994; Lamoureux & Rusness, 1993). In cell biochemistry, GSH serves as the non-protein thiol and along with AsA is a vital antioxidant involved in maintaining cellular redox homeostasis (Kunert & Foyer, 1993; Rennenberg, 1980). Synthesis of GSH occurs via two ATP-dependent

enzymes namely γ-glutamylcysteine synthetase and glutathione synthetase (R Hell & Bergmann, 1988; Rüdiger Hell & Bergmann, 1990). In cells, GSH represents a sulphur pool(Rennenberg, 1980); studies have shown that by applying H_2S via fumigation or by sulphates, cysteine contents increase which results in increased GSH levels (Buwalda, De Kok, & Stulen, 1993; Kok & Kuiper, 1986). When glutamate is applied, very minute increases in GSH is seen suggesting that cysteine is the rate limiting amino acid for tripeptide formation(Noctor, Jouanin, & Foyer, 1997). Sub-cellular localization of GSH synthesis are the cytosol and the chloroplast where its concentration ranges 1-4.5 mM (C. H. Foyer & Halliwell, 1976). In *Zea mays* GSH is found to be more present in bundle sheath cells when compared to mesophyll cells (Burnell, 1984; Doulis, Debian, Kingston-Smith, & Foyer, 1997).

2.5. The Effects of Heat Stress on SOD, CAT, APX, GPX, GR and DHAR activity in Plants

2.5.1. Overview

The section aims to review of studies published that have focused on determining the effects of high temperature stress on the effects on antioxidant activities in various plant species, mainly from the Poaceae family to which maize belongs. As performed in this study, antioxidant enzyme activities are measuredly mainly via two methods in the laboratory setting. Native polyacrylamide gel electrophoresis (PAGE) as well as the employment of spectrophotometric methods allows one to measure the activity of enzymes *in vitro.*

2.5.2. The Effects of Heat Stress on Antioxidant Enzymes in Plants

Kumar et al., 2012, performed experiments whereby two genotypes of maize (PMH 1 and 2) were grown hydroponically while being subjected to four day/night temperatures i.e. 30ºC/25 ºC, 35 ºC/30 ºC and 40 ºC/35 ºC and 45ºC/40ºC for 13hr/11hr where 30ºC/25 ºC served as controls. Upon measuring total antioxidant activities in shoots via spectrophotometric means they reported a non-significant total increase in SOD activities at 35 ºC/30 ºC, while at 40 ºC/35 ºC, SOD activity was significantly ($P < 0.05$) elevated to ~1.5-fold to that of controls in PMH1 and ~1.4-fold for PMH2. At 45ºC/40ºC total SOD activity was decreased to ~1.2 fold in PHM1 and ~1.3-fold suggesting some loss of enzyme activity due to intense heat treatment. With regards to CAT activity, a ~1.7-

fold increase .was seen in PMH1 plants grown at 40 ºC/35 ºC whereas in PMH2, there was a ~1, 4-fold increase. The highest heat treated plants, the activity of which was significantly higher when compared to controls, showed loss of CAT activity of 10% when compared to PMH1 grown at 40 ºC/35 ºC and 12% in PMH2. In the PMH1 genotype H_2O_2 increased significantly and ranged from 2 µmol/g at control temperatures to 3.9 µmol/g in the highest heat treatment; this was also seen in the PMH2 cultivar which ranged from 2.2 to 4.6 µmol/g although the increase observed in plants grown at 35 ºC/30 ºC was not significant. Ascorbate peroxidase activity (APX) results showed a ~1.5-fold increase in PMH1 and a ~1.7-fold increase in PMH2 when exposed to 40 ºC/35 ºC which decreased by 11% in both PMH1 and 2 when exposed to 45ºC/40ºC. Ascorbate content in PMH1 grown at 40 ºC/35 ºC increased from 80nmol/gram dry weight to 159 nmol/gram which represented a 2-fold induction in AsA content; in PMH2 ascorbate levels also increased significantly from 90nmol/gram to 160 nmol/gram. Glutathione Reductase (GR) activities followed the same pattern; in response to 40 ºC/35 ºC treatment GR activities increased significantly by ~1, 8-fold in PMH1 and ~1.4-fold in PMH2, however significant increases were also seen in plants that were grown at 35 ºC/30 ºC, PMH1 showed a ~1.5-fold increase in GR activity while PMH2 had an overall ~ 1.1-fold increase. This was not seen in SOD, APX or CAT activities which suggests that GR may be an important enzyme in thermotolerance mechanisms. Total GSH content increased from 30nmol/g to 53nmol/g in PMH1 plants grown at 40 ºC/35 ºC, a trend that was also seen in PMH2 plants and at 35 ºC/30 ºC, both maize genotypes present with significant increases in GSH which corroborated increases that were also seen in GR activity. As a measure of lipid peroxidation, MDA content was the same in both genotypes and significantly increased in the higher temperature treatment groups. At control temperatures MDA content per gram was 22nmol which increased to 40nmol in the 45ºC/40ºC treatment group (Kumar, et al., 2012) . In summary, all antioxidant enzymes measured in this study increased in activity at 40 ºC/35 ºC however at 45 ºC enzyme activity was compromised which suggests that this temperature is above the threshold temperature for these maize genotypes as it appears that enzyme denaturation or inactivation occurs.

In *Lens culinaris*, Chakraborty and Pradhan, 2010, subjected six varieties of lentil to temperatures ranging from 30ºC-50 ºC for 4 hours in a growth chamber and measured antioxidant responses as follows: Superoxide dismutase

activity in all cultivars was induced by heat stress; at 30ºC SOD activity increases ranged from ~1.4-fold to ~1.8fold when compared to control plants grown at 20 ºC; this was further exacerbated in plants exposed to 35ºC where SOD activity ranged from ~1.4-fold to 2.1-fold when compared to control plants and at 40ºC SOD activity ranged from ~1.8-fold to 2.42-fold in 4 of the six varieties. The Lv and Sehore cultivars displayed low tolerance to heat stress as 40ºC resulted in decreases in SOD activity to control levels suggesting some loss of enzyme function. At 45ºC and 50 ºC SOD activity was significantly halted to below control levels. Ascorbate peroxidase levels also increased at temperatures 30 ºC, 35 ºC and 40ºC in fix of the six cultivars the fold increases ranged from ~1.2-fold to 2-fold at 30 ºC, ~1.5-fold to 2.2-fold at 35 ºC and ~1.9-fold to ~3.4-fold at 40ºC when compared to control plants at 25 ºC. Unlike SOD, APX activity in three of the six cultivars was further induced at 45ºC with fold increases ranging from 2.0 to 3.8-fold suggesting that APX is thermostable at higher temperatures which was not seen in other enzymes measured in this study. Glutathione reductase activities was also measured by Chakraborty and Pradhan, 2010 who showed that the activity of this enzyme peaked at control temperatures and was compromised in response to heat stress. At 30ºC, GR activity decreased ranged from ~1.0 to 1.8-fold in all cultivars; this extended to ~1.2 fold to -3.5-fold decreases at 35 ºC and ~1.67 fold to 9-fold decreases at 40 ºC. At higher temperatures of 45ºC and 50ºC GR activity was almost negliglbe thus indicating that this enzyme is extremely heat sensitive in *Lens culinaris*; in maize GR activity increases in response to heat stress and is therefore thermally activated. Catalase activity in four of the six cultivars was induced by heat particularly at 30ºC & 35ºC ; CAT activity increases ranged from ~1.0-fold to 1.5-fold at 30 ºC and ~1.2-fold to 1.4-fold in three of the six cultivars at 35ºC. In the three remaining cultivars CAT activity was retarded at 40ºC to below control levels which was further exacerbated at 45-50 ºC. Hydrogen peroxide content decreased in four of the six cultivars to below control levels from temperatures ranging from 30 ºC to 40 ºC, however at 50 ºC due to enzyme inactivation, H_2O_2 increased to above control levels solidly indicating that APX/CAT activity was responsible for H_2O_2. Malondialdehyde levels increased steadily in all cultivars in response to increasing increments of heat stress but was mostly elevated at the 45 ºC and 50 ºC treatments. Lastly, AsA contents increased in three of the six cultivars at 30 ºC, 35 ºC and 40ºC but decreased during the hotter treatments (Chakraborty & Pradhan, 2011).

In abscisic acid deficient *Zea mays* seedlings, Hu et al., 2010, exposed these *vp5* mutant plants to heat shock by increasing the ambient temperature from 28ºC to 42°C for 1 hour an measured the effects on four antioxidant enzymes. The outcomes of these experiments showed a ~1.4-fold induction in SOD activity, no increase in CAT activity and a ~1.5-fold increase in both APX and GR activities in leaves of maize seedlings (X. Hu et al., 2010). To the determine the effects of heat stress on cucumber, Shi et al., 2006, exposed *Cucumis sativa* seedlings, which were pretreated with AsA and 1 mM salicylic acid (SA) for 12hours, to a 40 ºC high temperature stress treatment for 36 hours and measured parameters involved in oxidative balance. Their findings showed that the heat treatment in leaves resulted in a ~1.2-fold induction in SOD, CAT, GR and DHAR activity, a ~1.4- fold increase in APX activity and a ~2.1-fold induction in GPX activity when compared to control plants grown at 20°C-29ºC. Furthermore, H_2O_2 content increased from 25nmol to 190nmols per gram fresh weight which was reflected in as a ~1.6-fold increase in TBARS activity (Q. Shi, Bao, Zhu, Ying, & Qian, 2006).

Savicka and Skute, 2010 measured the effects of high temperature effects on *Triticum aestivum* seedlings and reported that a single heat shock of 42ºC for 24 hours resulted in a ~3.2-fold surge in superoxide radical concentration which was associated with a ~ 1.6-fold decrease in MDA content in the first leaf (Savicka & Škute, 2010).

Xu et al., 2010, performed similar experiments whereby two cool-season turfgrasses were exposed to high temperatures ranging from 38°C- 46°C for 14 hours and reported that in *Festuca arundinacea* , there was a ~4-, 12.5 and 39-fold increases in superoxide content, H_2O_2 and MDA content when compared to controls at 22ºC. Ascorbate and GSH contents decreased steadily in response to increasing temperatures where 46°C treatments caused a ~5, 2-fold and ~13-fold reductions in AsA and GSH respectively. In *Lolium perenne,* 46°C heat stress for 14 hours results in a ~9-, ~15.5- and ~18-fold reductions in in superoxide content, H_2O_2 and MDA content whereas AsA concentrations decreased ~7-fold and GSH concentrations decreased from 520nmol per gram to 20nmols per gram (Xu, et al., 2006).

Djanaguiraman et al., 2001, exposed flowering soybean plants to heat stress of 38ºC for 14 days and reported a ~1,1-fold reduction in SOD activity, a ~2-fold decrease in CAT activity and a ~1,3-fold attenuation in total peroxidase activity when compared to plants grown at optimum temperature at 28°C. This was accompanied by a ~2,7-fold exacerbation in MDA content, a ~2.1-fold increase in superoxide radical content and an overall ~2,8-fold induction in H_2O_2 (Djanaguiraman, et al., 2011).

Dat et al., 1998, exposed *Sinapis alba* seedlings to a non-lethal high temperature of 45ºC for 1 hour and reported a ~1.7-fold induction in APX activity, a doubling of GR activity and an increase in DHAR activity from 512 $\mu mol.h^{-1}.mg^{-`1}$ to 523 $\mu mol.h^{-1}.mg^{-`1}$ in young leaves when compared to non-stressed controls grown at 24ºC. Furthermore, they also showed that exposure to heat stress of an hour results in a ~2.9-fold reduction in AsA with a concomitant ~2-fold increase in DHA one hour post heat stress and a ~1.2-fold drop in oxidized GSH with a ~1.4-fold increase in GSSG (J. F. Dat, Foyer, & Scott, 1998).

Kochhar and Kochhar , 2005, exposed *Vigna mungo* seedlings to high temperature stress of 40ºC for 2 hours in a growth chamber and reported a slight decrease in leaf SOD activity from 6.45U to 5.98U/mg soluble protein as well as a ~3.8-fold induction in total peroxidase activity when compared to seedlings grown at room temperature. Upon further analysis of SOD activity via the use of Native-PAGE and post-electrophoretic activity staining for the enzyme, they also showed the presence of 3 bands of SOD activity at 45-, 25 and 17.8 kDa. Heat stress appeared to cause a reduction in the 25 kDa band which was identified primarily as a Cu-ZnSOD as revealed by staining with inhibitors (KCN and H_2O_2). This was corroborated via transcript analysis in the form RNA-blotting using a probe that was obtained from the sequence of Cu-ZnSOD which was also increased in response to heat stress (Kochhar & Kochhar, 2005).

Yin et al., 2007, exposed *Lilium longiflorum* plants to heat stress at 42ºC and 47ºC for 10 hours and showed that in leaves superoxide content increased by ~1.2-fold whilst H_2O_2 content was increased from 41 $\mu mol.g^{-1}$ to 70 $\mu mol.g^{-1}$ fresh weight. Both AsA and GSH content decreased significantly by ~1.3-fold and ~1.1-fold respectively. Regarding antioxidant enzyme activities, SOD activity was induced ~1.4-fold after 4 hours at 47ºC

while total peroxidase activity was induced during the first hour of heat stress by ~1.6-fold but then was inactivated below control levels by ~1.1-fold suggesting that longer exposure to heat renders the enzyme inactive. Catalase activity peaked after 2 hours at 47ºC where a ~1.6-fold induction was seen; after 10 hours CAT activity then decreased by ~1.1-fold when compared to control plants grown at 20ºC. Ascorbate peroxidase activity peaked 4 hours at 47ºC by ~1.4-fold and after 10 hours APX activity was only slightly higher than control plants. A similar trend was seen in GR where the activity of this enzyme peaked at 4 hours with a ~1.1 fold induction (Yin, Chen, & Yi, 2008).

At 42º C, superoxide radical content increased from 8.5nmol.min^{-1}.g^{-1} to 9.2 nmol.min^{-1}.g^{-1} after 2 hours under heat stress at while H_2O_2 content increased from 42 µmol.g^{-1} to 49 µmol.g^{-1}. Ascorbate content peaked 2 hours at 42ºC where a ~1.26-fold increase was observed while GSH levels peaked after 4 hours under heat stress where a ~1.75-fold increase was seen; after 10 hours both AsA and GSH levels were below control levels that were indicative of a temperature of 20 ºC. With regards to antioxidant enzyme activities, SOD activity peaked after 10 hours with a ~1.25-fold increase in activity whereas the maximum total peroxidase activity was after 6 hours at 42 ºC at 295U.g^{-1} compared to control levels at 175 U.g^{-1} fresh weight. Catalase activity increased steadily over 10 hours where a ~1.5-fold induction was observed at the end of the incubation periods; this was also for APX activity as its levels increased from 3.1 µmol AsA.g^{-1}.h^{-1}fresh weight to 4.4 µmol AsA.g^{-1}.h^{-1}fresh weight after 10 hours. Glutathione reductase activity increased from 5.831 µmol NADPH .g^{-1} .h^{-1} to 8 µ mol NADPH .g^{-1}. h^{-1} after 10 hours at 42ºC (Yin, et al., 2008).

Yin et al., 2008, also separated SOD isoforms via native-PAGE and detected 3 isoforms (SOD1, SOD2, and SOD3) which were subsequently further classified by the use of inhibitors KCN and H_2O_2 which revealed that SOD1 was a MnSOD whilst SOD2 and 3 were Cu-ZnSODs which accounted for 96% of total SOD activity at control temperatures. Under heat stress, MnSOD and Cu-ZnSODs were induced at 42ºC, however at 47ºC MnSOD activity was abolished when compared to 20ºC controls (Yin, et al., 2008).

Chapter Three

Materials and Methods

3.1. Growth of *Zea mays*

For this study, *Zea mays* cultivar CAP341NG was used. Maize seeds were first surface-sterilized using a weak sodium hypochlorite solution (NaClO) of 0.35% for 15 minutes. Following this period, the seeds were thoroughly washed and rinsed several times in distilled water. The seeds were then placed in a beaker containing 10 mM $CaSO_4$ for 12-16 hours and bubbled continuously using a Tetra ASP100 aeration pump. Studies have shown that priming seeds in a salt solution increases the total germination percentage as well as other parameters such as root/shoot length and root/shoot elongation rates (Soojani, 2007).

After imbibition, seeds were placed between layers of wet paper towel and allowed to germinate in the dark at room temperature in plastic tray covered with heavy duty aluminum foil. They were monitored for radical emergence, which took places 48-72 hours post-imbibition. Once enough seeds had germinated and had healthy intact radicals, they were sown in 20cm terracotta pots containing Promix® Organic plant growth media (Windell Hydroponics, Cape Town, RSA). Prior to sowing, the Promix® media was fortified with a nutrient solution that contained trace elements and macronutrients for healthy growth, The final concentrations of these nutrients were: 10 mM Ammonium Nitrate (NH_4NO_3), 10 mM 2-(N-morphlino) Ethanesulfonic Acid [(MES), pH 6.2], 5 mM Calcium Chloride ($CaCl_2$), 5 mM Potassium Nitrate (KNO_3), 2 mM Magnesium Sulphate ($MgSO_4$), 1 mM Potassium Sulphate (K_2SO_4), 1 mM Di-Potassium Hydrogen Phosphate (K_2HPO_4), 100 µM Iron-Sodium Ethylenediaminotetraacetate (Fe-NaEDTA), 5 µM Boric Acid (H_3BO_4), 5 µM Manganese Sulphate ($MnSO_4$), 2 µM Sodium Molybdate (Na_2MOO_4), 1 µM Zinc Sulphate ($ZnSO_4$), 1 µM Copper Sulphate ($CuSO_4$) and 1 uM Cobalt Sulphate ($CoSO_4$). Once the nutrient solution was added to the Promix®, water was also added to the pots

to ensure that the seeds were well watered and did not encounter water-stress. After sowing the 20cm pots were placed in 2 separate growth chambers (large incubators) set at 25ºC/18 ºC (day/night temperature cycle) containing fluorescent Narva® amps with a photosynthetic photo flux density of . 300 µmol. photons. $m^{-2.}$ s^{-1} during the day cycle. Maize seedlings were allowed to grow to vegetative stage 2 (V2).

3.2. Administration of Heat Stress

Once the maize seedlings had reached the V2 stage, administration of heat stress began. This was achieved by setting one of the growth chambers to 42ºC/25 ºC (day/night temperature cycle). The remaining growth chamber was kept at 25ºC/25 ºC which served as controls for the study. The heat treatments lasted for 5 days, however each day plants were weighed for the mass of water lost; the amount lost was replenished thereby not allowing any water stress to compete with the heat treatments; all plants were kept in a well-watered environment. After 5 days, all plants were harvested, leaf tissue was ground in liquid N_2 and stored at -80 ºC.

3.3. Total Protein Isolation

To isolate total protein (crude extract), fresh finely-ground leaf tissue was weighed (200mg) from both heat stressed and control plants and homogenized in 500 µl 40 mM potassium phosphate buffer (KPO_4) containing 1 mM EDTA (Sigma) and 5% polyvinylpolypyrrolidone (PVPP, Sigma). The tissue-buffer matrix was vortexed thoroughly for 30 seconds and then centrifuged (Eppendorf) at 13 200 rpm for 15 minutes at 4ºC. The supernatant was then transferred to a clean 1.5ml microcentrifuge tube and kept on ice for further processing.

3.4. Bradford Assay for Protein Concentration Determination

Protein concentration of homogenates were determined via the Bradford Assay: 1 µL of protein homogenate was aliquoted into 9 µL distilled water thereby creating a 10-fold dilution. One hundred and ninety microliters of Bradford reagent (0.02% Coomassie brilliant G250, 8.5% phosphoric acid, 4.75% ethanol, Bio-Rad) was added to the protein sample and allowed to incubate for 5 minutes for colour development. The absorbance was then read spectrophotometrically at 595 nm. Protein concentration was determined via a standard curve which was created using a series of Bovine Serum Albumin (BSA, Sigma) standards (Kruger, 1994).

3.4. Native Polyacrylamide Gel Electrophoresis (PAGE) for In-Gel Enzyme Activity Assays

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is the standard proteomic procedure biologists utilize to separate proteins, however if one desires to analyze a protein, such as an enzyme in its native state, non-denaturing electrophoresis is performed which does not make use of detergents such as SDS or common reducing agents such as dithiotheritol (DTT) or β-mercaptoethanol (Walker, 2002). For this study, 6 antioxidant enzymes were analyzed for their respective activities using non-denaturing PAGE and spectrophotometric assays, these are discussed below.

3.4.1. Superoxide Dismutase In-Gel Activity Assay (SOD)

The superoxide dismutase (SOD) in-gel activity assay was performed according to (Beauchamp & Fridovich, 1971). To differentiate between the different SOD isoforms, three 12% separating gels (vol/vol) and 5% stacking gels were prepared using the Mini-PROTEAN Handcast System from Biorad. Once gels were polymerized, aliquots of each protein sample was added to a 4X loading dye (pH 6.8) that contained 62.5 mM Tris-HCL, 40% glycerol and 0.01% bromophenol blue. One hundred micrograms of each sample was loaded in to the wells of each of the three gels and electrophoresis was performed at 90V for 7 hours in running buffer (pH8.3) containing 12 mM Tris and 95 mM Glycine.

After electrophoretic migration was completed two of the gels were incubate with 50 mM KPO_4 (pH 7.8) buffer containing 5 mM Potassium cyanide (KCN) and 6 mM Hydrogen peroxide (H_2O_2) respectively for 15 minutes. The remaining gel was incubated in only 50 mM KPO_4 buffer for 15 minutes. Potassium cyanide serves as an inhibitor for Cu/ZnSOD whereas H_2O_2 inhibits both Fe and Cu/ZnSODs isoforms thereby allowing the activity of all SOD isoforms to be determined when cross-referenced to the uninhibited gel which represents all three metallo-isoforms (Beyer & Fridovich, 1987).

After the 15 minute incubation that included inhibitors, each gel were incubated with 50mls of 0.4 mM Nitrotetrazolium Blue Chloride (NBT, Sigma) in 50 mM KPO_4 (pH 7.8) buffer for 10 minutes in the dark.

Thereafter the NBT solution was poured off and replaced with 50 mls of 12 µM Riboflavin (Sigma) and 28 mM N,N,N',N'-tetramethylethylenediamine (TEMED, Bio-Rad) for 10 minutes also in the dark. After the final incubation step the gels were exposed to light and bands were allowed to develop.

3.4.2. Catalase In-Gel Activity Assay (CAT)

An eight percent (vol/vol) native gel was prepared for the catalase assay according to (Manchenko, 2002), which was loaded with 150 µg of protein and subjected to electrophoresis as described in section 3.4.1. After the electrophoretic run the gel was washed thrice with distilled for 10 minutes. The gel was then incubated with 0.0003% H_2O_2 (vol/vol, Merck) for 10 minutes and stained with both a 2% Iron III chloride ($FeCl_3$, Sigma) and Potassium ferricyanide. The solutions were poured simultaneously directly on top of the gel and the achromatic bands were allowed to develop against a Prussian blue background.

3.4.3. Ascorbate Peroxidase In-Gel Activity Assay (APX)

For the determination of APX activity according to (Mittler & Zilinskas, 1993), 10% gels (vol/vol) were prepared, 150 µg of protein loaded and electrophoresis was performed in running buffer that contained 12 mM Tris and 95 mM Glycine and 2 mM Ascorbate (Sigma). After the electrophoretic run, gels were calibrated thrice in 50mls of 50 mM of KPO_4 buffer (pH7.0) containing 2 mM ascorbate in for 10 minutes. This calibration solution was poured off and the gels were then incubated in 50 mM KPO_4 containing 4mM ascorbate and 2 mM H_2O_2 for 20min. Following this step, gels were washed with 50 mM KPO_4 (pH 7.8) buffer for 1min and then exposed to a solution of 50 mM (pH 7.8), 2.45 mM NBT and 28 mM TEMED on a platform shaker. Activity of APX was observed as achromatic bands against a purple background.

3.4.4. Glutathione Peroxidase In-Gel Activity Assay (GPX)

Glutathione peroxidase (GPX) In-gel activity assay was performed according to (Lin, Chen, & Hou, 2002), where 10% (vol/vol) polyacrylamide gels were cast. One hundred micrograms of protein was loaded into the gel which underwent electrophoresis in running buffer containing 12 mM Tris and 95 mM Glycine and 2 mM reduced

glutathione (GSH; Sigma) Following electrophoresis, gels were equilibrated in 50 mM KPO_4 (pH 7.8) containing 13 mM GSH and 0.008% cumene hydroperoxide for 10minutes. Following this incubation step gels were washed with 50 mM KPO_4 twice and stained as described 3.4.2., with 2% iron III chloride ($FeCl_3$) and potassium ferricyanide $K_3[Fe(CN)_6]$ (Sigma); achromatic bands were observed and allowed to develop against a purple background.

3.4.5. Glutathione Reductase In-Gel Activity Assay (GR)

The glutathione reductase (GR) In-Gel activity assay was performed according to Hou et al., 2004. Approximately 7,5% (vol/vol) gels were prepared and loaded with 200-400 µg of protein (Hou, Liang, Wang, & Liu, 2004). Gels were subjected to non-denaturing electrophoresis as described in 3.4.1., thereafter gels were incubated in 10 mM Tris-HCl (pH 7.9) for 5 minutes. Following the equilibration step, gels were exposed to 4 mM Oxidized Glutathione (GSSG, Sigma) and 1.5 mM nicotinamide adenine dinucleotide (NAD^+, Sigma) in and 2 mM 5.5-dithiobis(2-nitrobenzioc acid) (DTNB, Sigma) 50 mM Tris-HCl buffer for 10 minutes. Thereafter gels were stained in the dark for 5-10 minutes in 0.6 mM Thiazolyl Blue Tetrazolium Bromide (MTT, Sigma) and 0.8 mM Phenazine methosulphate (PMS, Sigma). Clear bands of GR activity were observed against a purple background.

3.4.6. Dehydroascorbate Reductase In-Gel Activity Assay (DHAR)

Dehydroascorbate Reductase activity was assessed according to De Gara et al., 1994, using 7.5% (vol/vol) gels, which were loaded with 150 µg of protein and electrophoresed as described in 3.4.1 (De Gara, Paciolla, Tommasi, Liso, & Arrigoni, 1994). Following electrophoresis, gels were exposed to 4 mM GSH and 2 mM dehydroascorbate (DHA) in 0.1M KPO_4 buffer for 20 minutes and then developed using solutions of $FeCl_3$ and $K_3[Fe(CN)_6]$ in 0.125N HCl; bands of DHAR activity were observed as dark blue bands against a light blue background.

3.5. Spectrophotometric Determination of Antioxidant Enzyme Activities, Malondialdehyde and Hydrogen Peroxide Content.

3.5.1. Superoxide Dismutase (SOD)

Superoxide dismutase activity (SOD) was measured using a modified method that is based on Beauchamp and Fridovich, 1971 that incorporates the inhibition of photoreduction of NBT, a yellow tetrazole containing compound that produces purple monoformazan by the superoxide radical. The presence of SOD would therefore inhibit the formation of formazan thereby allowing one to quantitate the enzymes activity (Beauchamp & Fridovich, 1971).

This assay was performed in a 96-well plate where a 250 µL reaction mixture was created that contained 50 mM KPO_4 buffer (pH 7.8), 9.9 mM L-methionine, 2 mM EDTA, 55 µM NBT and 0.025% Triton-X100. The reaction mixture was then supplemented with 10 µg of crude protein isolates and 20 µL of 1 mM riboflavin and illuminated for 10 minutes. Identical reaction mixtures that contained no protein isolate and reaction mixtures that were kept in the dark served as blanks. The absorbance of all three reaction mixtures was read at 560 nm immediately and the activity of SOD was determined according the Beauchamp and Fridovich definition which states that 1 unit of SOD is the amount that initiates the inhibition of NBT photoreduction by 50%. This was represented in units of activity per grams of fresh weight (Beauchamp & Fridovich, 1971).

3.5.2. Catalase (CAT)

Catalase activity was measured according to Aebi and Lester (1984) where the disintegration of H_2O_2 is accompanied by a decrease in absorbance at 240 nm. In a quartz cuvette, a 1 ml reaction mixture containing 50 mM KPO_4 (pH 7.0) and 50 µg of protein sample was prepared. To initiate the reaction 10 mM of H_2O_2 was then added and the $A_{240\ nm}$ was recorded. Using the extinction coefficient of 40 $mM^{-1}.cm^{-1}$ at 240 nm, the amount of

CAT activity (deduced from the linear section of the curve) activity was determined and expressed in terms of millimoles of H_2O_2 .min^{-1} .g^{-1} FWT (fresh weight).

3.5.3. Ascorbate Peroxidase (APX)

Ascorbate peroxidase activity was assayed using an adapted method based of the protocol by Nakano and Asada, 1981, which measures the oxidation of ascorbate which is reflected by a decrease in absorbance at $A_{290\ nm}$. A reaction mixture containing 50mM KPO4 buffer (pH 7.0), 50 µg of protein sample, 0.5 mM ascorbate and 0.5 mM H_2O_2 to initiate the reaction. The absorbance at 290 nm was recorded for 3min and APX activity was calculating using the extinction coefficient of 2.8 $mM^{-1}.cm^{-1}$ for reduced ascorbate. Enzyme activity was represented in terms of mM Ascorbate.min^{-1}.g^{-1} FWT (Nakano & Asada, 1981).

3.5.4. Glutathione Peroxidase (GPX)

Glutathione peroxidase activity was measured using an indirect, coupled (two enzyme) reaction that detected the oxidation of Nicotinamide adenine dinucleotide phosphate (NADPH) by GR at 340 nm. Cumene hydroperoxide is said to be reduced by GPX, which uses GSH as an electron donor thus forming GSSG. Oxidized glutathione is then reduced back to GSH by GR which uses NADPH and thus provides a reflection of GPX activity (Paglia & Valentine, 1967). The 1 ml reaction mixture contained: 5 mM K_2HPO_4, 1 mM GSH, 0.5 mM cumene hydroperoxide, 0.2 mM EDTA, 0.2 mM Sodium Azide (NaN_3), 0.1 mM NADPH and 100 µg protein sample. The activity of GPX was determined via the linear portion of the curve using the extinction coefficient of 6.200$M^{-1}.cm^{-1}$ for NADPH at A_{340} and represented with units of nanomoles of NADPH. min^{-1} .mg^{-1} .

3.5.5. Glutathione Reductase (GR)

Glutathione reductase activity was assays according to Smith et al., 1988, which is based on the reduction of DTNB (Ellman's reagent) to 2-nitro-5-thiobenzoate (TNB) which is associated with a an concomitant increase in absorbance at 412 nm (I. K. Smith, Vierheller, & Thorne, 1988). The 1 ml reaction volume contained 50 mM KPO_4 buffer, 2 mM EDTA, 1 mM GSSG, 0.1 mM NADPH and 100 µg of protein sample. The total GR activity

of the sample was calculated from the extinction coefficient of TNB (14.15 $M^{-1}.cm^{-1}$) and expressed as millimoles of TNB minute per gram fresh weight.

3.5.6. Dehydroascorbate Reductase (DHAR)

The activity of DHAR was measured according to a modified method by Hossain an Asada (1984), where the appearance of ascorbate is measured by an increase in absorbance at 265 nm (Hossain & Asada, 1984; Kato, Urano, Maki, & Ushimaru, 1997). The 1ml assay mixture contained 50 mM KPO_4 buffer (pH 7.8), 2.5 mM GSH, and 0.2 mM DHAR (Sigma) and was initiated by the addition of 150 µg of protein. The absorbance was recorded for 2 minutes at 265 nm; DHAR activity was calculated from the extinction coefficient for ascorbate which was 14 mM^{-1} .$cm^{-1}$and expressed as nanomoles of DHA reduced per milligram protein per minute.

3.5.7. Thiobarbituric Acid Reactive Substances Assay (TBARS) as an Indicator of Lipid Peroxidation

To assess the amount of damage done to lipids by reactive oxygen species, MDA contents were assayed according to Buege and Aust, (1978). Approximately 100 µg of leaf tissue was homogenized in 500 µL of 6% trichloroacetic acid (TCA, Sigma) and then centrifuged for 15 minutes at 13 000 x g at 4ºC. Two-hundred microliters of the supernatant was aspirated and added to 400 µL of 0.5% 2-thiobarbituric acid (TBA, Sigma) in 20% TCA. The solution was vortexed and boiled at 90ºC for 20 minutes. After the incubation step the solution was then subjected to centrifugation again at 13 000 x g for 5minutes; the absorbance of the supernatant was read at 532 nm and at 600 nm wavelengths to correct for non-specific absorbance (Buege & Aust, 1978).

3.5.8. Hydrogen Peroxide Content (H_2O_2)

Hydrogen peroxide levels were measured according to Velikova et al., 2000; 75 µL of the remaining 6% TCA extract that was also used for the TBARS assay was mixed with a 50 mM K_2HPO_4 containing 0.5 M Potassium Iodide (KI, pH 5.0). Samples were then incubated at room temperature for 20 minutes and the absorbance of

solution was measured at 390 nm. The H_2O_2 content in these samples were calculated using a standard curve generated by preparing standard solutions of H_2O_2 which ranged from 0 nM to 2500 nM (Velikova, Yordanov, & Edreva, 2000)

3.6. Statistical Analysis

Data gathered from these experiments were represented as means ± standard error. Statistical differences between both temperature treatments were performed using the Students *t*-test using GraphPad Instat software. Statistical significance was accepted when $p < 0.05$.

Chapter Four

Results

To reiterate, the aim of this study was to determine the effects of heat stress on the enzymatic antioxidant machinery in *Zea mays* leaves. Six enzymes were chosen for investigation: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), ascorbate peroxidase (APX), glutathione reductase (GR) and dehydroascorbate reductase (DHAR) which was measured via native-polyacrylamide gel electrophoresis and spectrophotometric means. The results of these investigations are presented in this chapter.

4.1. Heat Stress Induces Total Superoxide Dismutase (SOD) Activity and Copper/Zinc-SOD in leaves of *Zea mays.*

Zea mays plants at the V2 developmental stage were grown in growth chambers at 42ºC/18ºC (day/night temperature cycle) for 5 days, which served as the heat stress treatments, and 25ºC/18ºC which served as controls. Figure 4.1A & B, shows that heat treatment resulted in a 13% induction in Cu/Zn SOD activity ($P < 0.01$) as well as a total upregulation in total SOD activity from 1 (Figure 4.1.C). Upon the addition of inhibitors of Cu/Zn-SODs and Fe-SODs namely potassium cyanide and hydrogen peroxide, no other SOD isoforms were found i.e. Mn-SODs thereby clearly distinguishing a role of Cu-ZnSODs in heat stress responses in *Zea mays*.

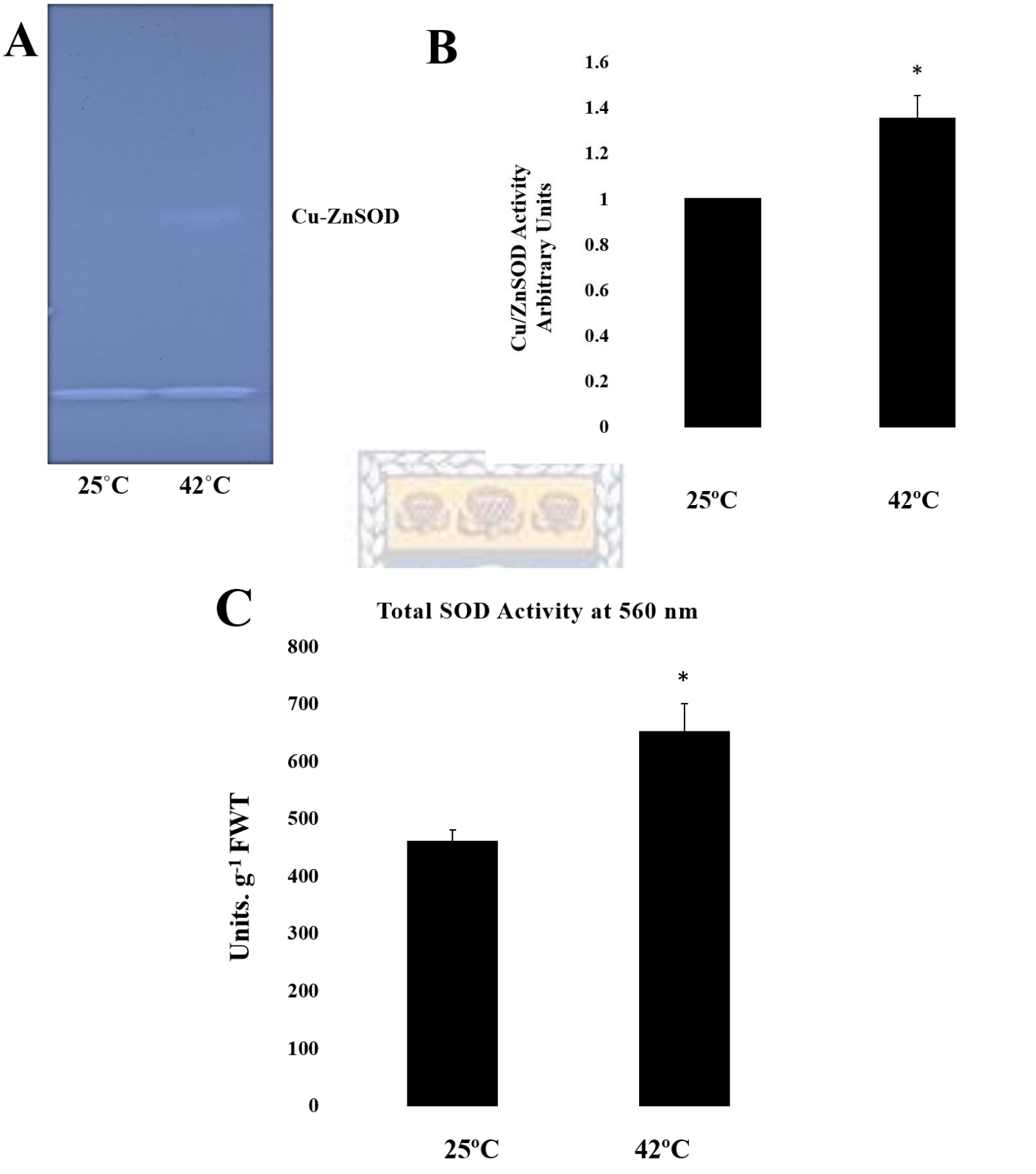


Figure 2. Effects of heat stress on the enzymatic activities of *Zea mays* leaf SOD isoforms represented as pixel intensities quantified from bands developed through Native-PAGE experiments. Illustrative gel (A), Cu-ZnSOD enzyme activity (B) and total SOD activity

in arbitrary units (C) as performed via spectrophotometric assay at 560nm in Units.g^{-1}.FWT. Error bars are indicative of standard error with significance accepted at P <0.05 (*) analyzed from 3-6 independent experiments.

4.2. Heat Stress Increases Hydrogen Peroxide and MDA contents in *Zea mays* leaves.

As shown by Figure 7A, total hydrogen peroxide content increased from 2 nanomoles per gram fresh weight to 4.02 nanomoles per gram fresh weight (P <0.01). Malondialdehyde content was also measures as an indicator of lipid peroxidation via the TBARS assay; heat stress was found to increase the amounts of MDA by ~1.2-fold (P< 0.1) when compared to non- heated controls indicating that there was more ROS-mediated lipidic oxidation

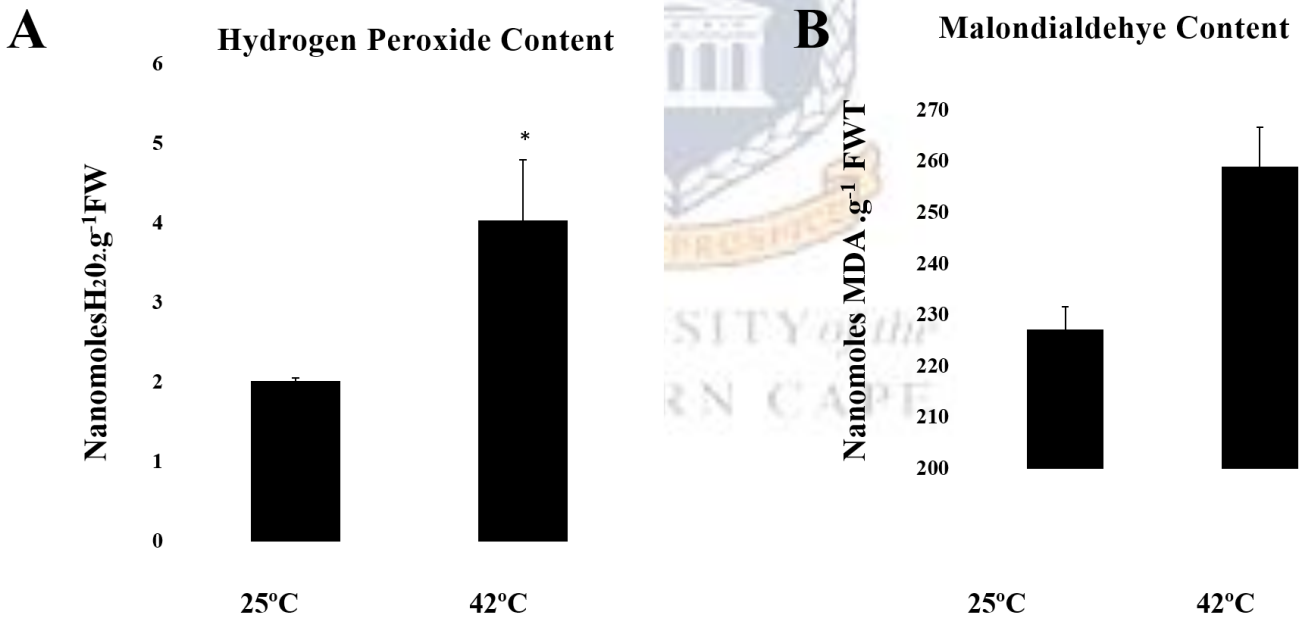
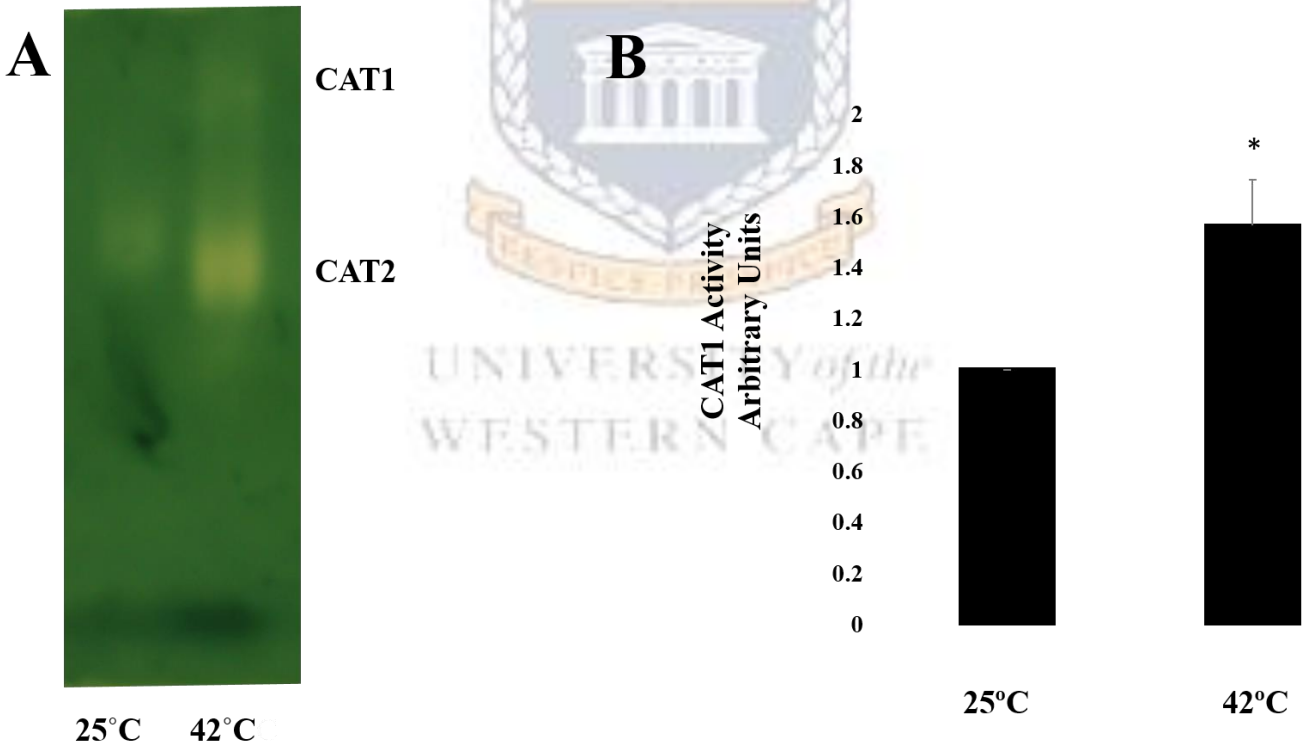


Figure 3. Effects of heat stress on H_2O_2 and MDA contents in *Zea mays* leaves performed via spectrophotometric assays. Hydrogen peroxide content (A) and malondialdehyde content (B) performed at 390nm and 532nm respectively. Error bars are indicative of standard error with significance accepted at P <0.05 (*) analyzed from 3-6 independent experiments.

4.3. Heat Stress Induces Total Catalase Activity and induces the activity of 2 CAT isoforms in leaves of *Zea mays.*

To determine the effects of heat stress on the activity of catalase activity, native-PAGE was performed; as shown by Figure 4.2.A, the representative gel showed the upregulation in the activity of 2 CAT isoforms. CAT1, which was not active in the 25ºC plants, was induced ~ 1.5-fold ($P < 0.001$) in response to heat stress at 42 ºC (Figure 4.2.B). Figure 4.2.C. is a representative graph depicting a ~2.5-fold increase ($P< 0.0001$) in the CAT2 isoform when compared to 25ºC controls. Spectrophotometric determination of total CAT activity (Figure 4.2.D) revealed that stress resulted in a 13% increase ($P < 0.001$) in enzymatic activity when compared to controls.



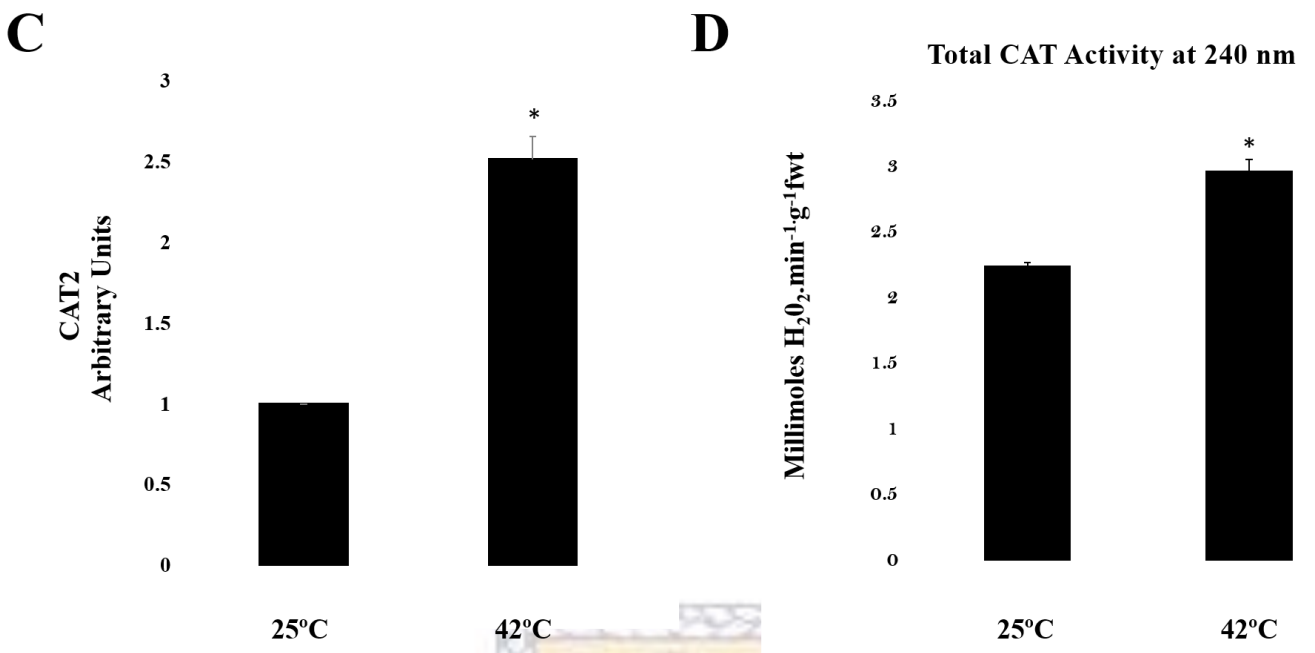


Figure 4. Effects of heat stress on the enzymatic activities of *Zea mays* leaf CAT isoforms represented as pixel intensities quantified from bands developed through Native-PAGE experiments. Illustrative gel (A), CAT1 enzyme activity (B), CAT2 enzyme activity (C) and total CAT activity (D) as performed via spectrophotometric assay at 240nm. Error bars are indicative of standard error with significance accepted at P <0.05 (*) analyzed from 3-6 independent experiments.

4.4. Heat Stress Induces Total Ascorbate Peroxidase Activity and induces the activity of 2 APX isoforms in leaves of *Zea mays.*

As shown by Figure 4.3.A, heat stress increased APX activity due to more intense staining of proteins in the 42 ºC lane. At least two isoforms of APX were seen to be active, these were quantified via densitometry in Figure 4.3.B & C, where APX1 activity was increase by ~2.3-fold (P < 0.5) and APX2 activity was also induced ~ 2.3-fold (P <0.001). Figure 4.3.D. where total APX activity at 290 nm was 13% higher (P <0.05) in heat stressed samples.

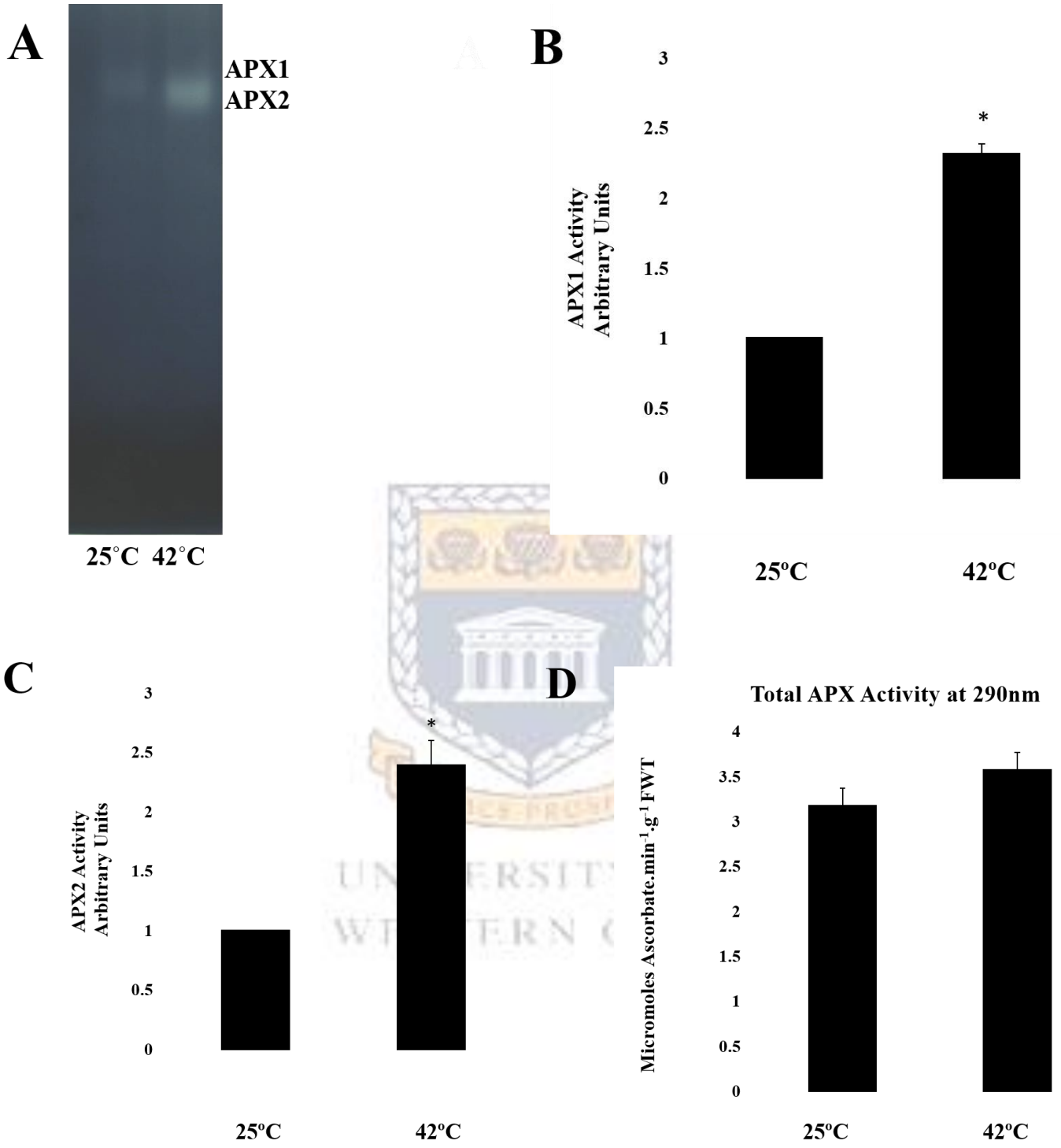


Figure 5. Effects of heat stress on the enzymatic activities of *Zea mays* leaf APX isoforms represented as pixel intensities quantified from bands developed through Native-PAGE experiments. Illustrative gel (A), APX1 enzyme activity (B), APX2 enzyme activity (C) and total APX activity (D) as performed via spectrophotometric assay at 290nm. Error bars are indicative of standard error with significance accepted at $P < 0.05$ (*) analyzed from 3-6 independent experiments.

4.5. Heat Stress Induces Total Glutathione Peroxidase Activity and induces the activity of 2 GPX isoforms in leaves of *Zea mays.*

Heat stress resulted in the upregulation of two GPX isoforms (Figure 4.4A-C): firstly GPX1 was induced ~1.4-fold ($P < 0.001$) whereas GPX2 activity was intensified ~1.5-fold ($P < 0.001$) when compared to their room temperature counterparts, as revealed by native-PAGE with specific staining for this enzyme. Spectrophotometric determination of total GPX activity at 340 nm (Figure 4.4D) showed that heat treatment resulted in a stimulation in total GPX activity from 5.2 to 6.2 ($P< 0.5$) millimoles of NADPH oxidized per gram fresh weight.

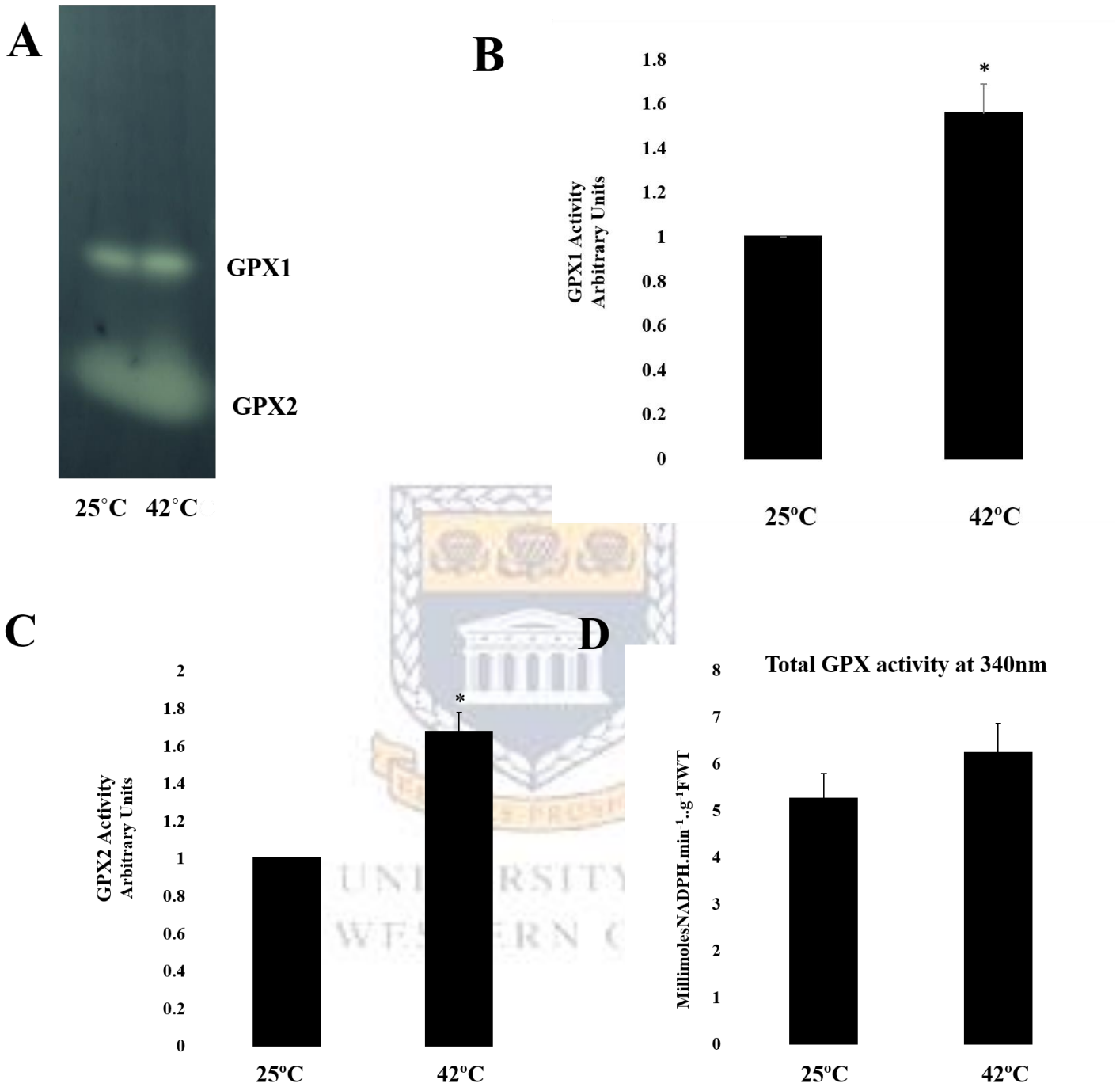
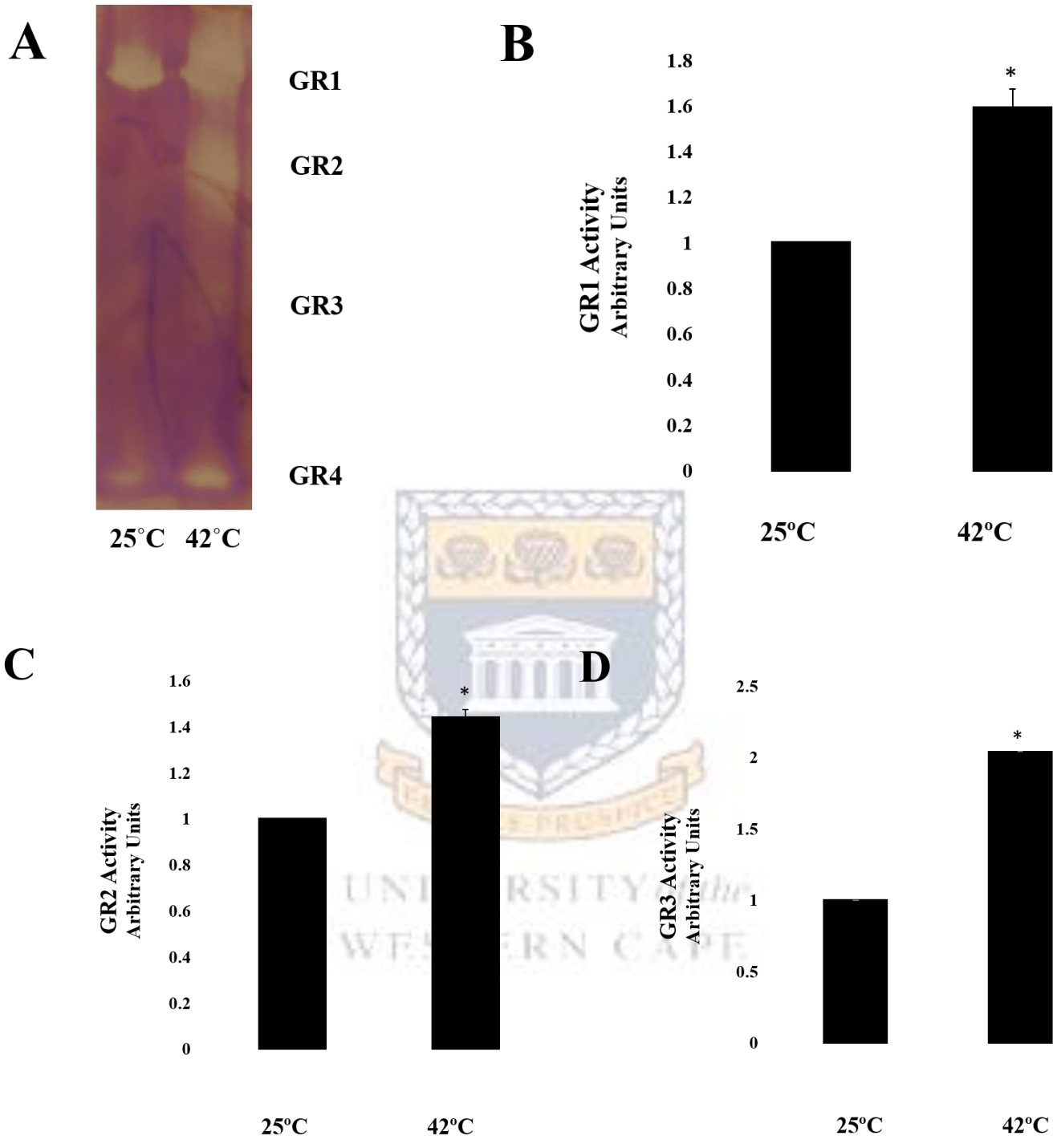


Figure 5. Effects of heat stress on the enzymatic activities of *Zea mays* leaf GPX isoforms represented as pixel intensities quantified from bands developed through Native-PAGE experiments. Illustrative gel (A), GPX1 enzyme activity (B), GPX2 enzyme activity (C) and total GPX activity (D) as performed via spectrophotometric assay at 340nm. Error bars are indicative of standard error with significance accepted at $P < 0.05$ (*) analyzed from 3-6 independent experiments.

Zea mays plants were exposed to heat stress at 42ºC for fives and analyzed for GPX activity via Native-PAGE and stained according to Lin, Chen, & Hou, 2002 (Figure 5A) and a spectrophotometric assay at 340 nm (Figure 5B). * $P < 0.05$ vs 25 ºC, n=3. Data presented as means **±** standard error; in the case of densitometry, data is normalized to 25ºC controls.

4.6. Heat Stress Induces the Activity of 4 GR isoforms and Total Glutathione Reductase activity in *Zea mays* leaves

Figure 4.5 indicates that heat stress resulted in a ~1.5-fold ($P < 0.1$) increase in total increase in the activity of the GR1 isoform furthermore 2 other GR isoforms namely GR2 and GR3 was more active by ~1.4-fold ($P < 0.01$) and 2-fold respectively ($P < 0.001$) when compared to 25˚C controls (Figure 4.5A-D). It appears that these two isoforms are largely activity in thermal responses as Native-PAGE analysis reveals a very low activity at room temperature. The GR4 isoform was induced ~1.4-fold ($P < 0.001$) in response to heat treatments while total GR increased by 11% ($P <0.2$) as indicated by the reduction of DTNB to TNB at 412 nm (Figure 4.5E).



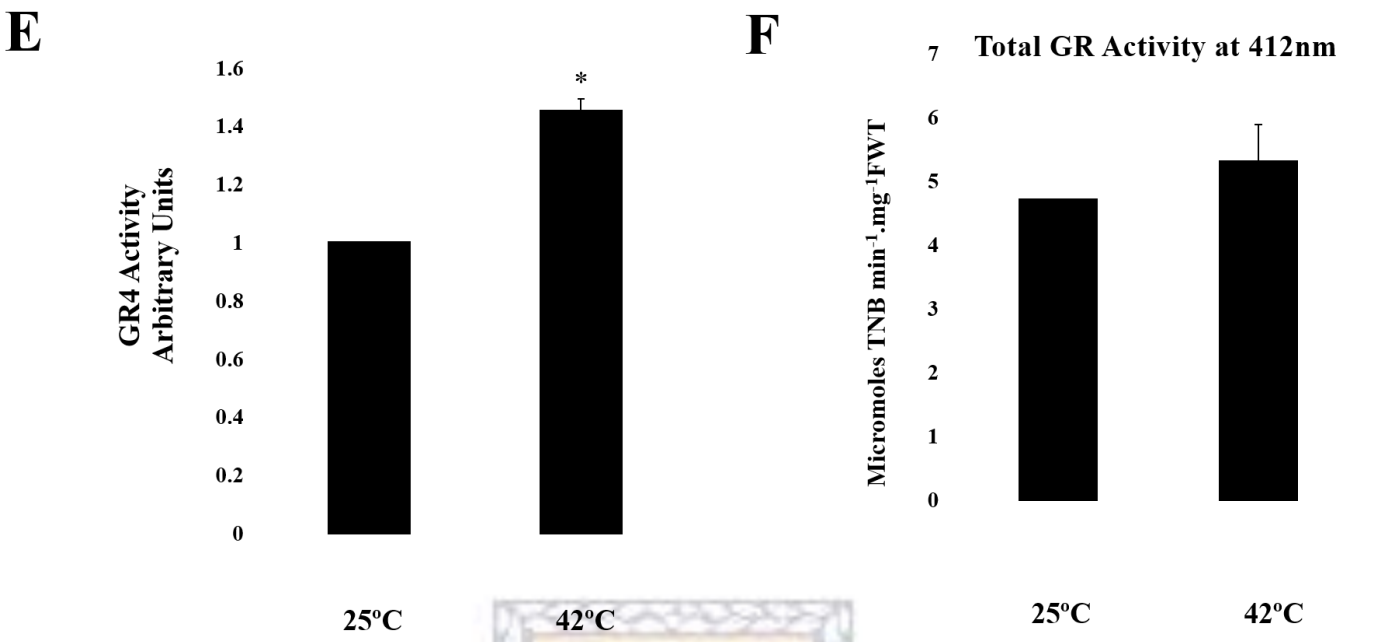
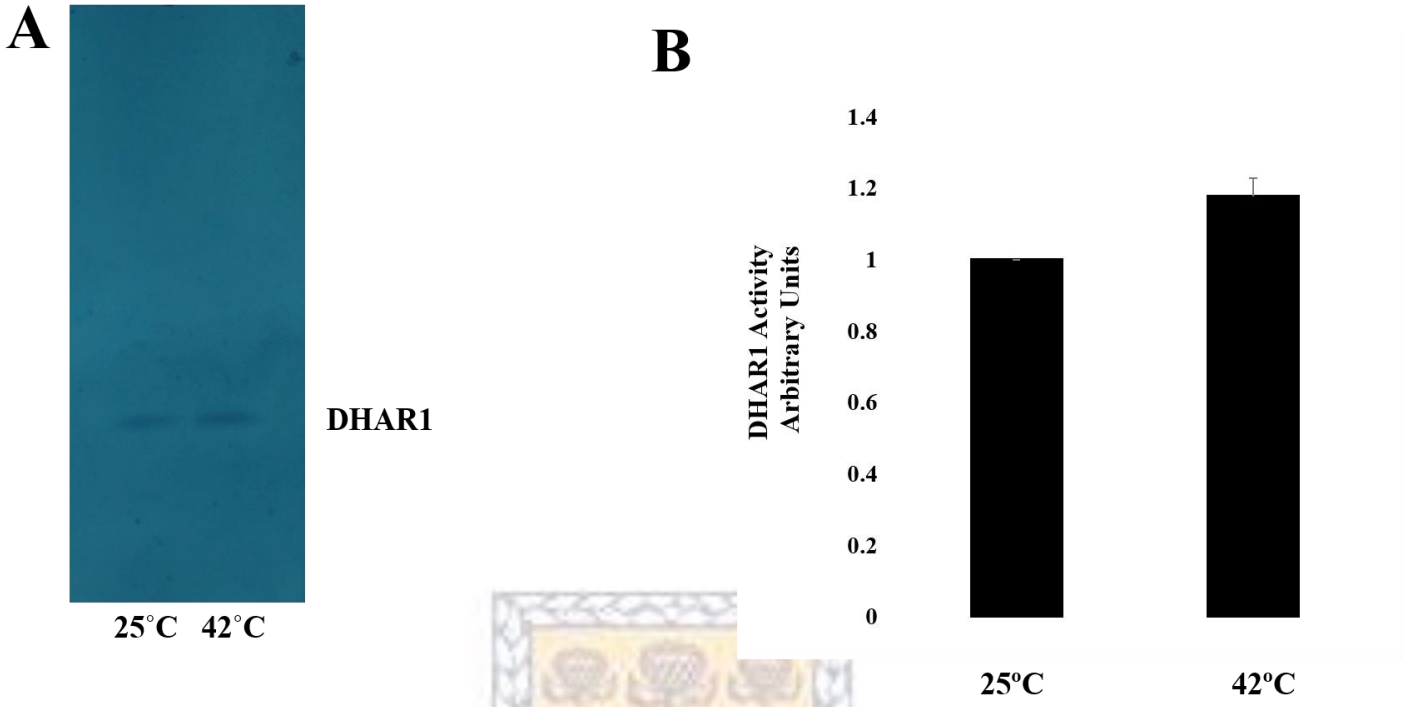


Figure 6. Effects of heat stress on the enzymatic activities of *Zea mays* leaf GR isoforms represented as pixel intensities quantified from bands developed through Native-PAGE experiments. Illustrative gel (A), GR1 enzyme activity (B), GR2 enzyme activity (C), GR3 enzyme activity (D), GR4 enzyme activity (E) and total GPX activity (F) as performed via spectrophotometric assay at 412nm. Error bars are indicative of standard error with significance accepted at P <0.05 (*) analyzed from 3-6 independent experiments.

4.7. Heat Stress Induces the Activity of DHAR and Total Dehydroascorbate Reductase activity in *Zea mays* leaves.

As revealed by native-PAGE (Figure 6A & B), one isoform of DHAR activity was found to be increased in responses to heat stress which presented with a ~1.2-fold (P <0.5) exacerbation when compared to 25ºC controls. The spectrophotometric assay also showed a 25% upregulation (P < 0.5) in DHAR activity as indicated by ascorbate formation at 265 nm (Figure 6C).



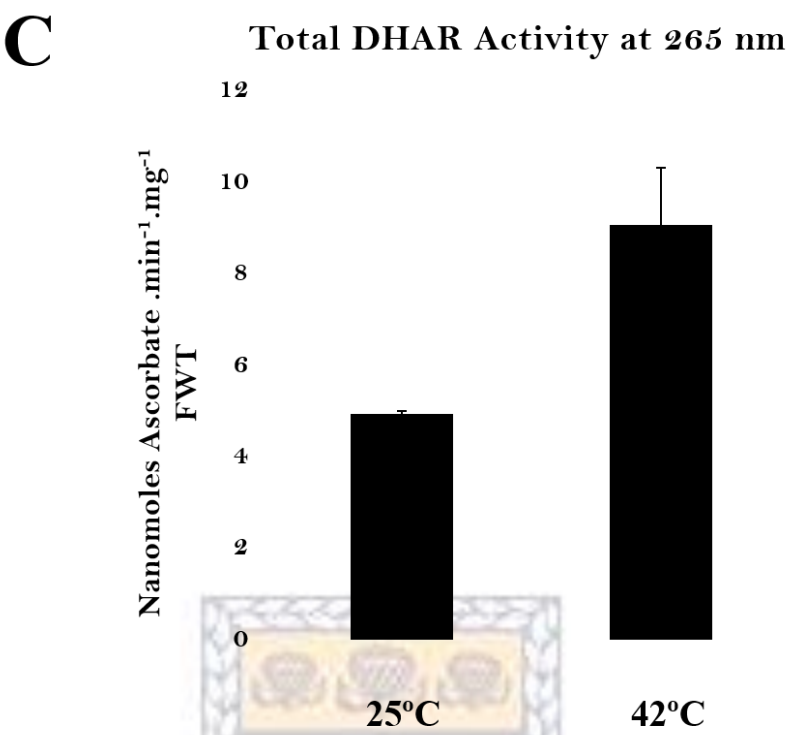


Figure 7. Effects of heat stress on the enzymatic activities of *Zea mays* leaf SOD isoforms represented as pixel intensities quantified from bands developed through Native-PAGE experiments. Illustrative gel (A), DHAR1 enzyme activity (B) and total DHAR activity (C) as performed via spectrophotometric assay at 265nm. Error bars are indicative of standard error with significance accepted at P <0.05 (*) analyzed from 3-6 independent experiments

Chapter Five

Discussion

This chapter serves to discuss the results of the study, which aimed to determine the effects of heat stress on several antioxidant enzymes in *Zea mays* leaves through the use of spectrophotometric assays and native polyacrylamide gel electrophoresis. This was achieved by subjecting maize plants to high temperature stress at 42ºC for 12 hours in a growth chamber for five days; plants grown at 25ºC served as controls. These plants were kept well-watered by daily replenishment of moisture-lost so as to not allow any water-deficit stress to compete with the heat stress treatments. The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), ascorbate peroxidase (APX), glutathione reductase (GR) and dehydroascorbate reductase (DHAR) were chosen for investigation as indicators of reactive oxygen species (ROS) defense, which play vital roles in plant viability in response to abiotic stresses such as high temperature stresses which addresses the larger aim of this thesis (Almeselmani, et al., 2006; Hasanuzzaman, et al., 2013).

5.1. High temperatures Stress Induces Total Superoxide Dismutase (SOD) Activity and Copper/Zinc-SOD in leaves of *Zea mays*.

Spectrophotometric determination of SOD activity in response to heat stress revealed a ~1.4-fold induction in total SOD activity (Figure 4.1C). This finding is in corroboration with earlier outputs in response to heat stress (X. Hu, et al., 2010; Kumar, et al., 2012). Although the measurement of leaf superoxide radical (O_2^-) content was not in the scope of this study, previous experiments by Djanaguiraman et al., 2010, have revealed that heat stress at 40ºC significantly ($P < 0.001$) and steadily induces the formation of O_2^- over a period of 28 days in *Glycine max* leaves (Djanaguiraman, et al., 2011). This phenomenon was further vindicated by Savicka et al., 2010, in *T.aestivum* seedlings which were exposed to 42ºC for 24 hours and presented with a ~3.3-fold increase in first leaf O_2^- content ($P < 0.01$) in early 4-day old seedlings and a ~1.65-fold increase in 7-day old seedlings after two

days (48hours) of heat stress. Increased levels of O_2^- content under heat stress is due downregulations in photosystem reaction centers that arise from increases in reactive oxygen species content (Grover, Sabat, & Mohanty, 1986). This results in the closing or blockage of the electron flow (Bibi, Oosterhuis, & Gonias, 2008) which is highly susceptible to heat or increased kinetic energy; as with all metabolic processes, thermal energy is at the heart of cellular biochemistry which validates the need for antioxidants both in enzymatic and non-enzymatic forms when cellular homeostasis, or in this scenario redox potential, remains elusive.

The term 'thermotolerance' describes the ability to withstand high temperature and in the case of plant antioxidant defense systems, the word is used to explain the protection that antioxidants offer in neutralizing harmful ROS species. In the case of SODs, they are deemed to be the first line of defense as they dismutate the primary superoxide radical to hydrogen peroxide and molecular oxygen.(McCord & Fridovich, 1969) Figure 4.1A and B shows, heat stress results in the up-regulation in a Cu-ZnSOD species. Copper-Zn SODs are mainly found both in the cytosol (Ogawa, et al., 1996) and the stromal flank of the thylakoidal membrane in close vicinity to photosystem one (Ogawa, et al., 1995). A comprehensive study by Tsang et al., 1991, which aimed to measure SOD transcript abundance using RNA gel blotting analysis, incorporating an isolated cDNA cytosolic Cu-Zn SOD clone, together with a previously isolated mitochondrial Mn-SOD and chloroplastic Fe-SOD, in response to heat stress in *Nicotania plumbaginifolia* leaves clearly indicated that Cu-ZnSODs are the most sensitive to thermal discrepancies; a response that was independent of light stress. Their data showed a 10-fold induction Cu-$ZnSOD_{cyt}$ mRNA in response to a heat stress regimen that exposed seedlings to 37 ºC for 5 hours. This group also subjected plants to a cold temperature which revealed that Cu-$ZnSOD_{cyt}$ was also induced 10-fold after 24 hours at 4ºC (Tsang, et al., 1991). From these experiments it appears that the finding of the study, is partially supported by those in Tsang et al., 1991, in that Cu-ZnSOD is the chief isoenzyme involved in mechanisms relating to thermotolerance and draws attention to the cytosol which may represent the principal source of superoxide radical formation under conditions of high and low temperature stress.

Copper-ZnSODs are not confined to the cytosol as studies have shown that there exists a soluble variant in the stroma (Ogawa, et al., 1995) as well the peroxisome (Sandalio & Del Río, 1987). To address this, Khanna-

Chopra and Sabarinath, 2004, performed experiments in the weed species *Chenopodium murale* that aimed to characterize the thermostability of SOD isoenzymes in respone to a high temperatures. Their experiments, which exposed thylakoidal and stromal protein fractions to temperatures from 50˚C to boiling, showed the presence of six SOD isoforms. SODV and SOD VI was later identified as a stromal and thylakoidal Cu-ZnSOD respectively and displayed catalysis under extreme temperatures. The stromal Cu-ZnSOD showed activity even after 10 minutes of boiling as revealed by Native-Page whereas the thylakoidal version which migrated fairly the same distance as the stromal Cu-ZnSOD was still active at 80˚C (Khanna-Chopra & Sabarinath, 2004). This study offers new insight into the indelibility of the chloroplastic Cu-ZnSODs in response to extreme temperatures as the results of this study may seem to border on fallacy; one must remember the materialization and the evolution of the Cu-ZnSODs and its uniqueness to those to the rest of the SOD family i.e. the replenishment of oxygen in the earth's atmosphere with subsequent reduction in iron atom valency and most importantly, the incorporation of copper and other transitional metal atoms into active centers of enzymes (Bannister, et al., 1991).

5.2. Heat Stress results in increased Hydrogen Peroxide (H_2O_2) and Malondialdehyde (MDA) content in *Zea mays* leaves.

As catalysis by SODs results in the formation of H_2O_2; we measured the concentration of this product according to Velikova et al., 2000 at 390 nm and witnessed a significant increase in peroxide content in response to heat stress. This finding is supported by previous outputs (Kumar, et al., 2012; Q. Shi, et al., 2006) which also displayed increase activity in SOD in response to heat stress therefore it is not wholly surprising that H_2O_2 content increases as a product of the superoxide radical dismutation reaction, however there are other scenarios in which H_2O_2 can be synthesized as is in the case of the NADPH oxidase and the lack of peroxidase activity, the latter which holds untrue in this study and will be explained in the next section.

The fate of hydrogen peroxide in cells appears to be manifold; firstly it is a powerful oxidizing agent with a relatively long half-life (one millisecond) when compared to other ROS species. Secondly due its ability to traverse through membranes via water channels known as aquaporins (Bienert, et al., 2006), adds to its lethality

in that it has the ability to inflict oxidative damage to biomolecules far from its site of generation. Thirdly, recent data has shown that H_2O_2 is a powerful signaling molecule modulating the expression of 1-2% of genes in *Arabidopsis* as revealed by microarray studies. Most of these genes were potentially involved in apoptosis (Swidzinski, Sweetlove, & Leaver, 2002) however this study also found increased expression of genes involved in calcium signaling such as calmodulin (T. Yang & Poovaiah, 2002), mitogen activated protein kinases (MAPKs) (Ren, Yang, & Zhang, 2002) and expression of transcription factors. Thus the presence of H_2O_2, its accumulation and its culmination of effects intracellularly are complex, its higher content in the 42˚C treated plants however, it does convey the message that that these plants were under oxidative stress.

As oxidative stress is known to result in damage to lipids, we measured levels of malondialdehyde (MDA) which is a final product in the peroxidation of polyunsaturated fatty acids in cells (Gaweł, Wardas, Niedworok, & Wardas, 2003). Our results show that heat stress result in an increase in MDA content which is consistent with other authors (Gong, Li, & Chen, 1998; Xu, et al., 2006) however the fold increases that were observed in our data was much less than in other reports. For example, Gong et al., 1998, exposed maize seedlings to 46˚C for 2 days are presented lipid peroxidation data showed a 2-fold increase in MDA content when compared to controls at 28˚C. In another study by Xu et al.., 2006, in cool season turf grasses, heat stress at 42˚C resulted in a 8-fold surge in MDA content (Xu, et al., 2006) whilst Song et al., 2006, exposed mustard seedlings to heat stress at 45˚C resulted in a 2-fold gain in MDA content. Our MDA content data show only a 1.1-fold increase in MDA content from 225 to 255 nanomoles MDA per gram of tissue indicating that there was indeed an increase in oxidative damage however there may have also been efficient ROS quenching by antioxidant enzymes that may have aided plant damage; this is discussed in the following section.

5.3. Heat Stress Increased total Catalase Activity and induces the activity of 2 CAT isoforms in leaves of *Zea mays.*

In response to heat stress, total CAT activity, which was determined spectrophotometrically at 240 nm, increased significantly ($P < 0.05$) from 2.2- to 2.9 mmols $H_2O_2.min^{-1}$.gram fresh weight (Figure 2D) when compared to 25˚C

controls. This is consistent with other reports where CAT activity was evaluated in under heat stress (W. Shi, et al., 2001; Yin, et al., 2008). Native-PAGE analysis for CAT activity revealed two isoforms that were upregulated in response to heat stress: A CAT1 isoform which was more active by ~1.6-fold and the CAT2 isoform that was heavily induced by ~2.5-fold when compared to room temperature control plants which only presented with activity of the CAT2 isoform. Out of all the antioxidant isoform analyses presented in this thesis, the fold-upregulation of the CAT2 isoform is the largest in response to heat stress.

In maize, there exits three well-characterized CAT isoenzymes that are encoded by three unlinked nuclear genes; however in etiolated leaves only two are present i.e. CAT1 and CAT3 that migrate at distinct positions when separated by electrophoresis. Catalase-3, which is found exclusively in mitochondrial fractions (Scandalios, et al., 1980), is probably 'CAT1' (represented by Figure 4.3A) , as the relative mobility of this isoform is congruent with zymograms and Native-PAGE gels that have been probed for CAT activity in previous reports (Chandlee & Scandalios, 1984; Prasad, 1997; SCANDALIOS, 1974). The upregulation of CAT3 in response to heat treatment is significant in that it alludes to a thermotolerance mechanism in *Zea mays* as the activity of this isoform was not received in room temperature plants. Biochemically, CAT3 is unique from its counterparts in that it presents with a much higher peroxidatic activity than CAT-2, approximately 70-fold (Havir & McHale, 1989). As stipulated earlier, the largest fold change in antioxidant activity in response to heat stress was the ~2.5-fold induction in CAT2 activity. This isoform possibly represents the maize CAT1 isoform that is chiefly found in peroxisomes where it converts glyoxysomal H_2O_2 to less toxic intermediaries. Several studies have measured the effects of heat stress in maize however these experiments were performed in maize seedlings, and limited to the scutellum which present with different isoforms to that of leaves. Nevertheless, scutella that were incubated at 35˚ and 40˚C for 24 hours presented with an increase in both CAT1 and CAT2 mRNA and protein (Matters & Scandalios, 1986). Contradictory results were found in *Nicotania plumbaginifolia* were *Cat1* and *Cat2* transcripts diminished following a 5-hour exposure of high temperature (37˚C). Upon return to 22˚C, transcript levels of both isoforms returned to control levels; interestingly, *Cat3* mRNA levels were unaffected by heat stress suggesting that CAT3 is the most thermostable isoform (Willekens, et al., 1994). It appears that acute heat stress alters CAT mRNA

transcript levels differently when compared to chronic heat treatment. To our knowledge, this is the first study, in *Zea mays*, that displays data showing the changes in CAT isoforms in response to a chronic heat stress regimen as and more importantly, in leaves.

5.4. Heat Stress Induces Total Ascorbate Peroxidase Activity and induces the activity of 2 APX isoforms in leaves of *Zea mays.*

In response to heat stress, APX activity measured spectrophotometrically at 290 nm increased significantly from 3.17- to 3.57 µmoles ascorbate.min^{-1}.g^{-1} fresh weight ($P < 0.05$). This was supported by Native-PAGE analysis and previous co-workers (Chakraborty & Pradhan, 2011; Kumar, et al., 2012; W. Shi, et al., 2001) where there was an overall increase in APX band intensity and the upregulation of an APX2 isoform that was not evident in the control room temperature samples. The fold increases of these isoforms were harmonious to that of the CAT isoforms in that there was a large ~2-fold increase in protein activity suggesting increases in hydrogen peroxide content indicative of oxidative imbalance due to high temperature.

Ascorbate peroxidase isoenzymes have been identified throughout the cell, however concerning its activity pattern in response to heat stress, Song et al., 2005, reported a heavy induction in cytosolic, stromal and microbody APX isoforms in cucumber plants that were exposed to temperatures of 40˚C for 4 days. This group also measured the response of a thylakoidal APX isoform which was not affected by heat treatment however their data showed that the stromal APX isoform was the most responsive to heat stress not only at the catalytic level but also at transcriptionally as revealed through northern blotting (Song, et al., 2005). This was also mirrored by Shi et al., 2001, who cloned a peroxisomal ascorbate peroxidase from *Hordeum vulgare* (barley) denoted *HvAPX1* and overexpressed it *Arabidopsis* under the control of the RNA 35S promoter of the cauliflower mosaic virus. Upon heat stress treatment, this group reported a ~2-fold increase in transcript levels thus corroborating the findings of Song et al., 2001(W. Shi, et al., 2001). In similar experiments, Tang et al., 2006, developed transgenic potato plants in which both Cu-ZnSOD and APX were expressed in chloroplasts and driven by the *SWPA2* promoter which is induced by oxidative stress in the form of methylviologen treatment (MV). Under conditions of high

temperature (42°C). Reverse transcriptase-polymerase chain reaction (RT-PCR) performed in both control and heat stressed plants for the expression levels for Cu-ZnSOD and APX showed that these enzymes were induced in response to MV when grown at 42°C (Tang, et al., 2006).

The work presented by Tang et al., 2006, does support the findings of this thesis in that Cu-ZnSOD was also upregulated in response to heat stress at 42˚C Unfortunately, isoforms of APX are not as well characterized to those of CAT in *Zea mays*; the APX isoforms upregulated in response to heat stress as presented in this study does offer some insight regarding the recruitment of this antioxidant under heat stress. Interestingly the APX2 isoform as represented by Figure 4.4A, does migrate slightly lower than APX1. It is well known that membrane bound APXs such as the thylakoidal APX present with a ~4.5 kDa domain that is essential for membrane binding, therefore it could be speculated that APX1 is a membrane-bound enzyme and APX2 is either cytosolic or stromal.

5.5 .Heat Stress Induces the Activity of 2 GPX isoforms and Total Glutathione Peroxidase activity in *Zea mays* leaves.

Heat stress resulted in a total ~1.2-fold increase in GPX activity when measured spectrophotometrically at 340 nm; this is consistent with previous reports (Hasanuzzaman, Nahar, Alam, & Fujita, 2012; Q. Shi, et al., 2006). Native-Page analyses revealed a ~1.4-fold induction in GPX1 activity and a ~1.5-fold increase in GPX2 activity, both of which were significant ($P < 0.05$). These increases were not as stark as those of APX and CAT however, they do suggest an increase in H_2O_2 scavenging ability. Thus, it appears that all three peroxidatic scavengers were recruited under heat stress. Although much classification of GPX enzymes have been made in animal cells, studies have revealed their presence in plants such as is in the case of data presented by Sabeh et al., 1992, where a 16 kDa tetrameric protein was found to exist in *Aloe vera* (Sabeh, et al., 1992). Nevertheless, in animal cells, four distinct groups of GPXs exist : these are the typical and cytosolic GPX (Rotruck et al., 1973), the isoform located in the extracellular fluid (Chu, Doroshow, & Esworthy, 1993), a cytosolic variant found in gastrointestinal cells (Chu, et al., 1993) and the phospholipid hydroperoxide GPX (PHGPX)

PHGPX is of particular significance as genes bearing striking similarity have been found in *Citrus sinesis*, *Nicotania sylvestris*, *Arabidopsis thaliana*, *Brassica campestrus*, *Oryza sativa* and *Avena fatua* (Criqui et al., 1992; Holland et al., 1993; Johnson, Cranston, Chaverra, & Dyer, 1995). These proteins are named as such as they have the ability to reduce lipid hydroperoxides. Furthermore, it has been proposed by Eshdat et al., 1997 that predominately APX and CAT catalyze the formation of H_2O_2 to O_2 and water, whereas PHGPX, a protein that has very low molecular weight of around 25 kDa, has an essential role in minimizing the lipid peroxidation chain reaction mediated by the hydroxyl radical through the non-enzymatic reduction of H_2O_2 via the Fenton reaction (Eshdat, Holland, Faltin, & Ben-Hayyim, 1997; Piette, 1979).

Using molecular cloning techniques, Sugimoto et al., 1997, cloned and sequenced a cDNA encoding a *PHGPX* from spinach that encoded a 171 amino acid peptide which shared 77% sequence homology to previous putative plant PHGPXs. More importantly, using primers that were synthesized from the citrus and tobacco sequences in PCR reactions, Sugimoto et al., 1999, revealed same-size products when maize DNA was included the reaction (Sugimoto, Furui, & Suzuki, 1997). Furthermore, Avsian-Kretchmer et al., 1999, published data showing that both PHPGPX and the *csa* gene encoding the protein was heavily upregulated by heat stress at 37˚C in *Citrus* (Avsian-Kretchmer, Eshdat, Gueta-Dahan, & Ben-Hayyim, 1999) . These findings suggests that *Zea mays* does possess the PHGPX protein which is also induced in heat stress both in terms of content and catalytic activity; therefore the possibility of its antioxidant capacity through the denouement of lipid peroxidation, its specific role as proposed by Eshdat et al., 1997, is justified : It is plausible that the high-migrating GPX2 isoform that is represented by Figure 5.4A is a maize PHGPX, as its relative migration is typical of a 25 kDa protein, and its upregulation under heat stress may have resulted in minor increases in MDA levels or lipid peroxidation as respresented by Figure 4.2A in the 42˚C treated plants. All peroxidatic activity measured in this study especially that of APX and CAT have revealed large fold-differences when subjected to heat stress which suggests that H_2O_2 was detoxified adequately and possible limited hydroxyl radical formation via the Fenton reaction(Piette, 1979). It is possible that the GPX1 isoform that was also induced in response to heat stress is involved in H_2O_2 subjugation, whereas the GPX2 is more involved in lipid peroxidation avoidance, which our MDA data suggests.

5.6. Heat Stress Induces the Activity of 4 GR isoforms and Total Glutathione Reductase activity in *Zea mays* leaves.

Heat stress resulted in the induction of total GR activity as measured spectrophotometrically at 412 nm which is in accordance with (Almeselmani, et al., 2006; Chakraborty & Pradhan, 2011; J. Dat et al., 2000). Native-PAGE analysis revealed an upregulation in the activity of 4 isoforms, 3 of which were heavily induced when compared to room temperature controls. The slow migrating GR1 isoform which was present in both treatments revealed a ~1.5-fold upregulation in response to heat stress. Multiple studies have measured the response of total GR activity in response to however very few have analyzed the respective changes in isoform pattern in *Zea mays*, and more specifically in response to heat stress Nevertheless, Pinhero et al., 1997, measured the change antioxidant enzyme isoform pattern in response to paclobutrazol induced chilling in maize leaves and reported three main GR isoforms when subjected to the Native-Page technique. Their results revealed three distinct isoforms that mirror GR1, GR2 and GR3 presented in Figure 4.6A (Pinhero, Rao, Paliyath, Murr, & Fletcher, 1997). Also, in mesocotyls of maize seedlings in response to chilling stress, Anderson et al., 1995, measured the isoenzyme pattern in GR and reported three cytosolic, one mitochondrial and one plastidic isoform in *Zea mays* as revealed by Native-page analyses performed on different cell fractionations achieved through differential centrifugation. The slow-migrating GR1 isoform was apparent in the plastidal fraction which also revealed a fast migrating isoform that appears to migrate at the same distance as GR4. However, Anderson and co-workers denounced this isoform as the band indicating this isoform stained at equal intensity when oxidized glutathione was negated from the incubation buffer. Therefore GR4 may not be a true glutathione reductase however this remains to be clarified. The three remaining isoforms GR1, GR2 and GR3 appears to be the plastidal, chloroplastic and mitochondrial respectively as subordinated by Anderson and coworkers and to Pinhero et al., 1997 in *Zea mays*.

Irrespective of where these enzymes occur, the message they infer regarding GR activity in response to heat stress is the upregulation in enzyme activity. Glutathione reductase is responsible for reducing oxidized glutathione (GSSG) back into its antioxidant form (GSH) and therefore is crucial in maintaining a high GSH: GSSG ratios (Edwards, Enard, Creissen, & Mullineaux, 1993). Therefore the upregulation seen in GR activity is significant

in terms of thermotolerance. Although we did not measure total GSH or GSSG levels previous studies have revealed that heats stress increases total GSH content (Kumar, et al., 2012; Yin, et al., 2008) whereas others have reported contradictory results (J. F. Dat, et al., 1998; Xu, et al., 2006). Furthermore, GPX, and enzyme that requires GSH as a reducing equivalent was found to be upregulated as presented in Figure 4.5. It appears that the upregulation in GR activity especially those of GR2 and GR3 which were not active in the control plants may have assisted in maintaining glutathione in its reduced state that may have allowed the increases in GPX activity we have observed with subsequent decrease of lipid peroxidation or MDA which has been previously speculated may be the function of GPX2, a possible PHGPX (McCay, Gibson, Kuo-Lan, & Roger, 1976; Thomas, Maiorino, Ursini, & Girotti, 1990).

5.7. Heat Stress Induces the Activity of DHAR and Total Dehydroascorbate Reductase activity in *Zea mays* leaves.

Spectrophotometric determination of total DHAR activity in response to heat stress revealed a ~1.6-fold induction when compared to room temperature controls, this finding is corroborated via previous studies (J. F. Dat, et al., 1998; Q. Shi, et al., 2006). Native-Page analyses with subsequent staining for DHAR activity also revealed a ~1.1-fold upregulation of a single DHAR1 isoform in response to heat stress; this isoform was present in both control and stressed plants. In maize leaves, as revealed by Zhang et al., 2015 there are to date, four possible DHAR isoforms all with a relative molecular weight of ~28 kDa therefore the position at which this enzyme migrates in a 7.5% acrylamide gel, as revealed by Figure 4.7A is typical of that of a low molecular weight protein. In terms of expression level, their RT-PCR data revealed that *ZmDHAR1* and *ZmDHAR2* are the most expressed in leaf tissue; furthermore, subcellular localization studies showed that *ZmDHAR1 and ZmDHAR3* are cytosolic while *ZmDHAR2* is chloroplastic and *ZmDHAR4* is present in vacuoles. Zhang et al., 2015, also measured the effects of temperature on each of the four DHAR isoforms that were expressed and purified: they reported that *Zea mays* enzymes showed wide optimal temperature ranges. Upon further analysis of their enzymatic data which was measured spectrophotometrically at 265 nm, the same protocol used in this study (Hossain & Asada, 1984), it appears that at 25˚C, our control temperature, ZmDHAR1 and ZMDHAR4 exhibited the highest residual

activity, however at 42˚C the activities of ZmDHAR3 decreases linearly while the activity ZmDHAR2 is activated exponentially. The activities of ZmDHAR1 peaks at 35 ˚C while the optimum temperature for ZmDHAR4 is 45 ˚C and thereafter decreases linearly (Y.-J. Zhang et al., 2015). These experiments reveal details about the thermotolerance of these enzymes however, in an *in vitro* scenario it does offer insight into the upregulation of the enzyme activity as presented in Figure 4.7.

What our data does suggests is that there was an attempt by the heat stressed plants to increase the content of AsA as revealed by the increase in DHAR activity. In addition, the measurement of APX activity, which was upregulated in response to heat stress, which is highly sensitive to availability of AsA, could have only been induced if its electron donor was available at sufficient concentrations. Therefore, it appears that heat-stress induced DHAR activity may have played a positive role in allowing AsA levels to be maintained therefore allowing efficient removal of H_2O_2, an occurrence that may also hold true for GPX and the contents of GSH.

Chapter Six

Conclusions and Future Research

The aim of this thesis was to determine the effects of high temperatures stress on the antioxidant machinery or enzymes in *Zea mays.* Antioxidant enzymes are vital components in maintaining cellular redox balance when concentrations of reactive oxygen species (ROS) reach toxic concentrations- a consequence of heat stress in plants (Mittler, 2002; Potters, Pasternak, Guisez, Palme, & Jansen, 2007). All six of the enzymes i.e. Superoxide dismutase (SOD), Catalase (CAT), Glutathione Peroxidase (GPX), Ascorbate Peroxidase (APX), Glutathione Reductase (GR) and Dehydroascorbate Reductase (DHAR) chosen for measurement in this study were induced in response to heat stress at 42˚C when compared to control plants grown at 25˚C when measured spectrophotometrically. These findings are not original as various other studies have reported such occurrences (Chakraborty & Pradhan, 2011; J. Dat, et al., 2000; Djanaguiraman, et al., 2011; X. Hu, et al., 2010; Y. X. Hu, Wang, Liu, & LI, 2004; Kochhar & Kochhar, 2005; Kumar, et al., 2012; Savicka & Škute, 2010; Q. Shi, et al., 2006; Xu, et al., 2006; Yin, et al., 2008). Spectrophotometric determination of the six enzymes were further analyzed through the technique of Native-Page to separate the various isoforms that make up the total antioxidant activities. These results offer new insights and are summarized below:

With respect to SOD activity, our data, in corroboration with previous studies (Perl et al., 1993; Tsang, et al., 1991), revealed the upregulation of a Cu-ZnSOD which appears to be highly involved in thermotolerance mechanisms. Multiple studies that have transgenically expressed this SOD species in tomato, tobacco and tall fescue have reported enhanced tolerance to paraquat and methylviologen (Gupta, Heinen, Holaday, Burke, & Allen, 1993; Lee et al., 2007; Perl, et al., 1993). In addition Tang et al., 2006, reported that in potato, overexpression of SOD displayed enhanced tolerance in chloroplasts in response to heat stress (Tang, et al., 2006).

The largest fold differences or activation of enzyme activity in response to heat stress was that of the CAT2 isoform which may represent the well-characterized peroxisomal CAT1 isoform in *Zea* mays (Scandalios, Guan, & Polidoros, 1997). Also, the upregulation of the CAT1 and its recruitment in response to heat stress which was not present in the room temperatures controls is of particular significance as it suggests that this isoform, which may be the CAT3 mitochondrial (Scandalios, et al., 1980) isoform and there reflects a thermotolerance mechanism or a thermal induction aspect of this isoform in maize.

Native-Page analyses for APX activity revealed the upregulation an isoform in response to heat stress that was not active in the room temperature controls. This isoform (APX2) also migrated a slightly faster than the APX1 isoform and therefore might allude to the upregulation of a stromal, matrix or cytosolic isoform that is not induced when compared to its membrane-bound counterpart. This is based on the realization that membrane-embedded isoforms possess a 4.5 kDa peptide that is not found in the free form. Our investigation into the effects of heat stress on GPX activity revealed the upregulation of two GPX isoforms both which were present in both heat-treated and control plants. The GPX2 isoform could be of significance in that it appeared to migrate much faster than the GPX1 isoform allowing for speculation that this isoform may be a phospholipid hydroperoxide GPX (PHGPX), as the molecular weight of these enzymes are known to be low. Furthermore, measurement of MDA content, a measure of lipid oxidation caused by ROS species did not differ significantly in the heat treated plants. As certain GPX isoforms are known to reduce phospholipid hydroperoxides specifically, it is highly possible that GPX2 has this ability.

All three hydrogen peroxide (H_2O_2) scavengers were upregulated in response to heat stress; in addition, H_2O_2 content which was also determined did increase significantly in the heat-treated plants when compared to room temperature controls however as mentioned previously, our MDA data suggested that lipid peroxidation was not as severe. Lipid peroxidation mainly occurs by the voracious, short-lived hydroxyl radical that is produced via a reaction involving H_2O_2 and iron in the Fenton reaction. Therefore, it appears that either lipid peroxidation was diminished in the heat-treated plants which implicates the GPX isoforms. However adequate conversion of H_2O_2

to water and molecular oxygen may have also occurred as large-fold increases which may have mediated by APX and CAT.

As GPX uses glutathione (GSH) as an electron donor, thus highlighting its importance in maintaining redox balance, we measured the conversion of the oxidized form (GSSG) to GSH via Native-Page analyses for GR. Heat stress resulted in the upregulation of 2 isoforms that were not exclusively active in control plants suggesting recruitment of other isoforms that are involved in thermotolerance mechanisms. These isoforms, when compared to other studies that have also measured GR activity is possibly found in either the cytoplasm or mitochondria. Nevertheless, our data shows that GSH availability was adequate in that, GPX activity was induced in response to heat stress.

We also measured the effects of heat stress on DHAR activity to determine whether ascorbate renewal was affected by high temperature. Only one DHAR isoform was identified which was upregulated in the heat–stressed plants. As the availability of ascorbate is of utmost importance in APX catalysis we can deduce that there was an increase in AsA regeneration in the heat-stress plants possibly due to increase in APX activity

Future Work

Firstly, as we have shown that heat stress induces the activity of certain antioxidant isoforms that appear to be involved in thermotolerance mechanisms, it would be wise to determine whether these upregulations in catalysis are due to increases in protein content or whether there are actual post-translational modifications such as phosphorylation of enzymes, which are known to be effectors of catalytic activity. To clarify this, immunoblotting for the different isoforms could be performed. For example, our data revealed that a Cu-ZnSOD was induced in response to heat stress; this isoform could have been upregulated due increase gene activity and therefore protein content. Furthermore, it has been confirmed in previous studies that this isoform is present in both the chloroplast and the cytosol therefore western blots for these proteins and preferably on plant tissues that have been separated

via differential centrifugation according to the subcellular fractions or in different plant organs will reveal exactly which SOD is induced in response to high temperature. These experiments should not only be performed using antibodies directed for SOD but for all six enzymes chosen for investigation as there are antibodies already available for CAT, APX, GPX, GR and DHAR. For isoforms that immunoblotting fail to give results, the use of mass spectrometry could be used to determine these protein sequences from which primers could be synthesized to determine gene expression at the mRNA level using either semi-quantitative PCR or real-time analysis techniques. These experiments would address the effects of heat stress on antioxidant gene expression which would lead onto clarifying whether there are any post-translational modifications made by other enzymes that may enhance or switch on enzyme catalysis. As heat stress is known to alter cell signaling pathways especially calcium-sensitive signaling (Mach, 2012) that lead to the activation of kinases which phosphorylate enzymes, experiments involving the blockage of these pathways (using chemical antagonists) could also provide information regarding catalytic activity.

Heat-stress has been shown to drastically induce the expression of heat shock factors (HSFs) which have been shown to be critical in heat tolerance. Heat shock protein 70 (HSP70) has been identified as a key facilitator of thermotolerance in plants as it higher expression in heat-treated plants results in the assistance in translocation, protein aggregation and folding, refolding of denatured proteins and proteolysis (Gorantla et al., 2007; Gurley, 2000). Experiments involving the measurement of HSP70 or other HSFs could be performed to determine its role in thermotolerance on antioxidant activities in maize in response to heat stress.

Finally, to fully elucidate the thermotolerance effects of enhanced antioxidant activity expression in response to heat stress, transgenic plants could be created using molecular cloning techniques that overexpress these proteins with subsequent transformation not only in maize but other major food crops.

Chapter Seven

References

Adams, S., Cockshull, K., & Cave, C. (2001). Effect of temperature on the growth and development of tomato fruits. *Annals of Botany, 88*(5), 869-877.

Almeselmani, M., Deshmukh, P., Sairam, R., Kushwaha, S., & Singh, T. (2006). Protective role of antioxidant enzymes under high temperature stress. *Plant Science, 171*(3), 382-388.

Apel, K., & Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol., 55*, 373-399.

Asada, K. (1987). Production and scavenging of active oxygen in photosynthesis. *Photoinhibition*.

Asada, K. (1994). Production and action of active oxygen species in photosynthetic tissues. *Causes of photooxidative stress and amelioration of defense systems in plants*, 77-104.

Asada, K. (2006). Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant physiology, 141*(2), 391-396.

Ashraf, M., & Foolad, M. (2007). Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environmental and Experimental Botany, 59*(2), 206-216.

Ashraf, M., & Hafeez, M. (2004). Thermotolerance of pearl millet and maize at early growth stages: growth and nutrient relations. *Biologia plantarum, 48*(1), 81-86.

Avsian-Kretchmer, O., Eshdat, Y., Gueta-Dahan, Y., & Ben-Hayyim, G. (1999). Regulation of stress-induced phospholipid hydroperoxide glutathione peroxidase expression in citrus. *Planta, 209*(4), 469-477.

Bannister, W., Bannister, J., Barra, D., Bond, J., & Bossa, F. (1991). Evolutionary aspects of superoxide dismutase: the copper/zinc enzyme. *Free radical research communications, 12*(1), 349-361.

Beauchamp, C., & Fridovich, I. (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Analytical biochemistry, 44*(1), 276-287.

Beevers, H. (1982). Glyoxysomes in higher plants. *Annals of the New York Academy of Sciences, 386*(1), 243-253.

Ben-Hayyim, G., Faltin, Z., Gepstein, S., Camoin, L., Strosberg, A. D., & Eshdat, Y. (1993). Isolation and characterization of salt-associated protein in Citrus. *Plant Science, 88*(2), 129-140.

Bernstein, L., Bosch, P., Canziani, O., Chen, Z., Christ, R., & Riahi, K. (2008). *IPCC, 2007: climate change 2007: synthesis report*: IPCC.

Beyer, W. F., & Fridovich, I. (1987). Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. *Analytical biochemistry, 161*(2), 559-566.

Bhattacharjee, S. (2005). Reactive oxygen species and oxidative burst: roles in stress, senescence and signal. *Curr Sci India, 89*, 1113-1121.

Bibi, A., Oosterhuis, D., & Gonias, E. (2008). Photosynthesis, quantum yield of photosystem II and membrane leakage as affected by high temperatures in cotton genotypes. *Journal of Cotton Science*.

Bienert, G. P., Schjoerring, J. K., & Jahn, T. P. (2006). Membrane transport of hydrogen peroxide. *Biochimica et Biophysica Acta (BBA)-Biomembranes, 1758*(8), 994-1003.

Bita, C., & Gerats, T. (2013). Plant tolerance to high temperature in a changing environment: scientific fundamentals and production of heat stress-tolerant crops. *Frontiers in plant science, 4*, 273.

Bolaños, J., & Edmeades, G. (1996). The importance of the anthesis-silking interval in breeding for drought tolerance in tropical maize. *Field Crops Research, 48*(1), 65-80.

Bolwell, G. P. (1999). Role of active oxygen species and NO in plant defence responses. *Current opinion in plant biology, 2*(4), 287-294.

Bordo, D., Djinovic, K., & Bolognesi, M. (1994). Conserved patterns in the Cu, Zn superoxide dismutase family. *Journal of molecular biology, 238*(3), 366-386.

Bowler, C., Montagu, M. v., & Inze, D. (1992). Superoxide dismutase and stress tolerance. *Annual review of plant biology, 43*(1), 83-116.

Bowler, C., Slooten, L., Vandenbranden, S., De Rycke, R., Botterman, J., Sybesma, C., . . . Inzé, D. (1991). Manganese superoxide dismutase can reduce cellular damage mediated by oxygen radicals in transgenic plants. *The EMBO journal, 10*(7), 1723.

Boyer, C. D., & Hannah, L. C. (2000). 1 Kernel Mutants of Corn. *Specialty corns*, 1.

Bryk, R., Griffin, P., & Nathan, C. (2000). Peroxynitrite reductase activity of bacterial peroxiredoxins. *Nature, 407*(6801), 211-215.

Buege, J. A., & Aust, S. D. (1978). [30] Microsomal lipid peroxidation. *Methods in enzymology, 52*, 302-310.

Buettner, G. R., & Jurkiewicz, A. (1996). Handbook of antioxidants.

Bukhov, N. G., Wiese, C., Neimanis, S., & Heber, U. (1999). Heat sensitivity of chloroplasts and leaves: leakage of protons from thylakoids and reversible activation of cyclic electron transport. *Photosynthesis Research, 59*(1), 81-93.

Burnell, J. N. (1984). Sulfate Assimilation in C4 Plants Intercellular and Intracellular Location of ATP Sulfurylase, Cysteine Synthase, and Cystathionine β-Lyase in Maize Leaves. *Plant physiology, 75*(3), 873-875.

Buwalda, F., De Kok, L., & Stulen, I. (1993). Effects of atmospheric H2S on thiol composition of crop plants. *Journal of plant physiology, 142*(3), 281-285.

Camejo, D., Jiménez, A., Alarcón, J. J., Torres, W., Gómez, J. M., & Sevilla, F. (2006). Changes in photosynthetic parameters and antioxidant activities following heat-shock treatment in tomato plants. *Functional Plant Biology, 33*(2), 177-187.

Camejo, D., Rodríguez, P., Morales, M. A., Dell'Amico, J. M., Torrecillas, A., & Alarcón, J. J. (2005). High temperature effects on photosynthetic activity of two tomato cultivars with different heat susceptibility. *Journal of plant physiology, 162*(3), 281-289.

Carberry, P., Muchow, R., & McCown, R. (1989). Testing the CERES-Maize simulation model in a semi-arid tropical environment. *Field Crops Research, 20*(4), 297-315.

Chae, H., Kim, I., Kim, K., & Rhee, S. (1993). Cloning, sequencing, and mutation of thiol-specific antioxidant gene of Saccharomyces cerevisiae. *Journal of Biological Chemistry, 268*(22), 16815-16821.

Chakraborty, U., & Pradhan, D. (2011). High temperature-induced oxidative stress in Lens culinaris, role of antioxidants and amelioration of stress by chemical pre-treatments. *Journal of Plant Interactions, 6*(1), 43-52.

Chandlee, J., & Scandalios, J. (1984). Analysis of variants affecting the catalase developmental program in maize scutellum. *Theoretical and applied genetics, 69*(1), 71-77.

Change, C. (2007). Synthesis Report. Contribution of Working Groups I, II and III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change: IPCC, Geneva, Switzerland.

Charles, S. A., & Halliwell, B. (1981). Light activation of fructose bisphosphatase in isolated spinach chloroplasts and deactivation by hydrogen peroxide. *Planta, 151*(3), 242-246.

Chen, G.-X., & Asada, K. (1989). Ascorbate peroxidase in tea leaves: occurrence of two isozymes and the differences in their enzymatic and molecular properties. *Plant and Cell Physiology, 30*(7), 987-998.

Chu, F.-F., Doroshow, J., and, & Esworthy, R. (1993). Expression, characterization, and tissue distribution of a new cellular selenium-dependent glutathione peroxidase, GSHPx-GI. *Journal of Biological Chemistry, 268*(4), 2571-2576.

Colless, J. (1992). Maize growing: Report.
Compass, G. (2011). Genetically modified plants: global cultivation area Maize.
Crafts-Brandner, S. J., & Salvucci, M. E. (2002). Sensitivity of photosynthesis in a C4 plant, maize, to heat stress. *Plant physiology, 129*(4), 1773-1780.
Creissen, G. P., Edwards, E. A., & Mullineaux, P. M. (1994). Glutathione reductase and ascorbate peroxidase. *Causes of photooxidative stress and amelioration of defense systems in plants. CRC Press, Boca Raton*, 343-364.
Criqui, M., Jamet, E., Parmentier, Y., Marbach, J., Durr, A., & Fleck, J. (1992). Isolation and characterization of a plant cDNA showing homology to animal glutathione peroxidases. *Plant molecular biology, 18*(3), 623-627.
Dat, J., Vandenabeele, S., Vranová, E., Van Montagu, M., Inzé, D., & Van Breusegem, F. (2000). Dual action of the active oxygen species during plant stress responses. *Cellular and Molecular Life Sciences CMLS, 57*(5), 779-795.
Dat, J. F., Foyer, C. H., & Scott, I. M. (1998). Changes in salicylic acid and antioxidants during induced thermotolerance in mustard seedlings. *Plant physiology, 118*(4), 1455-1461.
De Gara, L., Paciolla, C., Tommasi, F., Liso, R., & Arrigoni, O. (1994). «In vivo» Inhibition of Galactono-γ-Lactone Conversion to Ascorbate by Lycorine. *Journal of plant physiology, 144*(6), 649-653.
De Leonardis, S., Dipierro, N., & Dipierro, S. (2000). Purification and characterization of an ascorbate peroxidase from potato tuber mitochondria. *Plant Physiology and Biochemistry, 38*(10), 773-779.
De Ronde, J., Cress, W., Krüger, G., Strasser, R., & Van Staden, J. (2004). Photosynthetic response of transgenic soybean plants, containing an Arabidopsis P5CR gene, during heat and drought stress. *Journal of plant physiology, 161*(11), 1211-1224.
Delledonne, M., Zeier, J., Marocco, A., & Lamb, C. (2001). Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. *Proceedings of the National Academy of Sciences, 98*(23), 13454-13459.
Díaz, A., Loewen, P. C., Fita, I., & Carpena, X. (2012). Thirty years of heme catalases structural biology. *Archives of biochemistry and biophysics, 525*(2), 102-110.
Djanaguiraman, M., Prasad, P., & Al-Khatib, K. (2011). Ethylene perception inhibitor 1-MCP decreases oxidative damage of leaves through enhanced antioxidant defense mechanisms in soybean plants grown under high temperature stress. *Environmental and Experimental Botany, 71*(2), 215-223.
Doulis, A. G., Debian, N., Kingston-Smith, A. H., & Foyer, C. H. (1997). Differential localization of antioxidants in maize leaves. *Plant Physiology, 114*(3), 1031-1037.
Drotar, A., Phelps, P., & Fall, R. (1985). Evidence for glutathione peroxidase activities in cultured plant cells. *Plant Science, 42*(1), 35-40.
Edwards, E. A., Enard, C., Creissen, G. P., & Mullineaux, P. M. (1993). Synthesis and properties of glutathione reductase in stressed peas. *Planta, 192*(1), 137-143.
Edwards, E. A., Rawsthorne, S., & Mullineaux, P. M. (1990). Subcellular distribution of multiple forms of glutathione reductase in leaves of pea (Pisum sativum L.). *Planta, 180*(2), 278-284.
Ellyard, P. W., & Gibbs, M. (1969). Inhibition of photosynthesis by oxygen in isolated spinach chloroplasts. *Plant physiology, 44*(8), 1115-1121.
Esau, K. (1977). The stem: primary state of growth. *Anatomy of Seed Plants, Ed, 2*, 243-294.
Eshdat, Y., Holland, D., Faltin, Z., & Ben-Hayyim, G. (1997). Plant glutathione peroxidases. *Physiologia Plantarum, 100*(2), 234-240.
Evert, R. F. (2006). *Esau's plant anatomy: meristems, cells, and tissues of the plant body: their structure, function, and development*: John Wiley & Sons.
Farnham, D., Benson, G., Pearce, R., White, P., & Johnson, L. (2003). Corn perspective and culture. *Corn: chemistry and technology*(Ed. 2), 1-33.
Foyer, C., Rowell, J., & Walker, D. (1983). Measurement of the ascorbate content of spinach leaf protoplasts and chloroplasts during illumination. *Planta, 157*(3), 239-244.

Foyer, C. H., & Halliwell, B. (1976). The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. *Planta, 133*(1), 21-25.
Foyer, C. H., & Halliwell, B. (1977). Purification and properties of dehydroascorbate reductase from spinach leaves. *Phytochemistry, 16*(9), 1347-1350.
Foyer, C. H., & Lelandais, M. (1996). A comparison of the relative rates of transport of ascorbate and glucose across the thylakoid, chloroplast and plasmalemma membranes of pea leaf mesophyll cells. *Journal of Plant Physiology, 148*(3-4), 391-398.
Foyer, C. H., & Mullineaux, P. M. (1998). The presence of dehydroascorbate and dehydroascorbate reductase in plant tissues. *FEBS letters, 425*(3), 528-529.
Fridovich, I. (1986). Superoxide dismutases. *Adv Enzymol Relat Areas Mol Biol, 58*(6), 61-97.
Gaweł, S., Wardas, M., Niedworok, E., & Wardas, P. (2003). Malondialdehyde (MDA) as a lipid peroxidation marker. *Wiadomosci lekarskie (Warsaw, Poland: 1960), 57*(9-10), 453-455.
Gelhaye, E., Rouhier, N., Navrot, N., & Jacquot, J. (2005). The plant thioredoxin system. *Cellular and Molecular Life Sciences CMLS, 62*(1), 24-35.
Gibson, L., & Benson, G. (2002). Origin, History, and uses of corn (Zea mays). *Iowa State University*.
Gill, S. S., & Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry, 48*(12), 909-930.
Gong, M., Li, Y.-J., & Chen, S.-Z. (1998). Abscisic acid-induced thermotolerance in maize seedlings is mediated by calcium and associated with antioxidant systems. *Journal of Plant Physiology, 153*(3-4), 488-496.
Gorantla, M., Babu, P., Reddy Lachagari, V., Reddy, A., Wusirika, R., Bennetzen, J. L., & Reddy, A. R. (2007). Identification of stress-responsive genes in an indica rice (Oryza sativa L.) using ESTs generated from drought-stressed seedlings. *Journal of experimental botany, 58*(2), 253-265.
Greer, D. H., & Weedon, M. M. (2012). Modelling photosynthetic responses to temperature of grapevine (Vitis vinifera cv. Semillon) leaves on vines grown in a hot climate. *Plant, Cell & Environment, 35*(6), 1050-1064.
Grover, A., Sabat, S. C., & Mohanty, P. (1986). Effect of temperature on photosynthetic activities of senescing detached wheat leaves. *Plant and cell physiology, 27*(1), 117-126.
Guilioni, L., Wery, J., & Tardieu, F. (1997). Heat stress-induced abortion of buds and flowers in pea: is sensitivity linked to organ age or to relations between reproductive organs? *Annals of Botany, 80*(2), 159-168.
Gupta, A. S., Heinen, J. L., Holaday, A. S., Burke, J. J., & Allen, R. D. (1993). Increased resistance to oxidative stress in transgenic plants that overexpress chloroplastic Cu/Zn superoxide dismutase. *Proceedings of the National Academy of Sciences, 90*(4), 1629-1633.
Gurley, W. B. (2000). HSP101: a key component for the acquisition of thermotolerance in plants. *The Plant Cell, 12*(4), 457-460.
Hasanuzzaman, M., Nahar, K., Alam, M. M., & Fujita, M. (2012). Exogenous nitric oxide alleviates high temperature induced oxidative stress in wheat (Triticum aestivum L.) seedlings by modulating the antioxidant defense and glyoxalase system. *Australian Journal of Crop Science, 6*(8), 1314.
Hasanuzzaman, M., Nahar, K., Alam, M. M., Roychowdhury, R., & Fujita, M. (2013). Physiological, biochemical, and molecular mechanisms of heat stress tolerance in plants. *International Journal of Molecular Sciences, 14*(5), 9643-9684.
Havir, E. A., & McHale, N. A. (1989). Enhanced-peroxidatic activity in specific catalase isozymes of tobacco, barley, and maize. *Plant Physiology, 91*(3), 812-815.
Heisey, P. W., & Edmeades, G. O. (1999). *Maize production in drought-stressed environments: technical options and research allocation*: CIMMYT.
Hell, R., & Bergmann, L. (1988). Glutathione synthetase in tobacco suspension cultures: catalytic properties and localization. *Physiologia Plantarum, 72*(1), 70-76.
Hell, R., & Bergmann, L. (1990). λ-Glutamylcysteine synthetase in higher plants: catalytic properties and subcellular localization. *Planta, 180*(4), 603-612.
Herrero, M. P., & Johnson, R. (1980). High temperature stress and pollen viability of maize. *Crop science, 20*(6), 796-800.

Herschbach, C., & Rennenberg, H. (1994). Influence of glutathione (GSH) on net uptake of sulphate and sulphate transport in tobacco plants. *Journal of Experimental Botany, 45*(8), 1069-1076.

Hillas, P. J., del Alba, F. S., Oyarzabal, J., Wilks, A., & de Montellano, P. R. O. (2000). The AhpC and AhpD antioxidant defense system of Mycobacterium tuberculosis. *Journal of Biological Chemistry, 275*(25), 18801-18809.

Hoeft, R. G., Aldrich, S. R., Nafziger, E. D., & Johnson, R. R. (2000). Modern corn and soybean production.

Holland, D., Ben-Hayyim, G., Faltin, Z., Camoin, L., Strosberg, A. D., & Eshdat, Y. (1993). Molecular characterization of salt-stress-associated protein in citrus: protein and cDNA sequence homology to mammalian glutathione peroxidases. *Plant molecular biology, 21*(5), 923-927.

Hossain, M. A., & Asada, K. (1984). Purification of dehydroascorbate reductase from spinach and its characterization as a thiol enzyme. *Plant and Cell Physiology, 25*(1), 85-92.

Hou, W. C., Liang, H. J., Wang, C. C., & Liu, D. Z. (2004). Detection of glutathione reductase after electrophoresis on native or sodium dodecyl sulfate polyacrylamide gels. *Electrophoresis, 25*(17), 2926-2931.

Hu, X., Liu, R., Li, Y., Wang, W., Tai, F., Xue, R., & Li, C. (2010). Heat shock protein 70 regulates the abscisic acid-induced antioxidant response of maize to combined drought and heat stress. *Plant Growth Regulation, 60*(3), 225-235.

Hu, Y. X., Wang, Y. H., Liu, X. F., & LI, J. Y. (2004). Arabidopsis RAV1 is down-regulated by brassinosteroid and may act as a negative regulator during plant development. *Cell research, 14*(1), 8-15.

Ishikawa, T., Sakai, K., Yoshimura, K., Takeda, T., & Shigeoka, S. (1996). cDNAs encoding spinach stromal and thylakoid-bound ascorbate peroxidase, differing in the presence or absence of their 3′-coding regions. *FEBS letters, 384*(3), 289-293.

Ishikawa, T., Yoshimura, K., Sakai, K., Tamoi, M., Takeda, T., & Shigeoka, S. (1998). Molecular characterization and physiological role of a glyoxysome-bound ascorbate peroxidase from spinach. *Plant and Cell Physiology, 39*(1), 23-34.

Ismail, A. M., & Hall, A. E. (1999). Reproductive-stage heat tolerance, leaf membrane thermostability and plant morphology in cowpea. *Crop Science, 39*(6), 1762-1768.

Jiang, M., & Zhang, J. (2001). Effect of abscisic acid on active oxygen species, antioxidative defence system and oxidative damage in leaves of maize seedlings. *Plant and Cell Physiology, 42*(11), 1265-1273.

Jimenez, A., Hernandez, J. A., del Rio, L. A., & Sevilla, F. (1997). Evidence for the presence of the ascorbate-glutathione cycle in mitochondria and peroxisomes of pea leaves. *Plant physiology, 114*(1), 275-284.

Johnson, R. R., Cranston, H. J., Chaverra, M. E., & Dyer, W. E. (1995). Characterization of cDNA clones for differentially expressed genes in embryos of dormant and nondormant Avena fatua L. caryopses. *Plant Molecular Biology, 28*(1), 113-122.

Jones, C. A., Kiniry, J. R., & Dyke, P. (1986). *CERES-Maize: A simulation model of maize growth and development*: Texas AandM University Press.

Joo, J. H., Bae, Y. S., & Lee, J. S. (2001). Role of auxin-induced reactive oxygen species in root gravitropism. *Plant Physiology, 126*(3), 1055-1060.

Kaiser, A. G. (1997). *Australian Maize*: Kondinin Group.

Kanematsu, S., & Asada, K. (1990). Characteristic amino acid sequences of chloroplast and cytosol isozymes of CuZn-superoxide dismutase in spinach, rice and horsetail. *Plant and Cell physiology, 31*(1), 99-112.

Karim, M., Fracheboud, Y., & Stamp, P. (1997). Heat tolerance of maize with reference of some physiological characteristics. *Ann. Bangladesh Agri, 7*(2).

Karim, M., Fracheboud, Y., & Stamp, P. (1999). Photosynthetic activity of developing leaves of Zea mays is less affected by heat stress than that of developed leaves. *Physiologia Plantarum, 105*(4), 685-693.

Kato, Y., Urano, J. i., Maki, Y., & Ushimaru, T. (1997). Purification and characterization of dehydroascorbate reductase from rice. *Plant and Cell Physiology, 38*(2), 173-178.

Khanna-Chopra, R., & Sabarinath, S. (2004). Heat-stable chloroplastic Cu/Zn superoxide dismutase in Chenopodium murale. *Biochemical and biophysical research communications, 320*(4), 1187-1192.

Kim, I. H., Kim, K., & Rhee, S. G. (1989). Induction of an antioxidant protein of Saccharomyces cerevisiae by O2, Fe3+, or 2-mercaptoethanol. *Proceedings of the National Academy of Sciences, 86*(16), 6018-6022.
Kindl, H., & Lazarow, P. B. (1982). *Peroxisomes and glyoxysomes* (Vol. 386): New York Academy of Sciences.
Kishor, P. K., Sangam, S., Amrutha, R., Laxmi, P. S., Naidu, K., Rao, K., . . . Sreenivasulu, N. (2005). Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: its implications in plant growth and abiotic stress tolerance. *Curr Sci, 88*(3), 424-438.
Kochhar, S., & Kochhar, V. (2005). Expression of antioxidant enzymes and heat shock proteins in relation to combined stress of cadmium and heat in Vigna mungo seedlings. *Plant science, 168*(4), 921-929.
Kok, L. J., & Kuiper, P. J. (1986). Effect of short-term dark incubation with sulfate, chloride and selenate on the glutathione content of spinach leaf discs. *Physiologia Plantarum, 68*(3), 477-482.
Kruger, N. J. (1994). The Bradford method for protein quantitation. *Basic protein and peptide protocols*, 9-15.
Kumar, S., Gupta, D., & Nayyar, H. (2012). Comparative response of maize and rice genotypes to heat stress: status of oxidative stress and antioxidants. *Acta Physiologiae Plantarum, 34*(1), 75-86.
Kunert, K., & Foyer, C. (1993). Thiol/disulphide exchange in plants. *Sulfur Nutrition and Assimilation in Higher Plants. Regulatory Agricultural and Environmental Aspects. SPB Academic Publishers, The Hague, The Netherlands*, 139-151.
Kusunose, E., ICHIHARA, K., NODA, Y., & KUSUNOSE, M. (1976). Superoxide dismutase from Mycobacterium tuberculosis. *The Journal of Biochemistry, 80*(6), 1343-1352.
Lamoureux, G. L., & Rusness, D. G. (1993). Glutathione in the metabolism and detoxification of xenobiotics in plants. *Sulfur Nutrition and Assimilation in Higher Plants. The Hague, The Netherlands: SPB Academic Publishing*, 221-237.
Lee, S.-H., Ahsan, N., Lee, K.-W., Kim, D.-H., Lee, D.-G., Kwak, S.-S., . . . Lee, B.-H. (2007). Simultaneous overexpression of both CuZn superoxide dismutase and ascorbate peroxidase in transgenic tall fescue plants confers increased tolerance to a wide range of abiotic stresses. *Journal of plant physiology, 164*(12), 1626-1638.
Lim, Y.-S., Cha, M.-K., Kim, H.-K., & Kim, I.-H. (1994). The thiol-specific antioxidant protein from human brain: gene cloning and analysis of conserved cysteine regions. *Gene, 140*(2), 279-284.
Lin, C. L., Chen, H. J., & Hou, W. C. (2002). Activity staining of glutathione peroxidase after electrophoresis on native and sodium dodecyl sulfate polyacrylamide gels. *Electrophoresis, 23*(4), 513-516.
LOEWUS, F. A. (2012). Ascorbic Acid and I ts 3 Metabolic Products. *The Biochemistry of Plants: Carbohydrates, 14*, 85.
Mach, J. (2012). Calcium channels and acquired thermotolerance: here comes the sun and it's all right: Am Soc Plant Biol.
Mahan, J. R., & Burke, J. J. (1987). Purification and characterization of glutathione reductase from corn mesophyll chloroplasts. *Physiologia Plantarum, 71*(3), 352-358.
Manchenko, G. P. (2002). *Handbook of detection of enzymes on electrophoretic gels*: CRC press.
Mano, J. i. (2002). Early events in environmental stresses in plants. Induction mechanisms of oxidative stress. *Oxidative stress in plants*, 217-245.
Marchand, F. L., Mertens, S., Kockelbergh, F., Beyens, L., & Nijs, I. (2005). Performance of High Arctic tundra plants improved during but deteriorated after exposure to a simulated extreme temperature event. *Global Change Biology, 11*(12), 2078-2089.
Matters, G. L., & Scandalios, J. G. (1986). Effect of elevated temperature on catalase and superoxide dismutase during maize development. *Differentiation, 30*(3), 190-196.
McAinsh, M. R., Clayton, H., Mansfield, T. A., & Hetherington, A. M. (1996). Changes in stomatal behavior and guard cell cytosolic free calcium in response to oxidative stress. *Plant Physiology, 111*(4), 1031-1042.
McCay, P. B., Gibson, D. D., Kuo-Lan, F., & Roger, H. K. (1976). Effect of glutathione peroxidase activity on lipid peroxidation in biological membranes. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism, 431*(3), 459-468.
McCord, J. M., & Fridovich, I. (1969). Superoxide dismutase an enzymic function for erythrocuprein (hemocuprein). *Journal of Biological chemistry, 244*(22), 6049-6055.

Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. *Trends in plant science, 7*(9), 405-410.
Mittler, R., & Zilinskas, B. A. (1991). Purification and characterization of pea cytosolic ascorbate peroxidase. *Plant Physiology, 97*(3), 962-968.
Mittler, R., & Zilinskas, B. A. (1993). Detection of ascorbate peroxidase activity in native gels by inhibition of the ascorbate-dependent reduction of nitroblue tetrazolium. *Analytical biochemistry, 212*(2), 540-546.
Miyake, C., Cao, W.-H., & Asada, K. (1993). Purification and molecular properties of the thylakoid-bound ascorbate peroxidase in spinach chloroplasts. *Plant and cell physiology, 34*(6), 881-889.
Morell, S., Follmann, H., De Tullio, M., & Häberlein, I. (1997). Dehydroascorbate and dehydroascorbate reductase are phantom indicators of oxidative stress in plants. *FEBS letters, 414*(3), 567-570.
Mori, I. C., & Schroeder, J. I. (2004). Reactive oxygen species activation of plant Ca2+ channels. A signaling mechanism in polar growth, hormone transduction, stress signaling, and hypothetically mechanotransduction. *Plant Physiology, 135*(2), 702-708.
Nagesh Babu, R., & Devaraj, V. (2008). High temperature and salt stress response in French bean (Phaseolus vulgaris). *Aust J Crop Sci, 2*(2), 40-48.
Nakano, Y., & Asada, K. (1981). Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant and cell physiology, 22*(5), 867-880.
Nakano, Y., & Asada, K. (1987). Purification of ascorbate peroxidase in spinach chloroplasts; its inactivation in ascorbate-depleted medium and reactivation by monodehydroascorbate radical. *Plant and cell physiology, 28*(1), 131-140.
Navrot, N., Collin, V., Gualberto, J., Gelhaye, E., Hirasawa, M., Rey, P., . . . Rouhier, N. (2006). Plant glutathione peroxidases are functional peroxiredoxins distributed in several subcellular compartments and regulated during biotic and abiotic stresses. *Plant Physiology, 142*(4), 1364-1379.
Noctor, G., & Foyer, C. H. (1998). Ascorbate and glutathione: keeping active oxygen under control. *Annual review of plant biology, 49*(1), 249-279.
Noctor, G., Jouanin, L., & Foyer, C. H. (1997). The biosynthesis of glutathione explored in transformed plants. *Regulation of Enzymatic Systems Detoxifying Xenobiotics in Plants. NATO ASI Series. Dordrecht, The Netherlands: Kluwer*, 109-124.
Nogoceke, E., Gommel, D. U., Kieß, M., Kalisz, H. M., & Flohé, L. (1997). A unique cascade of oxidoreductases catalyses trypanothione-mediated peroxide metabolism in Crithidia fasciculata. *Biological chemistry, 378*(8), 827-836.
Ôba, K., Ishikawa, S., Nishikawa, M., Mizuno, H., & Yamamoto, T. (1995). Purification and properties of L-galactono-γ-lactone dehydrogenase, a key enzyme for ascorbic acid biosynthesis, from sweet potato roots. *Journal of Biochemistry, 117*(1), 120-124.
Ogawa, K. i., Kanematsu, S., & Asada, K. (1996). Intra-and extra-cellular localization of "cytosolic" CuZn-superoxide dismutase in spinach leaf and hypocotyl. *Plant and Cell Physiology, 37*(6), 790-799.
Ogawa, K. i., Kanematsu, S., Takabe, K., & Asada, K. (1995). Attachment of CuZn-superoxide dismutase to thylakoid membranes at the site of superoxide generation (PSI) in spinach chloroplasts: detection by immuno-gold labeling after rapid freezing and substitution method. *Plant and Cell Physiology, 36*(4), 565-573.
Ortiz, R., Braun, H.-J., Crossa, J., Crouch, J. H., Davenport, G., Dixon, J., . . . Huerta, J. (2008). Wheat genetic resources enhancement by the International Maize and Wheat Improvement Center (CIMMYT). *Genetic Resources and Crop Evolution, 55*(7), 1095-1140.
Paglia, D. E., & Valentine, W. N. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *Translational Research, 70*(1), 158-169.
Paliwal, R. (2000). Origin, evolution and spread of maize. Tropical Maize Improvement and Production.
Paliwal, R. L., Granados, G., Lafitte, H. R., Violic, A. D., & Marathée, J. P. (2000). *Tropical maize: improvement and production*.
Pastori, G. M., Mullineaux, P. M., & Foyer, C. H. (2000). Post-transcriptional regulation prevents accumulation of glutathione reductase protein and activity in the bundle sheath cells of maize. *Plant Physiology, 122*(3), 667-676.

Pei, Z.-M., Murata, Y., Benning, G., Thomine, S., Klüsener, B., Allen, G. J., . . . Schroeder, J. I. (2000). Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. *Nature, 406*(6797), 731-734.

Perl, A., Perl-Treves, R., Galili, S., Aviv, D., Shalgi, E., Malkin, S., & Galun, E. (1993). Enhanced oxidative-stress defense in transgenic potato expressing tomato Cu, Zn superoxide dismutases. *Theoretical and Applied Genetics, 85*(5), 568-576.

Piette, L. H. (1979). Further evidence for OH radical production in Fenton's reagent. *Tetrahedron Letters, 20*(9), 775-778.

Pinhero, R. G., Rao, M. V., Paliyath, G., Murr, D. P., & Fletcher, R. A. (1997). Changes in activities of antioxidant enzymes and their relationship to genetic and paclobutrazol-induced chilling tolerance of maize seedlings. *Plant Physiology, 114*(2), 695-704.

Piperno, D. R., & Flannery, K. V. (2001). The earliest archaeological maize (Zea mays L.) from highland Mexico: new accelerator mass spectrometry dates and their implications. *Proceedings of the National Academy of Sciences, 98*(4), 2101-2103.

Potters, G., Pasternak, T. P., Guisez, Y., Palme, K. J., & Jansen, M. A. (2007). Stress-induced morphogenic responses: growing out of trouble? *Trends in plant science, 12*(3), 98-105.

Prasad, T. K. (1997). Role of catalase in inducing chilling tolerance in pre-emergent maize seedlings. *Plant Physiology, 114*(4), 1369-1376.

Quan, R., Shang, M., Zhang, H., Zhao, Y., & Zhang, J. (2004). Engineering of enhanced glycine betaine synthesis improves drought tolerance in maize. *Plant Biotechnology Journal, 2*(6), 477-486.

Rao, K. M., Raghavendra, A., & Reddy, K. J. (2006). *Physiology and molecular biology of stress tolerance in plants*: Springer Science & Business Media.

Reddy, A. R., Chaitanya, K. V., & Vivekanandan, M. (2004). Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. *Journal of plant physiology, 161*(11), 1189-1202.

Ren, D., Yang, H., & Zhang, S. (2002). Cell death mediated by MAPK is associated with hydrogen peroxide production in Arabidopsis. *Journal of Biological Chemistry, 277*(1), 559-565.

Rennenberg, H. (1980). Glutathione metabolism and possible biological roles in higher plants. *Phytochemistry, 21*(12), 2771-2781.

Ritchie, S., Hanway, J., & Benson, G. (1992). How a corn plant grows. *Iowa State Univ Sci Tech Coop Ext Serv Rep, 48*.

Romero-Puertas, M. C., Corpas, F. J., Sandalio, L. M., Leterrier, M., Rodríguez-Serrano, M., Del Río, L. A., & Palma, J. M. (2006). Glutathione reductase from pea leaves: response to abiotic stress and characterization of the peroxisomal isozyme. *New phytologist, 170*(1), 43-52.

Rotruck, J., Pope, A., Ganther, H., Swanson, A., Hafeman, D. G., & Hoekstra, W. (1973). Selenium: biochemical role as a component of glutathione peroxidase. *Science, 179*(4073), 588-590.

Sabeh, F., Wright, T., & Norton, S. (1992). Purification and characterization of a glutathione peroxidase from the Aloe vera plant. *Enzyme & protein, 47*(2), 92-98.

Salin, M. L., & Bridges, S. M. (1981). Absence of the iron-containing superoxide dismutase in mitochondria from mustard (Brassica campestris). *Biochemical Journal, 195*(1), 229-233.

Sánchez, B., Rasmussen, A., & Porter, J. R. (2014). Temperatures and the growth and development of maize and rice: a review. *Global change biology, 20*(2), 408-417.

Sandalio, L. M., & Del Río, L. A. (1987). Localization of superoxide dismutase in glyoxysomes from Citrullus vulgaris. Functional implications in cellular metabolism. *Journal of Plant Physiology, 127*(5), 395-409.

Savchenko, G., Klyuchareva, E., Abramchik, L., & Serdyuchenko, E. (2002). Effect of periodic heat shock on the inner membrane system of etioplasts. *Russian journal of plant physiology, 49*(3), 349-359.

Savicka, M., & Škute, N. (2010). Effects of high temperature on malondialdehyde content, superoxide production and growth changes in wheat seedlings (Triticum aestivum L.). *Ekologija, 56*(1), 26-33.

Sayed, O. (1996). Adaptational responses ofZygophyllum qatarenseHadidi to stress conditions in a desert environment. *Journal of Arid Environments, 32*(4), 445-452.

SCANDALIOS, J. G. (1974). Subcellular localization of catalase variants coded by two genetic loci during maize development. *Journal of Heredity, 65*(1), 28-32.

Scandalios, J. G., Guan, L., & Polidoros, A. N. (1997). Catalases in plants: gene structure, properties, regulation, and expression. *Cold Spring Harbor Monograph Series, 34*, 343-406.

Scandalios, J. G., Tong, W.-F., & Roupakias, D. G. (1980). Cat3, a third gene locus coding for a tissue-specific catalase in maize: genetics, intracellular location, and some biochemical properties. *Molecular and General Genetics MGG, 179*(1), 33-41.

Schoper, J., Lambert, R., & Vasilas, B. (1987). Pollen viability, pollen shedding, and combining ability for tassel heat tolerance in maize. *Crop science, 27*(1), 27-31.

Sharkova, V. (2001). The effect of heat shock on the capacity of wheat plants to restore their photosynthetic electron transport after photoinhibition or repeated heating. *Russian Journal of Plant Physiology, 48*(6), 793-797.

Shi, Q., Bao, Z., Zhu, Z., Ying, Q., & Qian, Q. (2006). Effects of different treatments of salicylic acid on heat tolerance, chlorophyll fluorescence, and antioxidant enzyme activity in seedlings of Cucumis sativa L. *Plant growth regulation, 48*(2), 127-135.

Shi, W., Muramoto, Y., Ueda, A., & Takabe, T. (2001). Cloning of peroxisomal ascorbate peroxidase gene from barley and enhanced thermotolerance by overexpressing in Arabidopsis thaliana. *Gene, 273*(1), 23-27.

Shigeoka, S., Ishikawa, T., Tamoi, M., Miyagawa, Y., Takeda, T., Yabuta, Y., & Yoshimura, K. (2002). Regulation and function of ascorbate peroxidase isoenzymes. *Journal of experimental Botany, 53*(372), 1305-1319.

Shigeoka, S., Nakano, Y., & Kitaoka, S. (1980). Purification and some properties of L-ascorbic acid-specific peroxidase in Euglena gracilis Z. *Archives of Biochemistry and Biophysics, 201*(1), 121-127.

Smith, I. K., Vierheller, T. L., & Thorne, C. A. (1988). Assay of glutathione reductase in crude tissue homogenates using 5, 5′-dithiobis (2-nitrobenzoic acid). *Analytical biochemistry, 175*(2), 408-413.

Smith, M. W., & Doolittle, R. F. (1992). A comparison of evolutionary rates of the two major kinds of superoxide dismutase. *Journal of molecular evolution, 34*(2), 175-184.

Soliman, W. S., Fujimori, M., Tase, K., & Sugiyama, S. i. (2011). Oxidative stress and physiological damage under prolonged heat stress in C3 grass Lolium perenne. *Grassland science, 57*(2), 101-106.

Song, X. S., Hu, W. H., Mao, W. H., Ogweno, J. O., Zhou, Y. H., & Yu, J. Q. (2005). Response of ascorbate peroxidase isoenzymes and ascorbate regeneration system to abiotic stresses in Cucumis sativus L. *Plant Physiology and Biochemistry, 43*(12), 1082-1088.

Soojani, H. (2007). Effect of calcium sulphate (CaSO4) on seed germination and seedling growth of wheat (Triticum aestivum L.).

Sugimoto, M., Furui, S., & Suzuki, Y. (1997). Molecular cloning and characterization of a cDNA encoding putative phospholipid hydroperoxide glutathione peroxidase from spinach. *Bioscience, biotechnology, and biochemistry, 61*(8), 1379-1381.

Swidzinski, J. A., Sweetlove, L. J., & Leaver, C. J. (2002). A custom microarray analysis of gene expression during programmed cell death in Arabidopsis thaliana. *The Plant Journal, 30*(4), 431-446.

Takahashi, M., & Asada, K. (1988). Superoxide production in aprotic interior of chloroplast thylakoids. *Archives of biochemistry and biophysics, 267*(2), 714-722.

Tang, L., Kwon, S.-Y., Kim, S.-H., Kim, J.-S., Choi, J. S., Cho, K. Y., . . . Lee, H.-S. (2006). Enhanced tolerance of transgenic potato plants expressing both superoxide dismutase and ascorbate peroxidase in chloroplasts against oxidative stress and high temperature. *Plant Cell Reports, 25*(12), 1380-1386.

Thomas, J., Maiorino, M., Ursini, F., & Girotti, A. (1990). Protective action of phospholipid hydroperoxide glutathione peroxidase against membrane-damaging lipid peroxidation. In situ reduction of phospholipid and cholesterol hydroperoxides. *Journal of Biological Chemistry, 265*(1), 454-461.

Tiwari, B. S., Belenghi, B., & Levine, A. (2002). Oxidative stress increased respiration and generation of reactive oxygen species, resulting in ATP depletion, opening of mitochondrial permeability transition, and programmed cell death. *Plant physiology, 128*(4), 1271-1281.

Torres, M. A., Jones, J. D., & Dangl, J. L. (2006). Reactive oxygen species signaling in response to pathogens. *Plant physiology, 141*(2), 373-378.

Triantaphylidès, C., & Havaux, M. (2009). Singlet oxygen in plants: production, detoxification and signaling. *Trends in plant science, 14*(4), 219-228.

Triantaphylidès, C., Krischke, M., Hoeberichts, F. A., Ksas, B., Gresser, G., Havaux, M., . . . Mueller, M. J. (2008). Singlet oxygen is the major reactive oxygen species involved in photooxidative damage to plants. *Plant physiology, 148*(2), 960-968.

Tsaftaris, A., & Scandalios, J. (1986). Spatial pattern of catalase (Cat2) gene activation in scutella during postgerminative development in maize. *Proceedings of the National Academy of Sciences, 83*(15), 5549-5553.

Tsang, E., Bowler, C., Hérouart, D., Van Camp, W., Villarroel, R., Genetello, C., . . . Inze, D. (1991). Differential regulation of superoxide dismutases in plants exposed to environmental stress. *The Plant Cell, 3*(8), 783-792.

Tsukaguchi, T., Kawamitsu, Y., Takeda, H., Suzuki, K., & Egawa, Y. (2003). Water status of flower buds and leaves as affected by high temperature in heat-tolerant and heat-sensitive cultivars of snap bean (Phaseolus vulgaris L.). *Plant Production Science, 6*(1), 24-27.

Velikova, V., Yordanov, I., & Edreva, A. (2000). Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. *Plant Science, 151*(1), 59-66.

Vollenweider, P., & Günthardt-Goerg, M. S. (2005). Diagnosis of abiotic and biotic stress factors using the visible symptoms in foliage. *Environmental Pollution, 137*(3), 455-465.

Wahid, A., Gelani, S., Ashraf, M., & Foolad, M. R. (2007). Heat tolerance in plants: an overview. *Environmental and experimental botany, 61*(3), 199-223.

Wahid, A., & Ghazanfar, A. (2006). Possible involvement of some secondary metabolites in salt tolerance of sugarcane. *Journal of plant physiology, 163*(7), 723-730.

Walker, J. M. (2002). Nondenaturing polyacrylamide gel electrophoresis of proteins. *The protein protocols handbook*, 57-60.

Wang, J., Cui, L., Wang, Y., & Li, J. (2009). Growth, lipid peroxidation and photosynthesis in two tall fescue cultivars differing in heat tolerance. *Biologia Plantarum, 53*(2), 237-242.

Wang, W., Vinocur, B., & Altman, A. (2003). Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta, 218*(1), 1-14.

Wells, W. W., & Xu, D. P. (1994). Dehydroascorbate reduction. *Journal of bioenergetics and biomembranes, 26*(4), 369-377.

White, P. J. (2000). Properties of corn starch. *Specialty corns, 2*, 34-55.

Willekens, H., Inzé, D., Van Montagu, M., & Van Camp, W. (1995). Catalases in plants. *Molecular Breeding, 1*(3), 207-228.

Willekens, H., Langebartels, C., Tire, C., Van Montagu, M., Inze, D., & Van Camp, W. (1994). Differential expression of catalase genes in Nicotiana plumbaginifolia (L.). *Proceedings of the National Academy of Sciences, 91*(22), 10450-10454.

Willits, D., & Peet, M. (1998). The effect of night temperature on greenhouse grown tomato yields in warm climates. *Agricultural and Forest meteorology, 92*(3), 191-202.

Xu, S., Li, J., Zhang, X., Wei, H., & Cui, L. (2006). Effects of heat acclimation pretreatment on changes of membrane lipid peroxidation, antioxidant metabolites, and ultrastructure of chloroplasts in two cool-season turfgrass species under heat stress. *Environmental and Experimental Botany, 56*(3), 274-285.

Yamada, M., Hidaka, T., & Fukamachi, H. (1996). Heat tolerance in leaves of tropical fruit crops as measured by chlorophyll fluorescence. *Scientia Horticulturae, 67*(1-2), 39-48.

Yamaguchi, K., Mori, H., & Nishimura, M. (1995). A novel isoenzyme of ascorbate peroxidase localized on glyoxysomal and leaf peroxisomal membranes in pumpkin. *Plant and Cell Physiology, 36*(6), 1157-1162.

Yamaguchi, M., & Joslyn, M. (1952). Purification and properties of dehydroascorbic acid reductase of peas (Pisum sativum). *Archives of biochemistry and biophysics, 38*(1), 451-465.

Yang, T., & Poovaiah, B. (2002). Hydrogen peroxide homeostasis: activation of plant catalase by calcium/calmodulin. *Proceedings of the National Academy of Sciences, 99*(6), 4097-4102.

Yang, X., Chen, X., Ge, Q., Li, B., Tong, Y., Zhang, A., . . . Lu, C. (2006). Tolerance of photosynthesis to photoinhibition, high temperature and drought stress in flag leaves of wheat: A comparison between a hybridization line and its parents grown under field conditions. *Plant Science, 171*(3), 389-397.

Yin, H., Chen, Q., & Yi, M. (2008). Effects of short-term heat stress on oxidative damage and responses of antioxidant system in Lilium longiflorum. *Plant Growth Regulation, 54*(1), 45-54.

Yost, F. J., & Fridovich, I. (1973). An iron-containing superoxide dismutase from Escherichia coli. *Journal of Biological Chemistry, 248*(14), 4905-4908.

Yousuf, P. Y., Hakeem, K. U. R., Chandna, R., & Ahmad, P. (2012). Role of glutathione reductase in plant abiotic stress *Abiotic Stress Responses in Plants* (pp. 149-158): Springer.

ZHANG, J. H., HUANG, W. D., LIU, Y. P., & PAN, Q. H. (2005). Effects of Temperature Acclimation Pretreatment on the Ultrastructure of Mesophyll Cells in Young Grape Plants (Vitis vinifera L. cv. Jingxiu) Under Cross-Temperature Stresses. *Journal of Integrative Plant Biology, 47*(8), 959-970.

Zhang, Y.-J., Wang, W., Yang, H.-L., Li, Y., Kang, X.-Y., Wang, X.-R., & Yang, Z.-L. (2015). Molecular properties and functional divergence of the dehydroascorbate reductase gene family in lower and higher plants. *PloS one, 10*(12), e0145038.