RESPONSES OF MAIZE ANTIOXIDANT ENZYMES TO DROUGHT STRESS

By

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A thesis submitted in partial fulfilment of the requirement for the degree of Master of Science in the Department of Biotechnology, University of the Western Cape.

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KEY WORDS

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Catalase (CAT)

Ascorbate peroxidase (APX)

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Cell death

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ABSTRACT

Maize (*Zea mays* L) was subjected to drought stress for 28 days. The effects of the drought stress on growth, H_2O_2 content and lipid peroxidation were investigated and the activities of antioxidant enzymes [superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT)], were measured. Reverse transcription quantitative PCR (RT-qPCR) was used to measure relative expression of *APX* and *CAT* genes in response to drought stress. Maize biomass was significantly reduced and cell death was higher in drought-stressed plants. Similarly, the activities of antioxidant enzymes (SOD, APX and CAT) increased in drought-stressed plants. Lipid peroxidation (measured as malondialdehyde content) and hydrogen peroxide content increased in drought-stressed plants. The expression of *APX* genes in drought-stressed (water-deprived) leaves was significantly higher than the well-watered control. *CAT* gene expression showed differential response between the leaves and roots.

DECLARATION

I declare that "Responses of maize antioxidant enzymes to drought stress" 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.....

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To my wife, thank you for your patience, support and understanding.

ABBREVIATIONS

ABA	Abscisic acid
ANOVA	Analysis of variance
AOS	Active oxygen species
APS	Ammonium persulfate
APX	Ascorbate peroxidase
AsA	Ascorbic acid
AsA-GSH cycle	Ascorbate-glutathione cycle
BSA	Bovine serum albumin
CAT	Catalase
cm	Centimeters
Cu/Zn-SOD	Copper zinc superoxide dismutase
dH_2O	Distilled water
DHAR	Dehydroascorbate reductase
DHA	Dehydroascorbate
DNA	Deoxyribonucleic acid
DTNB	Nitrobenzoic acid
EDTA	Ethylenediaminetetraacetic acid
ETC	Electron transport chain

FAO	Food and Agricultural Organization of the United Nations
FDA	Food and Drug Administration
Fe-SOD	Iron superoxide dismutase
FW	Fresh weight
GPx	Glutathione peroxidase
GSH	Glutathione
GR	Glutathione reductase
KCN	Potassium cyanide
LP	Lipid peroxidation
MDA	Malondialdehyde
mg	Milligrams
MDHAR	Monodehydroascorbate Reductase (MDHAR)
mm	Millimeter
ml	Milliliter
mM	Millimolar
Mn-SOD	Manganese superoxide dismutase
MTT	(3-(4, 5-Dimethylthiazol-2-yl)-2, 5- Diphenyltetrazollium Bromide)
NADPH	Nicotinamide adenine dinucleotide phosphate

NBT	Nitro blue tetrazolium chloride
NO	Nitric Oxide
PAGE	Polyacrylamide gel electrophoresis
PCD	Programmed cell death
PMS	Phenazine methosulfate
POD	Peroxidase
PSI	Photosystem I
PSII	Photosystem II
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
RT- qPCR	Real-time quantitative polymerase chain reaction
RWC	Relative water content
SA	South Africa
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid

TEMED	N,N,N',N'-Tetramethylethylenediamine
µl	Microlitre
USA	United States of America
WDL	Water-deprived leaves
WDR	Water-deprived roots
WWL	Well-watered Leaves
WWR	Well-watered roots
w/v	Weight by volume

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AIMS AND OBJECTIVES

The aim of the study was to determine the effect of drought on the antioxidant activities of maize in respect of the activity of selected enzymes involved in the scavenging of reactive oxygen species. This is achieved by addressing the following objectives:

1) To determine the impact of drought stress on the growth of maize by measuring biomass and cell viability.

2) To determine the impact of drought stress by measuring the levels of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA).

3) To determine the responses of antioxidant enzymes, namely superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) to drought stress by in-gel native PAGE activity analysis and spectrophotometry.

4) To analyse *APX* and *CAT* gene expression levels in response to drought stress by RT-qPCR.

CHAPTER 1

Literature Review

1.1 Introduction

In nature, plants are constantly faced with environmental stress factors such as drought, salinity and extreme temperatures. These stresses have a negative effect on growth and development of plants. This becomes a serious concern for agriculture and food security where crop plants, such as maize, a staple food in the developing world, is involved where production and yield are negatively affected by these stress factors. This is so because maize provides for at least 30% of the food calories of over 4.5 billion people in 94 developing countries (Shiferaw *et al*., 2011). Maize is easy to grow in different environments, easy to harvest and store. This makes it the most widely distributed crop in the world, with America leading in its production, followed by Brazil, China, Mexico and India respectively (Chaudhary *et al*., 2014). Maize can be used for multiple purposes but, in the developing world, it is mainly grown for consumption directly as a food source for both humans and animals. Nutritionally, maize is rich in starch, proteins to some limited extent and contains some phosphorus, riboflavin and edible oil (Chaudhary *et al*., 2014).

However, as mentioned above, environmental stresses are the biggest threat to crop growth and yield worldwide. Fifty to seventy percent in crop yield reduction due to the combination of drought, salinity and extreme temperatures have been recorded in the past (Mittler, 2006). Of these stress factors (figure 1), drought is a major growth limiting factor, resulting in reduction in crop productivity worldwide (Aksoy, 2008; Mahajan and Tuteja, 2005; Ghannoum, 2008; Shao *et al*., 2009). It is predicted that drought will increase in severity and frequency,

drastically affecting global agricultural systems (Cutforth *et al.*, 2007; IPCC, 2007 and 2008; Mittler and Blumwald, 2010).

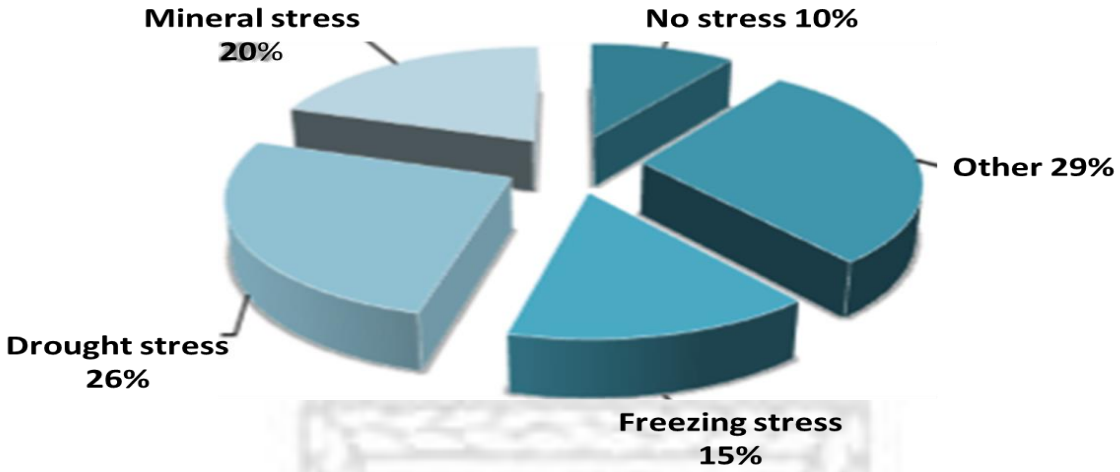

Figure 1.1: The effects of abiotic stress on crop production (Blum *et al*., 1996).

It has been estimated that over 24 million tonnes of annual maize yield has been lost due to drought stress (Maiti *et al*., 1996; Heisey and Edmeades, 1999). Drought stress negatively affects plant growth and development by inducing morphological, biochemical and physiological changes resulting in decreased transpiration and photosynthetic rate and leaf senescence (Manavalan *et al*., 2009, Chaves *et al*., 2003). As a result of exposure to drought stress, (including other abiotic and biotic stress), plants excessively produce reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot -}$), singlet oxygen ($^1O_2$) and the hydroxyl radical ($^{\cdot}OH$). Reactive oxygen species are produced, under normal cellular metabolism as by-products in mitochondria, chloroplast and perixomes (Ahmad *et al*., 2008). Under these non-stressful growth conditions, ROS are thought to be of benefit to the plant by acting as signal molecules for modulation of processes such as growth and development, stomatal closure, regulation of gene expression and in stress defence pathways against pathogens (Sunkar and Zhu, 2004; Mittler, 2002; Kwak *et al*., 2003; Overmyer *et al.*, 2003). To keep ROS production at low levels that are not harmful to the plant cell, ROS are continuously

removed to maintain the balance between production and removal (Gupta *et al*., 2015). While ROS can play a beneficial role to the plant, their overproduction due to stress factors can lead to oxidative stress damage, resulting in lipid peroxidation, protein oxidation, DNA damage and finally plant death (Mittler, 2002; Mahajan and Tuteja, 2005; Gill and Tuteja, 2010; Sharma *et al.*, 2012). To counteract the oxidative stress imposed by excessive ROS, plants have developed antioxidative defence strategies that include enzymatic and no-enzymatic mechanisms (Tang *et al*., 2006). The enzymatic defence system is comprised of superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), including enzymes of the glutathione-ascorbate cycle such as glutathione reductase (GR), dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR) (Bowler *et al*., 1992; Willekens *et al*., 1997; Noctor and Foyer, 1998; Smirnoff, 2005). Defence against the excessive production of ROS during drought requires that plants utilise these diverse and complicated antioxidant defence mechanisms as a way to escape, avoid or tolerate drought stress-induced oxidative stress (Avramova *et al*., 2017). Considering that drought is predicted to pose a major challenge due to near-future global climate conditions (IPCC, 2014; Lobell *et al*., 2011; Burke *et al*., 2009), it is important for plant biotechnologists and breeders to improve maize tolerance to drought and thereby sustain production and yield. For this to happen, the complex mechanisms of drought tolerance, avoidance and escape in maize must be elucidated in order to construct drought tolerant maize. To this end, this study sought to analyse how selected antioxidant enzymes behave in response to drought stress in maize. In addition, indicators of oxidative stress, such as malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) concentration levels were measured, together with biomass and cell death. The analysis utilised biochemical assays such as H_2O_2 content, MDA measurement as a marker for lipid peroxidation together with spectrophotometric and native PAGE in-gel activity measurements of SOD, APX and CAT isoforms.

1.2 The agro-economic importance of maize

Maize (*Zea mays,* L), otherwise known as corn, originated in Mexico some 7000 years ago as a wild grass (Ranum *et al*., 2014). However, the Native Americans managed to convert it to a nutritional food. Maize is considered to be one of the first crops to be cultivated by farmers 7000 to 10000 years ago (Smith, 2001; Piperno and Flannery, 2001; FAO, 2000; the International Plant Genetic Resources Institute, 2002). Maize is among the most efficient plants for capturing the energy of the sun and converts it to food. It is versatile and can adapt to various conditions of humidity, sunlight and altitude (OECD, 2003). Owing to its versatility, ease of growth, harvest and storage, maize is produced across the world (figure 1. 2) in different climatic conditions such as tropical, sub-tropical, temperate and semi-arid zones (Paliwal, 2000a; Farnham *et al*., 2003). It has the highest yield per hector amongst all major grain crops worldwide (Du Plessis, 2003). Maize is relatively low in price when compared to other major staple crops (DAFF, 2017).

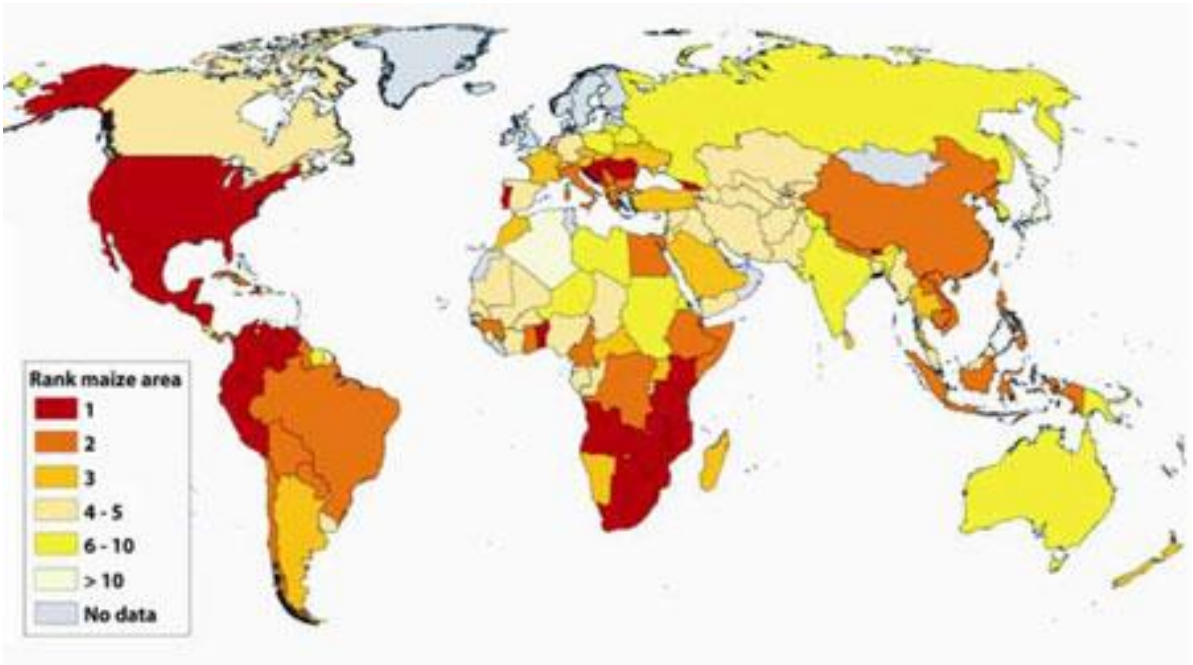

Figure1. 2: Rank of maize by area sown worldwide (Source: FAOSTAT, 2010).

Nutritionally, maize contains about 72% starch, 10% protein and 4% fat with an energy of 365 Kcal/100 g, making it a reliable staple food source (FAO, 2009). It has more riboflavin (vitamin B_3) than wheat or rice and is rich in phosphorus (Okoruwa and Kling, 1996). It contains vitamin B-complexes such as B_1 (thiamine), B_2 (niacin), B_5 (pantothenic acid) and B_6. Maize contains some vitamin C, K and A, and significant quantities of beta-carotene for yellow maize. Vitamin B-complex is crucial for healthy hair and skin, proper functioning of the brain, heart and digestive system (Kumar and Jhariya, 2013). It also contains selenium that is beneficial to human immune system (Kumar and Jhariya, 2013). It is a poor source of calcium, folate and iron (Ranum *et al.*, 2014). According to CIMMYT, maize makes up close to two-thirds of the world's food energy intake and contributing fifty to seventy percent (50-70 %) of the total calories in the diets of people living in developing countries (Ekpa *et al.*, 2018). Maize is equally an economically valuable crop. It is multifunctional with many applications in food (for animals and humans) and biofuel production. These products include starch, ethanol, oil, beverages and sweeteners. Starch from maize is further processed, through enzymatic mechanisms, to produce products such as glue, fireworks, paint, syrup, shoe polish, cosmetics, aspirin, beer, ice cream and batteries (Ranum *et al*., 2014). Maize can also be used as filler in products such as plastic, cigarette papers, adhesives, paper and insulation. Its use can also be found in the production of synthetic rubber, dyes, nylon and explosives (Nuss and Tanumihardjo, 2010). However, maize utilisation and application varies between the developing and the developed countries. In sub-Saharan Africa, Latin America, China and India (figure 1.3), maize is the staple food for the majority of the population and a major feed grain for livestock (Morris, 1998; DAFF, 2013 and 2016). Over 300 million people in Africa alone depend on maize as their main staple food. Seventy-seven percent (77%) of maize produced in Sub-Saharan Africa is used as food and only twelve percent (12%) as animal feed (TIAPD, 2005; Shiferaw *et al.*, 2011).

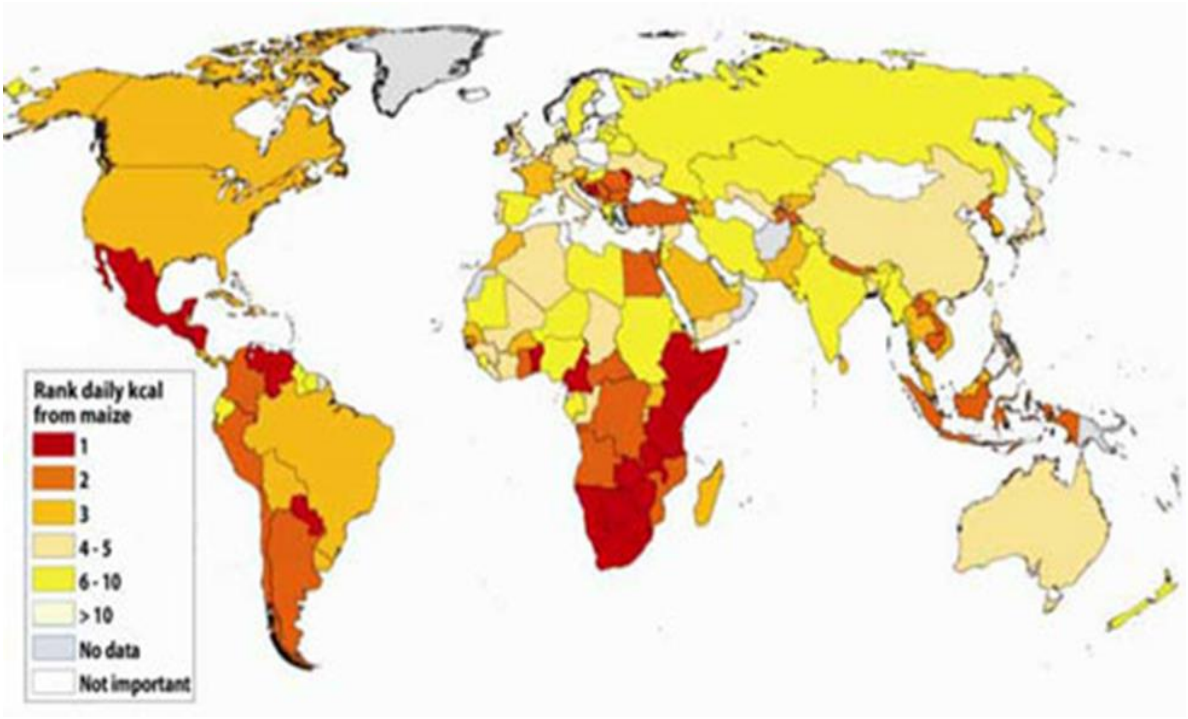

Figure 1.3: Rank of maize as a food crop worldwide (FAOSTAT, 2010).

Maize is processed to produce food such as porridges, bread and alcoholic beverages (Table 1). By contrast, in Asia, 70% of the total maize produced is used for animal feed purposes, 23% as food and 7% for other uses (Prasanna, 2011).

Table1: Staple food that is prepared from maize in different countries in Africa. (Ranum *et al*., 2014).

FOOD	COUNTRY
Porridges, thin fermented:	
Ogi	Nigeria
Uji	East Africa
Mahewu	South Africa
Porridges, thick:	
To, tuwo, asido, akoume	West Africa, North Africa, Horn of Africa
Ugali	Kenya, Tanzania
Steamed food:	
Couscous, cuzcuz	Africa
Bread, unfermented:	
Corn bread	Africa
Bread, fermented:	
Injani	Ethiopia
Fermented Dough	
Kenkey, ablo	Ghana, Benin, Togo
Alcoholic beverages:	
Urawga, mwenge	Kenya, Uganda
Chibuko	South Africa
Pito	Nigeria
Tella	Ethiopia
Busas	Kenya
Opaque beer	Zambia
Munkoyo	Zambia

However, in developed countries, approximately seventy percent (70%) of maize is used for animal feed and only three percent (3%) is consumed by humans. The remaining portion is used for biofuel production, seeds and industrial products (TIAPD, 2005). It is processed mainly to produce products such as glue, fuel ethanol and industrial ethanol (Ranum *et al.*, 2014).

Maize is grown in over 170 million hectares the world over, with a yearly production of approximately 790 million tons (Chulze, 2010). It is a major source of income for many farmers

and the second most important staple crop in the world (FAO, 2008). As such, an increase in maize productivity correlates positively with an increase in employment, resulting in increase in household incomes (Jayne *et al*., 2006). In Southern Africa, maize production depends heavily on rainfall, thus it is affected by changes in climate conditions such as flooding and drought. These changing climate conditions and rainfall patterns may, in any particular season, determine the length of the growing season and planting period. In turn, this affects production and yield.

Table 2: Plantings, production and yields of commercial maize in South Africa from 2011/12 to 2015/16. (Source: DAFF, 2016).

Season	2011/12	2012/13	2013/14	2014/15	2015/16
Plantings (ha)	2 699 200	2 781 200	2 688 200	2 652 850	1 946 750
Production (t)	12 120 656	11 810 600	14 250 000	9 955 000	7 536 875
Yield (t/ha)	4,49	4,25	5,30	3,75	3,87

Clearly, the drought condition of the 2015/16 period resulted in a decline in maize yield (Table 2). In the last five planting seasons, maize was the largest contributor (48%) towards the gross value of agricultural production (quantity produced and prices received by producers) of field crop compared to other crops in South Africa (DAFF, 2016).

In October 2016, the United States Foreign Agricultural Services reported that the maize world production for 2016/17 was estimated to be 2014 million tons while South Africa, the major producer of maize in Africa produced 14 million tons of maize (FAO, 2016). This decline has been attributed to rising global temperatures that results in drought.

A major challenge in the production of maize worldwide is drought. Of the seventy percent (70%) fresh water that is used in agriculture worldwide, developing countries account for 86% (Edmeades, 2013). Water constitutes about 80 – 95 % mass of growing plant tissues, making it

the most vital requirement for plant growth and development. Losses of approximately twenty-four million tons in yield were reported annually due to drought stress in maize (Maiti *et al.*, 1996; Heisey and Edmeades, 1999).

1.3 The broad agro-economic impact of drought

Drought can be described as a period of below average precipitation in a given region at a given time, causing long shortages of water supply, whether atmospheric, surface water or ground water. It occurs when soil moisture level and relative humidity in the air are low while atmospheric temperature is high. It may last for months or even years. Thus, the natural evaporation cycle between the earth and atmosphere that contributes to rainfall is affected.

Drought is considered to be amongst the most destructive natural disasters all over the world with devastating socio-economic impact. Areas of the world that are most vulnerable to drought are those that have annual rainfall below 500 mm. South Africa, being a country with a semi-arid to arid and highly variable weather pattern, is highly prone to drought conditions. Drought, which has recently devastated crop planting areas of the country, is a recurrent characteristic feature of the country's highly variable climate and weather extremes.

South Africa's annual average rainfall is approximately 450 mm and this makes the country prone to recurrent droughts (WRC, 2015). South Africa’s climate is characterised by periods of wet spells also called La Nina (years receiving above-normal rainfall) and dry spells also called El Niño (years receiving below-normal rainfall). Between 1991 and 1992 Southern Africa experienced what was regarded as the worst drought of the 20th century (WRC, 2015). This drought was driven by a powerful El Niño event that was associated with below normal rainfall. The agricultural sector was the first to feel the effects of the drought through decreased water quality and quantity, crop destruction and huge agricultural losses. Groundwater reservoirs

were depleted, fresh water availability was reduced, resulting in the use of unsafe water (Ujenza and Abiodun, 2015). Across the Southern African region, the drought resulted in food insecurity forcing about 30 million people to the brink of famine (Holloway, 2000; Kandji *et al*., 2006). Maize production was at its lowest with a 40% reduction (Glantz *et al*., 1997, Unganai and Kogan, 1998; UNISDR, 2002). In excess of five million metric tons of cereal had to be imported to compensate for the deficit. It is estimated that over 49 000 jobs in the agricultural sector were lost. However, the 1991-1992 drought was surpassed by the recent 2015/16 drought phenomenon (FAO, 2015). All nine South African provinces were affected and the five major maize crop producing regions (figure 4) were declared disaster areas (BFAP, 2016). The South African Weather Services recorded 2015 as the driest year in South Africa dating back to 1904. The average yearly rainfall between 1904 and 2015 was 608 mm across South Africa, whereas in 2015 alone an average of 406 mm was reported. By contrast, 1945 was the year with the lowest recorded rainfall of 437mm (AgriSA, 2016).

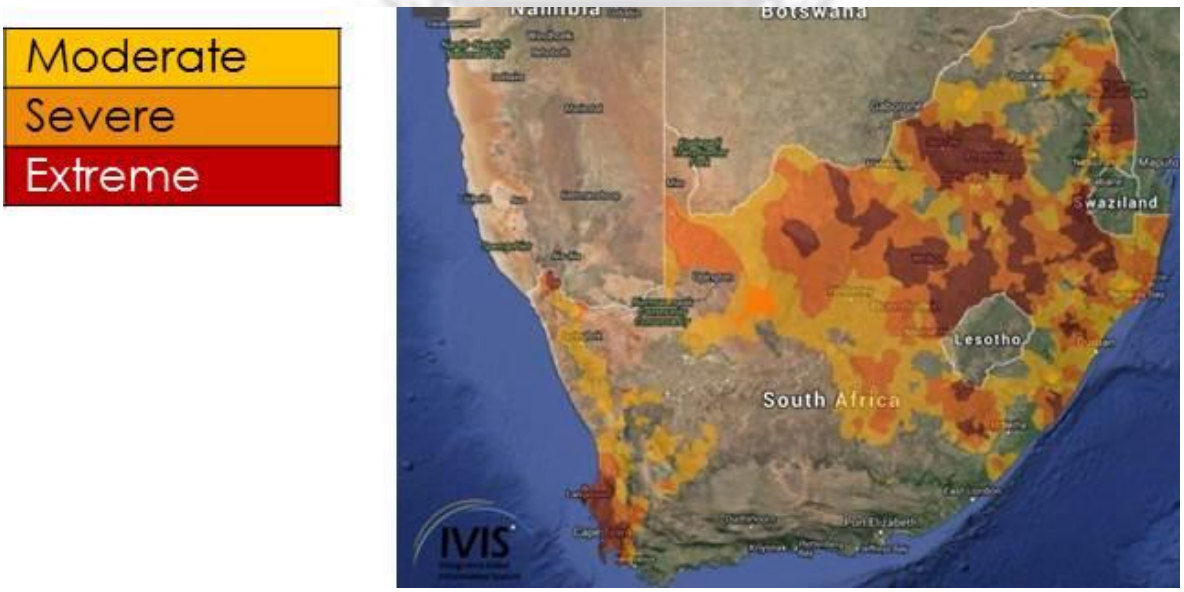

Figure 1.4: Standard Precipitation Index (SPI) and drought, 2015. Major maize production areas that were affected by extreme drought in 2015 (BFAP, 2016).

The negative impact of drought can be on everything from the environment, employment, economy, human health and agriculture (Kang *et al*., 2009). The year 2016 has already recorded reduced harvest due to the on-going drought. To this day, the Western Cape government is still implementing water restrictions to businesses and communities. The on-going drought has resulted in high unemployment rates, less consumer purchasing power and a rise in debt servicing costs for the farmers (AgriSA, 2016). Figure 5 provide the links that exist between interdependent economic sectors that rely on agriculture and the impact that drought may have on them. For example, the Sundays River Citrus Company (SRCC), just outside Port Elizabeth in the Eastern Cape Province of South Africa has reportedly lost between R1 billion to R1.5 billion in revenue because of the 2017/18 drought (Matavire, 2018). The SRCC is the largest grower, packer and exporter of South African Citrus. It supplies retailers and wholesalers across South Africa and exports internationally. As a consequence of drought, the company's exports and employee numbers were reduced by 30% and 20% or more, respectively (Matavire, 2018). Farmers and grazing land were also directly affected by the drought. Out of 967 farmers, 10% were commercial farmers, 58 % small scale farmers and 32 % were subsistence farmers (Matavire, 2018). Since South Africa is a major producer and net exporter of crops in Southern Africa, Southern African countries such as Zimbabwe, eSwatini, Lesotho, Namibia and Botswana, have been negatively affected by the low levels of food production in South Africa consequent to drought. This has pushed food prices up, resulting in food insecurity in those countries (UNOCHA, 2016).

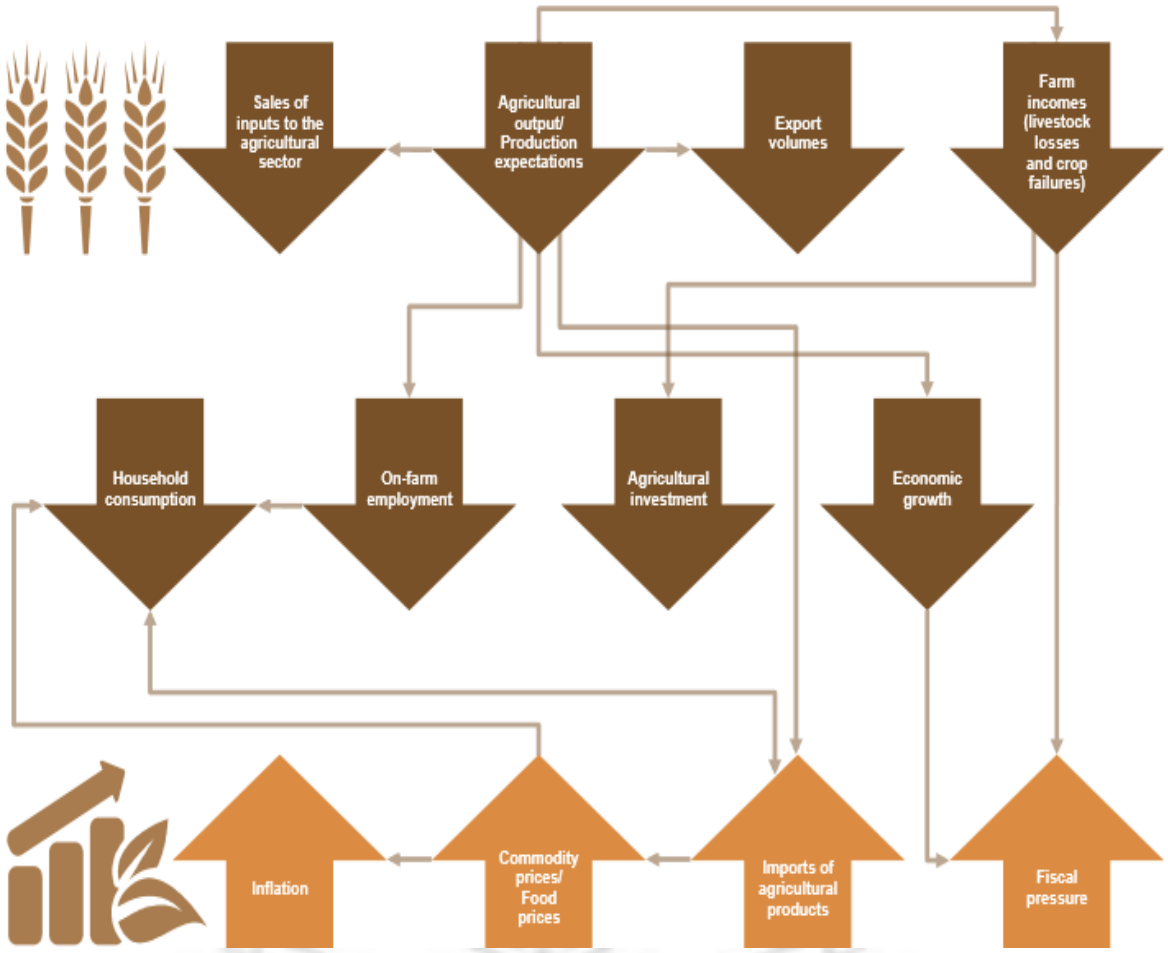

Figure 1.5: The broader economic impact of drought (AgriSA, 2016).

1.4 The impact of drought on maize

As all maize farmers around the world would know, especially in rain-fed farms, that any lack of water would greatly affect maize production and yield. This is because maize is a drought sensitive crop and, consequently, drought stress affects its growth and development, from seedling emergence until grain maturity (figure 1.6). Maize growth and development steps refer to parameters such as height, biomass, fresh weight, leaf area, root length and stem diameter. All of these parameters are negatively affected during drought (Khan *et al*., 2001;

Zhao *et al*., 2006). Any amount of yield loss due to drought depends on the severity of the drought and maize growth stage (Heinigre, 2000). For example, closer to pollination, drought stress can result in significant yield losses than when drought stress occurs before or after this stage (Lee, 2018). However, severe drought stress at the early vegetative stage may lead to complete crop failure because the maize plant may not even reach the reproductive stage (Aslam *et al*., 2015). Maize growth and development can be divided into two main stages: the vegetative and reproductive stages. These stages can be further divided into sub-stages like emergence, tassel and silk development. As it is with most other crop plants, there are certain growth stages in maize that are more sensitive to drought stress than others. At early growth stages, water requirement is low but it increases when maize growth reaches reproductive stages but decreases again during terminal growth stages (Aslam *et al*., 2015). For example, at specific physiological stages such as wilting just before tasselling, moderate drought can reduce maize yield by 10 – 25 percent (Lauer, 2003). Maize yield is severely affected when drought stress sets in during flowering and pollination, followed by grain-filling (kernel development) and during vegetative growth stages (Lauer, 2003). The growth and development stages at which drought stress has a significant negative impact on maize productivity and yield are briefly introduced below.

1.4.1 The Impact of drought on maize during vegetative growth stages

The vegetative stage is made up of different stages collectively referred to as crop stand establishment. Crop stand establishment include seed germination, emergence and establishment until tasselling. At this stage of development, drought can lead to poor maize stand and may further lead to total failure of seedling establishment (Jaleel *et al*., 2007). Growth, development and maize crop survival depends heavily on seedling establishment efficacy (Hadas, 2004). Seed germination requires water in order to activate metabolism for

the breakdown of seed dormancy and mobilization of nutrient reserves (Aslam, 2015). However, drought stress hinders this process and thereby reducing seed viability. During plant growth from the emergence stage, drought stress can reduce plant and leaf size, resulting in reduced maize height and leaf area. Furthermore, plumule and radicle growth are reduced following seed germination as a result of drought stress, resulting in abnormal seedling growth (Gharoobi *et al*., 2012).

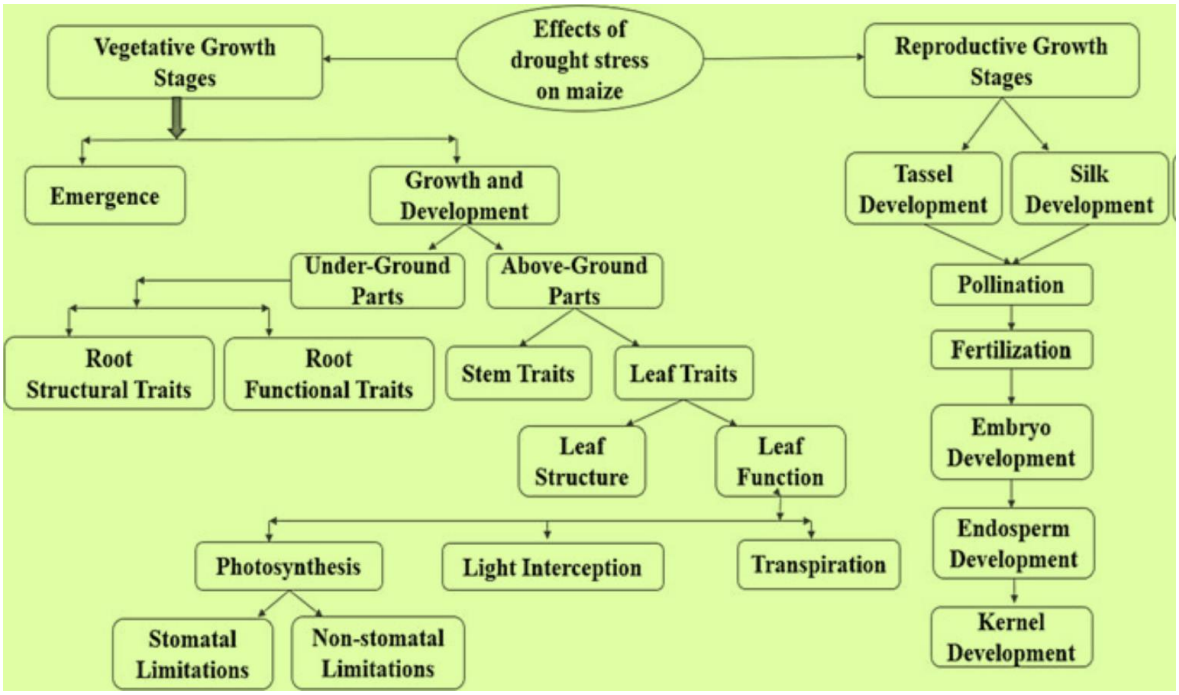

Figure 1.6: Maize vegetative and reproductive growth stages that are negatively affected by drought (Aslam *et al*., 2015).

1.4.2 The impact of drought on maize during reproductive growth stages

The reproductive growth phase is comprised of stages such as tassel and silk development, pollination, fertilization, embryo, endosperm and kernel development and is the most sensitive to drought stress (Aslam *et al*., 2015). Drought stress has the greatest impact during tasselling and pollination resulting in significant yield reduction of about 3-8% on each day of drought stress (Lee, 2015). The development of the embryo after pollination is also severely delayed by

drought. Furthermore, during kernel development, drought stress can reduce yield by up to 2.5 to 5.8 % with each day of stress (Lauer, 2003). During this stage, drought stress may also lead to an increase in leaf death rate, low kernel weight and a shorter period of grain-filling.

1.5 The effects of drought-induced stress on plants

When the water supply to the roots of a plant becomes limiting or the transpiration rate becomes intense, plants will experience water stress. Therefore, water stress is mainly caused by water deficit (Lisar *et al.*, 2012). Water stress also changes the physical environment for plant growth and physiology (Kramer, 1980). Every chemical process in the growing period of a plant requires water, directly or indirectly. Water shortage has profound effects on plant growth and development. Even plants with an optimum water supply experience transient water shortage periods, where water absorption cannot compensate for water loss by transpiration (Simonneau and Habib, 1994). Without water, seeds will not germinate. In fact, a total of approximately 250 litres of water is used by the maize plant during its growth and development until maturity (Du Plessis, 2003). In response to drought stress, plants have developed many stress tolerance and adaptation mechanisms (Gockay, 2012). These response mechanisms are regulated by the period, intensity, the progression rate of the imposed drought (Pinheiro and Chavez, 2011) and inherent genetics of the plant species (Mattos and Moretti, 2015). The different levels of drought and the corresponding effects and responses are summarised in figure 1.7**.** At the morpho-anatomical and physiological level, mild drought stress is characterised by a decrease in cell extension and growth, closure of stomata, reduction of water content and turgor loss, while severe drought stress on the other hand, results in the disturbance of metabolism, damage to photosynthetic processes and finally to plant death (Jaleel *et al*., 2009). Various physiological and biochemical processes, such as nutrient metabolism, ion uptake, carbohydrate content and protein synthesis are altered by

drought stress (Farooq *et al*., 2008). Thus, the chemical and physical composition of plant tissues may also be modified, leading to changes in plant quality such as the taste of the fruits (Akinci and Losel, 2012).

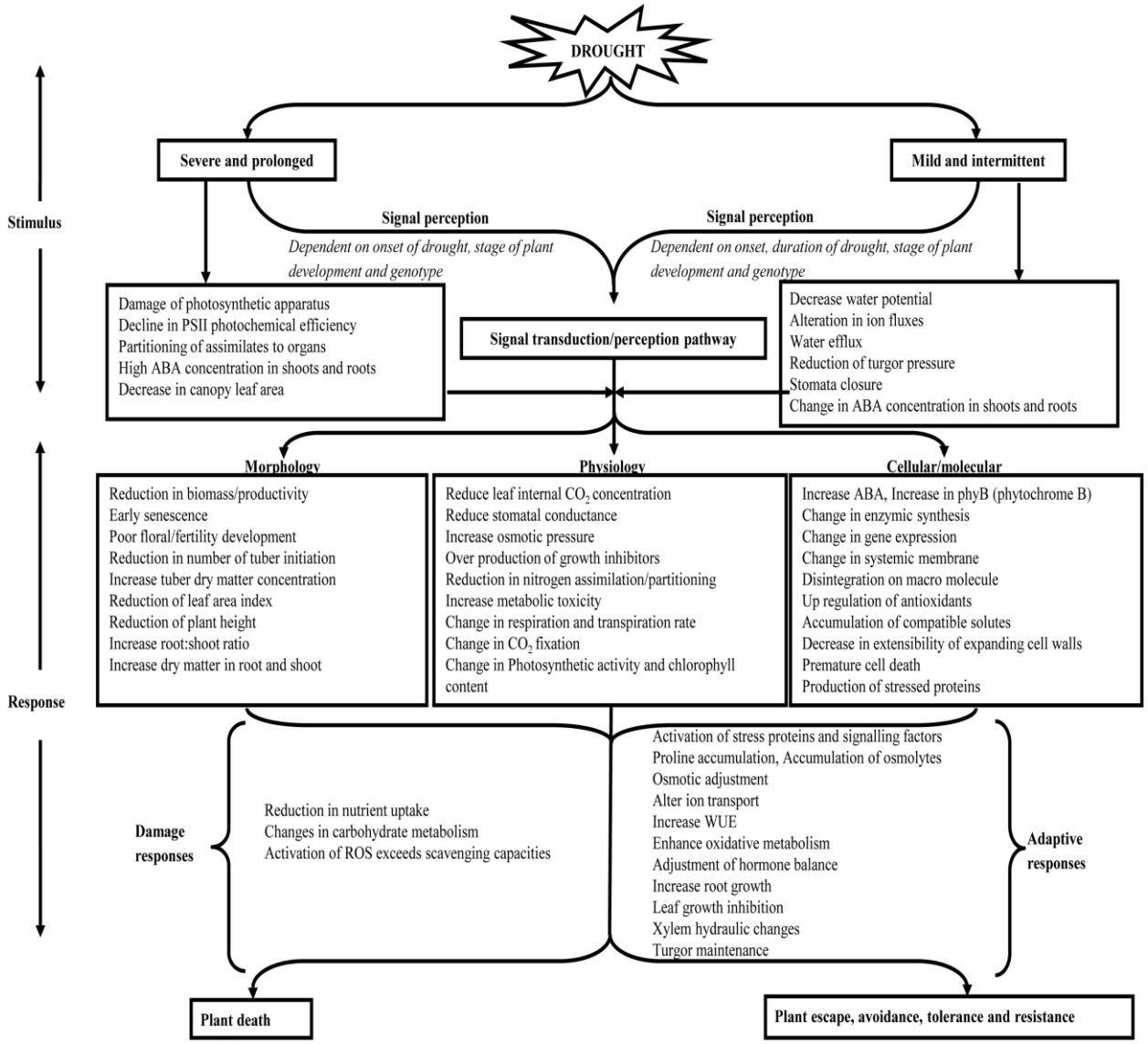

Figure 1.7: Flow diagram detailing the effects of drought and how plants are affected at morphological, physiological and molecular level. (Obidiegwu *et al*., 2015).

At the molecular and cellular levels, severe drought induces oxidative stress damage in plant cells, which can be detected by the accumulation of lipid peroxides (due to peroxidation of unsaturated fatty acids in membranes), oxidized proteins (denatured proteins) or modified

DNA. Oxidative stress occurs when there is excessive production of reactive oxygen species (ROS) such as O_2^-, $^1O_2^-$, H_2O_2 and OH^- (Cruz de Carvalho, 2008; Fridovich, 1995; Bolwell *et al.*, 2002) in plants that are experiencing severe drought, resulting in the disturbance of a balance between ROS production and antioxidant defences. However, in order to protect themselves against oxidative stress damage, plants have evolved enzymatic and non-enzymatic antioxidant systems in order to control the production levels of ROS. The enzymatic antioxidant systems include enzymes such as catalases (CAT), superoxide dismutase (SOD), ascorbate peroxidases (APX) and other components of the ascorbate-glutathione cycle; whereas the non-enzymatic antioxidant system include ascorbic acid, carotenoids, osmolytes and polyphenols (Apel and Hirt, 2004; Xiong and Zhu, 2002, Rodrigues, 2002). Superoxide dismutase (SOD) is a class of metalloenzymes that catalyse the detoxification of superoxide (O_2^-) to hydrogen peroxide (H_2O_2) and oxygen (O_2) and are considered as the first line of defence against ROS. Superoxide is a precursor of the highly oxidizing hydroxyl radical that gets formed when O_2^- reacts with H_2O_2 and therefore SOD is a critical defence enzyme against oxidative stress in plants. Ascorbate peroxidases (APX) use reduced ascorbate to detoxify H_2O_2 into O_2 and water and are thus crucial antioxidant enzymes towards prevention of oxidative stress in plants. The reduced ascorbate used by APX is regenerated by dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase using glutathione, which is regenerated by glutathione reductase (GR). This system of enzymes and non-enzymatic antioxidants contributes to the redox state of the plant and is thus crucial for the regulation of the level of reactive oxygen species (ROS, such as O_2^- and H_2O_2) in plant cells. Some of the effects of drought stress on various chemical processes, molecules and morpho-physiological growth of plants are discussed below.

1.5.1 Physiological effects of drought stress on plants

1.5.1.1 The effect of drought-induced stress on root signalling

Roots are a very important integral part of plants without which plants would not survive. In addition to anchoring the plant in the soil, a plants' extensive root system extracts water from deep in the soil (Boyer, 1996). Studies on water uptake in lettuce (Johnson *et al*., 2000) and rice (Yadav *et al*., 1997) have shown that the depth of roots is positively correlated with the ability of plants to extract water from deep within the soil. During water deficit stress, roots begin a signal cascade to the shoots through the xylem resulting in physiological changes that determine the level of tolerance to water stress. One of the most important signals involves abscisic acid (ABA) as a root to shoot stress chemical signal in plants that are exposed to drought (Hey *et al*., 2010; Obidiegwu *et al.*, 2015). ABA is a plant stress hormone that plays an important role in response to abiotic stress factors including regulation of stomatal closure, optimization of transpiration and by inducing the activation of many stress-related genes (Cutler *et al*., 2010; Lindemose *et al*., 2013). The levels of ABA increase dramatically in response to drought stress, leading to activation of the expression of a number of drought response genes (Chaves *et al.*, 2003). A substantial increase of ABA has also been documented in the xylem saps during drought, and this leads to an increase in the amount of ABA in different areas of the leaf (Lisar *et al.*, 2016), resulting in the reduction of transpiration and growth (Schachman and Goodger, 2008). It is well-established that high amounts of ABA near guard cells causes the stomata to close in order to conserve water. Other chemical signals that act in conjunction with ABA to control drought-induced stomatal closure include jasmonic acid (Mahouachi *et al.*, 2007; Du *et al*., 2013; Lee *et al.*, 2013), ethylene and gibberellic acid (Skirycz *et al*.,2010; Verelst *et al*., 2010).

1.5.1.2 The effect of drought-induced stress on photosynthesis

Photosynthesis can be defined as a process by which green plants use sunlight as a sole source of energy to produce nutrients and oxygen from carbon dioxide and water. Without photosynthesis, life on Earth would be impossible. There would be no food to support life and the Earth's atmosphere would lose its oxygen. Thus, any lack of adequate water supply to the plants results in reduced photosynthesis as a result of decreased leaf area, premature leaf senescence and impaired photosynthetic machinery (Farooq *et al*., 2009). Essentially all the important components of photosynthesis, including the carbon reduction cycle, thylakoid electron transport and the stomatal regulation of CO_2 supply are disturbed during water deficit (Anjum, *et al*., 2011). Stomatal closure is one of the first responses to drought stress which results in reduced rate of photosynthesis. Stomata are the entry points of water loss and CO_2. Plants that have been exposed to drought conditions tend to have lower stomatal conductance which results in water conservation, thus maintaining an adequate leaf water status (Chaves *et al*., 2002). However, when the stomata are closed, the leaves are deprived of CO_2, leading to a decreased photosynthetic carbon assimilation in favour of photorespiration (Anjum *et al*., 2011). With regards to electron transport, the limited supply of CO_2 to the leaf mesophyll causes a reduction in the regeneration of $NADP^+$ in the Calvin cycle (Cruz de Carvalho, 2008), resulting in the generation of ROS.

1.5.1.3 The effect of drought-induced stress on leaf morphology

Leaves make up one of the most important parts of the plant shoot. Through the process of photosynthesis, leaves capture the light energy and convert it to chemical energy which is required by the plant for growth and development. In addition to photosynthesis, leaves are sites of transpiration, guttation, water and nutrient storage. It is therefore not surprising that

the initial signs of drought stress are usually seen on the leaves. Drought stress can have a negatively impact leaf characteristics such as leaf area, leaf mass, leaf size, turgor, leaf bud emergence and chlorophyll content (Hussain and Ali, 2015). Leaf area is an index of stress, plant growth and productivity.

1.5.1.4 The effect of drought-induced stress on root morphology

Roots are responsible for anchoring the plant in the soil and for water and nutrient up-take (Brunner and Godbold, 2007). Most importantly, they sense drought stress and send signals to the shoots (Hamanishi and Campbell, 2011; Spollen and Sharp, 1991). During drought stress, the growth of lateral roots decreases significantly, while that of primary roots is not affected (Deak and Malamy, 2005). The reduction in lateral root formation during drought stress correlated positively with enhanced drought tolerance in Arabidopsis (Seo and Park, 2009). The reduction in lateral roots formation is also accompanied by small branching roots in order to increase the surface area for water absorption (Basu *et al*., 2016), a strategy considered to be an adaptive mechanism for drought tolerance. In another study carried out by Sengupta and Reddy (2011) the expression of xyloglucan endotransglucosylase, an enzyme related to root morphology was induced in response to drought stress while other structural proteins were down-regulated. The authors also found that the alteration in the expression of the proteins under drought stress correlated positively with lateral root development that in turn affects photosynthesis.

1.5.1.5 The effect of drought-induced stress on chlorophyll content

Chlorophyll is a green pigment found in plant cells, algae cells and cyanobacteria. The primary function of chlorophyll molecules is the absorption of energy from light, which provides energy that is essential for photosynthesis (Wright *et al*., 1994). There are three main functions of

chlorophyll in plants, namely to absorb light, to transfer that light energy by resonance energy transfer to a specific chlorophyll pair in the reaction centre of photosystems and finally, charge separation leading to biosynthesis (Farooq *et al*., 2009). The quantity of chlorophyll in the leaves is one of the major factors that affects a plants' photosynthetic ability (Arjenaki *et al.*, 2012; Nageswara *et al.*, 2001; Wright *et al*., 1994). This is so because the amount of leaf chlorophyll in a plant is influenced by environmental stresses such as drought, salinity, heat and cold (Farooq *et al*., 2009). Drought stress inhibits Chl *a/b* synthesis and decreases the content of Chlorophyll *a/b* binding proteins, leading to reduction of the light-harvesting pigment proteins associated with photosystem II (Sayed, 2003). Several other studies on different crops have also reported a reduction in leaf chlorophyll contents due to severe drought stress (IturbeOrmaetxe *et al*., 1998; Ommen *et al*., 1999; Manivannan *et al*., 2007a). The reduction in chlorophyll content as a result of exposure to drought stress is mainly the result of damage to chloroplasts caused by ROS (Smirnoff, 1995).

1.6 The effects of drought stress on cellular structures and macromolecules

1.6.1 Reactive Oxygen Species (ROS)

Reactive oxygen species play an important signalling role in plants; controlling processes such as development, growth, response to biotic and abiotic environmental stress and programmed cell death (Bailey-Serres and Mittler, 2006). During normal plant growth, ROS production and removal is under the control of different antioxidative defence mechanisms comprising of non-enzymatic as well as enzymatic, so that plants are protected from the harmful effects of the active oxygen molecules (GÖKÇAY, 2012; Noctor and Foyer, 1998). However, when plants are subjected to stressful conditions such as drought, salinity, chilling, metal toxicity, UV-B radiation as well as pathogen attack, ROS are produced at higher levels than normal (Sharma *et*

al., 2012). When ROS levels exceeds the defence mechanisms, a cell is said to be in a state of oxidative stress, and this may cause lipid peroxidation, protein oxidation, damage to DNA, inhibition of enzymes and activation of programmed cell death (Shar *et al*., 2001; Mishra *et al*., 2011; Meriga *et al*., 2004). Thus, ROS can be both beneficial and harmful (see figure 1.8 for the targets of ROS) depending on their concentration.

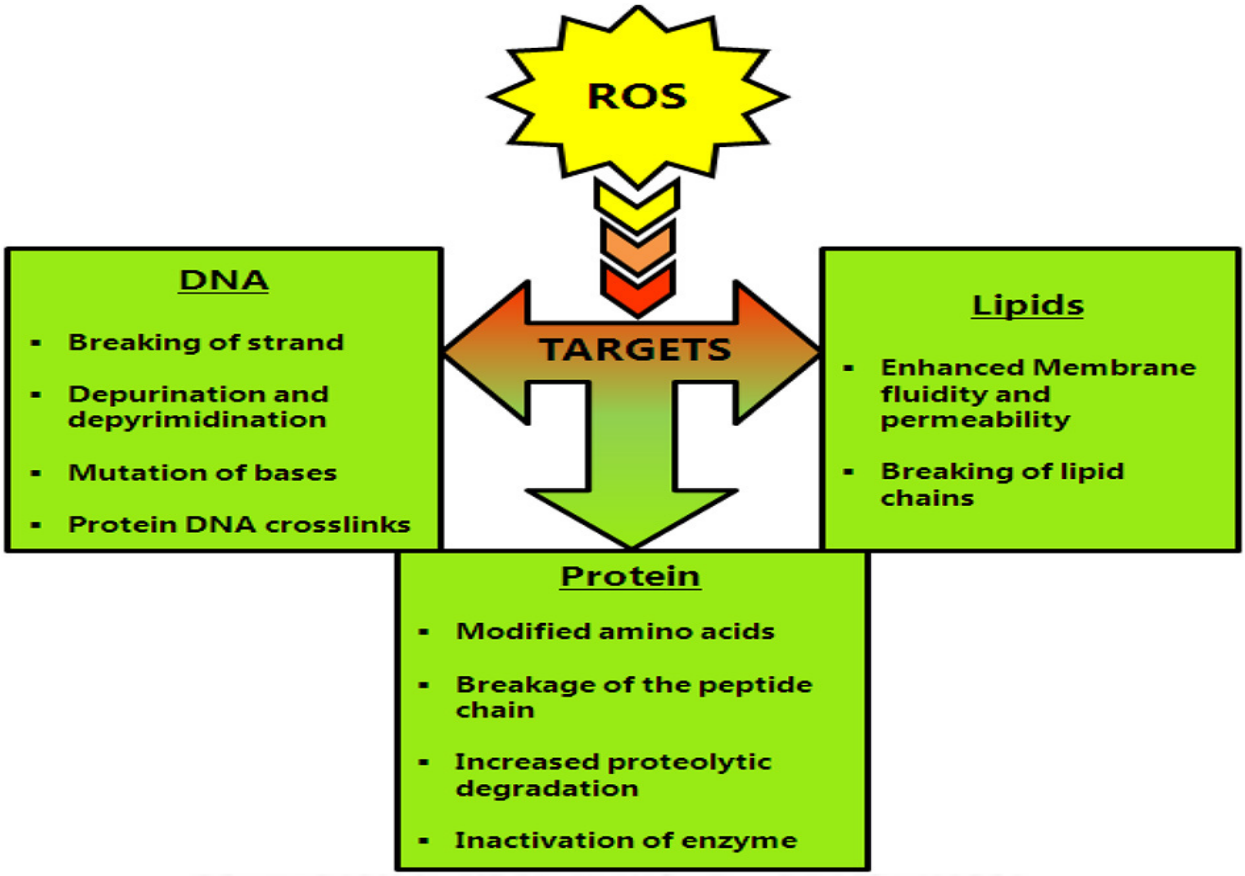

Figure 1.8: The different targets of ROS (Das and Roychoudhury, 2014).

1.6.2 Protein oxidation

Oxidative stress in plants leads to protein oxidation. Consequently, different types of modifications occur. These include direct modifications such as carboxylation, nitrosylation, glutathionylation and disulphide bond formation. Indirect modification of proteins can also occur by interaction with the products of lipid peroxidation (Yamauchi *et al*., 2008). High levels of modified proteins have been reported in plants undergoing oxidative stress (Sharma and Dubey, 2005; Maheshwari and Dubey, 2009; Tanou *et al*., 2009) including protein carboxylation which is often used as a marker of protein oxidation (Moller *et al*., 2007; Moller and Kristensen, 2004). Overproduction of ROS can also lead to site-specific modification of amino acids like Trp, Pro, Lys, Arg and Thr and aggregation of protease resistant cross-linked reaction products (Barlett and Stadtman 1997), fragmentation of the peptide chain, altered electric charge and increased susceptability to proteolytic degradation (Moller *et al*., 2007). The degree of susceptibility of amino acids to ROS attack depends on side group composition of an amino acid. The amino acids that contain sulphur and thiol groups are the most susceptible to ROS attack (Rinalduci *et al*., 2008). Proteolytic digestion is highly increased in oxidized peptides (Cabiscol and Piulats, 2000). The depletion of protein bound thiol groups in the presence of metals such as Cd, Hg and Pb have been reported (Stohs and Bagchi, 1995). $^1O_2$ and OH^- are well-known for oxidizing methionine and cysteine residues in proteins (Das and Roychoudhury, 2014). A number of reports have shown that oxidised methionine or sulfhydryl groups of proteins leads to degradation, conformational changes and protein unfolding (Lyras *et al*., 1997; Keck, 1996; Davies, 1987). The presence of metals such as iron on or near enzyme active sites leads to an irreversible inactivation of the enzyme on oxidation by O_2^- (Fucci *et al*., 1983).

1.6.3 Lipid peroxidation (LP)

Lipid peroxidation is the oxidative degradation of lipids. During oxidative stress, ROS such as O_2^- , H_2O_2 and OH^- radicals can attack the membrane lipid bilayer and induce lipid peroxidation resulting in cell damage (Girotti, 1985; Mittler, 2002; Halliwell, 2006). The primary target for ROS is often the polyunsaturated fatty acids (PUFA) that possess multiple double bonds between two carbon atoms that contain reactive hydrogen atoms and ester linkages. One of the final products of lipid peroxidation is malondialdehyde (MDA). Malondialdehyde accumulation in the cell causes damage to the cell membrane, resulting in changes to membrane fluidity, ion transport, protein cross-linking and enzyme inactivity, ultimately resulting in cell death (Sharma *et al*., 2012; Han *et al*., 2009; Tanou *et al*., 2009; Mishra *et al*., 2011). There are three main mechanisms by which lipid peroxidation takes place: initiation, progression and termination (Smirnoff, 1995). In the initiation phase, a fatty acid radical is produced through a reaction of O_2^- , H_2O_2 or $OH^\cdot$ with PUFA methylene groups, resulting in the formation of hydroperoxides and lipid peroxyl radicals. Highly reactive peroxyl radicals undergo a series of chain reactions leading to propagation of the oxidation of PUFA.

1.6.4 Damage to DNA

ROS oxidatively damage all types of DNA including nuclear, chloroplastic and mitochondrial DNA. These damages are characterised by strand breakage, deoxyribose oxidation, nucleotide removal and modifications that further result in mismatches during replication (Sharma *et al*., 2012; Dizdaroglu, 1993). The damage on the DNA bases occurs via the reaction of OH^- with the double bonds in purine and pyrimidine bases. The removal of hydrogen from the deoxyribose causes sugar damage (Dizdaroglu, 1993). In the process, $^1O_2$ reacts with the guanine base while

O_2^- and H_2O_2 do not react with any bases (Gill and Tuteja, 2010; Dizdaroğlu, 1993; Halliwell and Aruoma, 1991).

1.7 Plant defence and tolerance strategies in response to ROS overproduction due to drought stress

The antioxidant defence system in plants is made up of both enzymatic and non-enzymatic mechanisms. There are generally three ways by which plants respond to drought: (1) drought escape (e.g., short life cycle), (2) drought avoidance (i.e., maintenance of favourable water status during drought using different mechanisms such as stomatal closure and senescence of older leaves), and (3) drought tolerance (i.e., plant's ability to function at low water potential, including the ability to recover after stress) as a result of osmotic adjustment (OA), rigid cell walls, small cells and reactive oxygen species scavenging (Barnabas *et al*., 2008).

1.7.1 Enzymatic defence mechanisms

1.7.1.1 Superoxide dismutase (SOD)

The first line of defence against ROS is superoxide dismutases (SOD), a family of metaloenzymes that catalyses the dismutation of O_2^- to toxic H_2O_2 and molecular oxygen. Superoxide dismutase exists in three isoforms, namely Fe-SOD, Cu/Zn-SOD and Mn-SOD. Most of the SOD isoforms have been found in most of the plant cell compartments (Gomez *et al*., 2004; Veljovic-jovanovic *et al*., 2006). Of all the isoforms, CuZn-SOD is the most abundant and is found in chloroplast, peroxisome, cytosol and apoplast. Mn-SOD is found in mitochondria and in peroxisomes. Fe-SOD is located in chloroplasts. O_2^- is constantly produced in the cell where electron transport chain reactions are present and the phospholipid bilayer is

impermeable to charged O_2^- molecules (Takashani and Asada, 1983). Thus, SODs is important for the scavenging of O_2^- in the cell where O_2^- radicals are produced. Superoxide dismutase activity increases under stressful conditions and this high activity has been used as a marker for resistance to stress (Zaefyzadeh *et al*., 2009). The product of O_2^- dismutation, H_2O_2, may also react with ferric and cupric metal ions, leading to the production of hydroxyl radicals such as hydroxyl. The hydroxyl radical will eventually react with cellular molecules leading to lipid peroxidation, DNA mutations and protein denaturation, ultimately having detrimental effects on the plant (Bowler *et al*., 1991; Salin, 1988). However, H_2O_2 can be scavenged by ascorbate peroxidase (APX, EC 1.11.1.1) or catalase (Alsher *et al*., 2002).

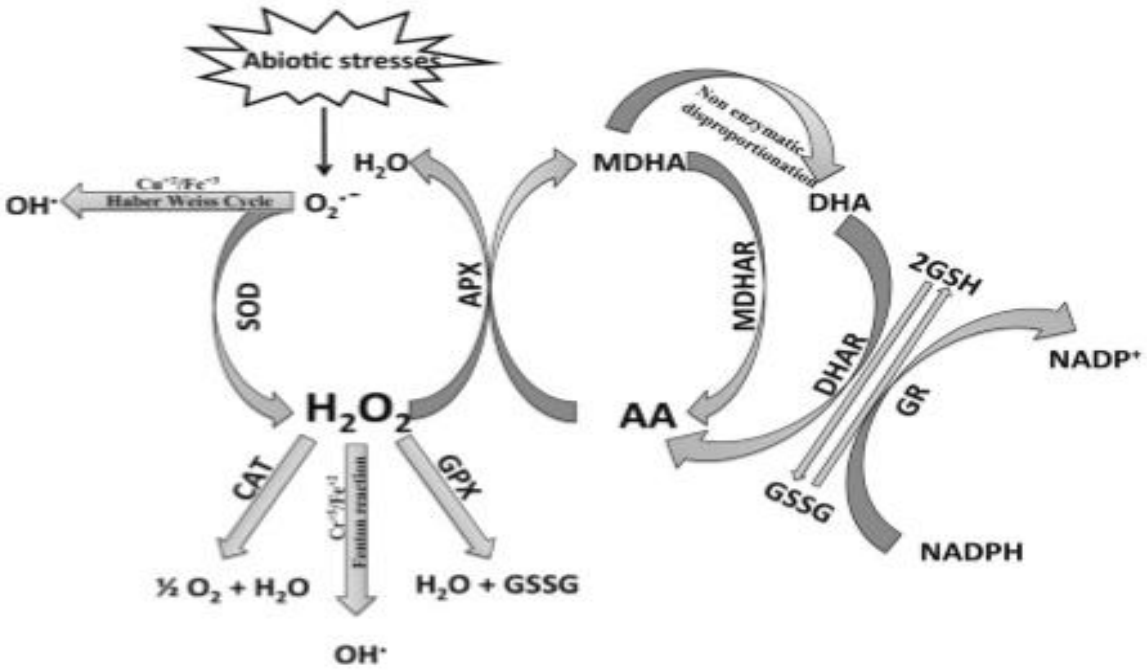

Figure 1.9: Reactive oxygen species and the antioxidant defence mechanism (Gill and Tuteja, 2010).

1.7.1.2 Ascorbate peroxidase (APX)

Ascorbate peroxidase is a class 1 heme peroxidase and a major part of the Ascorbate Glutathione (ASC-GSH) cycle. Using ascorbic acid (AA) as a reducing agent, APX catalyses the

reduction of H_2O_2 to water and two molecules of monodehydroascorbate (MDHA) from two molecules of ascorbate. The MDHA radical is further converted to ascorbate via onodehydroascorbate reductase (MDHAR) enzyme by using NADPH as an electron donor (Sakihama *et al*., 2002). Ascorbate peroxidase can be found in the stroma, mitochondria, thylakoid, perixomes and cytosol of plant cells. It exists in five isoforms based on the amino acid sequences (Jimenez *et al.*, 1997; Madhusudhan *et al.*, 2003; Sharma and Dubey, 2004; Nakano and Asada, 2004). Ascorbate peroxidase isoforms have been shown to possess high affinity for H_2O_2 than CAT (Wang *et al*., 1999) and that APX is the most widely distributed antioxidant enzyme in plant cells (Sharma and Dubey, 2004). This fact renders APX as an important scavenger of H_2O_2 during stressful conditions in plants.

1.7.1.3 Catalase (CAT)

Catalase is among the earliest antioxidant enzymes to be discovered and characterised. It is a ubiquitous tetrameric heme-containing enzyme with high specificity for H_2O_2 (Sharma and Dubey, 2004). It catalyses the dismutation of two molecules of H_2O_2 into water and oxygen. Catalase is located in peroxisomes, which are major organelles of H_2O_2 production. CAT removes the H_2O_2 generated during photorespiratory oxidation, B-oxidation of fatty acids and other enzyme sytems such as xanthine oxidase (XOD) coupled to SOD (De Rio *et al*., 2006; Scandalios *et al*., 1997; Corpas *et al*., 2008).

1.7.2 Non-enzymatic antioxidants

1.7.2.1 Ascorbic acid (AsA)

The most abundant non-enzymatic antioxidant in plants is ascorbate (ascorbic acid; AsA). Although it can be found in cellular compartments such as chloroplast and apoplast, it is produced mainly in the mitochondria (Desikan *et al.*, 2003). On account of its ability to donate

electrons to both enzymatic and non-enzymatic reactions, ascorbate is regarded as a powerful antioxidant (Sharma *et al.*, 2012). In the chloroplast, AsA is found in a reduced state under normal physiological conditions and is a cofactor of violaxanthin de-epoxidase (Smirnoff, 2000). It removes H_2O_2 via the ascorbate-glutathione cycle by reacting directly with H_2O_2 (Zaefyadeh *et al.*, 2009; Foyer *et al.*, 1997; Pinto *et al*., 2003). The oxidation of ascorbate follows two steps – during the first step, MDHA is produced by the utilization of two molecules of ascorbate by APX to reduce H_2O_2 to water. In the second step, owing to its short life time, MDHA can spontaneously dismutate to DHA and AsA. (Sharma *et al*., 2012). The levels of AsA change in response to different environmental stresses (Sharma and Dubey, 2005; Maheshwari and Dubey, 2009; Mishra *et al*., 2011; Srivastava and Dubey, 2011; Hernande *et al*., 2001; Radyuk *et al*., 2009).

1.7.2.2 Carotenoids

Carotenoids are lipophilic antioxidants which remove, neutralize and scavenge different types of ROS (Elstner, 1991). In plants, they are found in both photosynthetic and non-photosynthetic plastids where they protect the photosynthetic machinery by (1) reacting with lipid peroxidation products to terminate the peroxidation chain reactions, (2) preventing the formation of $^1O_2$ where they react with excited chlorophyll, and (3) scavenging $^1O_2$ where it is formed (Das and Roychoudhury, 2014).

1.7.2.3 Osmolytes

Osmolytes (also known as compatible solutes) are highly soluble, low molecular weight organic compounds that are produced by plants experiencing environmental stress such as drought. They include polyols, glycine betaine, alanine betaine, proline and sucrose. During oxidative stress, plants decrease their osmotic potential by overproducing or accumulating osmolytes

(Ashraf and Foolad, 2007). These osmolytes help detoxify ROS, maintain membrane integrity and protein/enzyme stabilization and cell turgor or osmotic balance (Serraj and Sinclair, 2002). Cell turgor has been shown to contribute to maintaining physiological processes such as photosynthesis, stomatal opening and expansion growth (Serraj and Sinclair, 2002). One of the highly studied osmolytes is proline. It plays a highly beneficial role to plants that are experiencing oxidative stress.

1.7.2.3.1 Proline

Proline is considered to be a powerful antioxidant. Proline is synthesized from glutamic acid via a pyroline 5-carboxylate synthetase intermediate and pyroline-5-carboxylate reductase enzyme (Ashraf and Foolad, 2007). Its accumulation during drought stress is increased not only by the activation of proline synthesis but also by the deactivation of proline degradation. It plays three important roles during drought-induced oxidative stress – it acts as a metal chelator, a signaling molecule and an antioxidative defense molecule (Hayat *et al.*, 2012). During drought stress, proline concentration increases in the cytosol where it provides osmotic adjustment (Caballero, 2005; Oliveira-Neto *et al*., 2009). Free proline levels increase significantly in maize seedling in response to drought-induced stress (Garcia *et al*., 1987). Similar results have been reported in wheat (Naidoo *et al*., 1990) and cotton cultivars (Ronde *et al*., 2000). Proline also prevents the harmful effects of lipid peroxidation by stabilizing proteins and membranes (Bartels and Souer, 2004; Smirnoff, 1998) and can efficiently remove OH^- and $^1O_2$ (Verbruggen and Hermans, 2008).

CHAPTER 2

MATERIALS AND METHODS

2.1 Plant Growth

Maize (*Zea mays L.*) cv Kalahari Early Pearl seeds (Capstone Seeds, South Africa) were surface sterilized in 0.35% (v/v) sodium hypochlorite (bleach) for 10 minutes and then rinsed five times with sterile distilled water. The seeds were imbibed in 10 mM calcium sulphate ($CaSO_4$) solution for 16 hours at room temperature with good aeration. Once again, the seeds were washed five times with sterile distilled water. The seeds were then germinated on petri dishes (9 cm diameter) with five seeds per petri dish - the Petri dishes contained a sheet of paper towel pre-soaked in sterile distilled water on which the seeds were placed. The seeds were then over-laid with another sheet of paper towel pre-soaked in sterile distilled water and then covered with the petri dish lead. Dishes with seeds were incubated at room temperature for 5 days with regular monitoring for radicle development. The paper towel was kept moist by watering with sterile distilled water during the germination period. After five days of germination, germinated seeds (defined as seeds with radicle measuring 3 mm or more in length) were selected and sown (8-10 seeds per pot) in 20 cm diameter plastic pots containing a 2:1 mixture of Landscapers Choice compost soil and potting soil (Checkers® Brackenfell, South Africa), that was pre-soaked with sterile distilled water, under regulated environmental conditions (25/19°C day/night temperature cycle under a 16/8 h light/dark cycle) in a greenhouse. Plant growth was maintained by regularly watering with 250 ml sterile dH_2O until V1 stage (when the collar of the first true leaf is visible).

2.1.1 Plant treatment

Plant watering continued until V2 stage, at which point all plants that were of good health and similar height (at equal developmental stage) were selected for use in control (12 plants) and treatment (12 plants) experiments. For control plants (well-watered), 250 ml of sterile distilled water was supplied every third day for 28 days while the treatment (water-deprived) plants received no water for the same period. At 28 days of drought treatment, plants were harvested from the soil.

2.1.2 Plant storage

The plants were immediately used for fresh weight measurements and cell viability assay, or frozen in liquid nitrogen, leaves and roots grounded into fine powder and kept at -80 °C for all other experiments.

2.2 Measurement of biomass (fresh weight)

Fresh weight analysis was performed by weighing 8 well-watered and 8 water-deprived maize shoots immediately after harvesting at day 28 of treatment.

2.3 Protein Isolation

Total soluble protein was isolated from leaves and roots of maize tissue (from four different plants for each of well-watered or water-deprived plants) by homogenising 500 mg of frozen plant tissue in 1ml of cold (4 °C) extraction buffer [40 mM K_2HPO_4 at pH 7.4; 1 mM EDTA and 5% (w/v) Polyvinylpolypyrrolidone (PVPP)]. After separating insoluble material by centrifugation at 13 000 X *g* for 1 minute at 4°C, the protein-containing supernatant was carefully removed and quantified or stored at -20˚C.

2.4 Quantification of protein by Bradford assay

The concentration of protein was determined according to Bradford (1976) by using the BioRad (BioRad, Hercules, USA) protein assay with bovine serum albumin as a standard. All protein standards and samples were prepared in triplicates and the determination of the protein concentration was obtained on the basis of the standard curve. Each protein standard was diluted with distilled water accordingly from a stock concentration of 1 mg/ml to give a final volume of 10 µl. The protein sample was diluted similarly. The 10 µl of the diluted standard or sample protein was mixed with 190 µl of 1X BioRad dye in a 96-well plate. Following incubation at room temperature for 10 minutes, the absorbance was read at 595 nm using the Floustar Omega UV-visible spectrophotometer (BMG LabTech GmbH, Ortenberg, Germany).

2.5 Estimation of hydrogen peroxide (H_2O_2) content

In order to establish if drought stress induces the production of high levels of H_2O_2, H_2O_2 content was measured in leaves and roots of maize treatments (from four different plants for each of well-watered and water-deprived plants frozen above) by a modified method of Velikova *et al*. (2000). Ground leaf and root tissue (100 mg each) was homogenized in 400 µl of cold 6% (w/v) TCA. The homogenates were centrifuged at 13 200 X *g* for 30 minutes at 4°C. The resulting supernatant (containing H_2O_2) was used (50 µl) to start a reaction. The reaction mixture (total volume of 200 µl) contained 50 µl leaf or root extracts, 5 mM dipotassium phosphate ($K_2HPO_{4)}$, pH 5.0 and 0.5 M potassium iodide (KI). Samples were incubated at 25°C for 20 minutes and the absorbance measured for each sample at 390 nm. H_2O_2 content was calculated based on a standard curve constructed from the absorbance (390 nm) of H_2O_2 standards and a H_2O_2 extinction coefficient of 39.4 mM^{-1} cm^{-1}.

2.6 Measurement of malondialdehyde (MDA) content

The level of lipid peroxidation in the leaves and roots was measured in terms of malondialdehyde content (a product of lipid peroxidation), using the thiobarbituric acid (TBA) method as previously described by Dhindsa and Matowe, 1981. The same extract as used in the H_2O_2 assay above was used for MDA measurement. Aliquots (200 µl) of the extract were mixed vigorously with 400 µl of 0.5% thiobarbituric acid (prepared in 20% TCA). The mixture was then heated at 95°C for 30 minutes and quickly placed on ice. The mixture was further centrifuged at 13000 x *g* for 5 minutes at 4°C. The absorbance of the supernatants (200 µl) was measured in triplicates at 532 nm and 600 nm wavelengths. Following the subtraction of the non-specific absorbance values (at 600 nm) from the 532 nm absorbance values, the concentration of MDA was determined using an extinction coefficient of 155 $Mm^{-1}cm^{-1}$ and expressed as $nmol.g^{-1}$ of fresh weight.

2.7 Measurement of cell death

Cell viability measurement was carried out on well-watered and water-deprived maize leaves from 3 separate plants (from the second youngest leaf) using a modified method of Sanevas and Sza, 2007. The assay was conducted separately for well-watered and water-deprived leaves. A 1 cm^2 section of the 2nd youngest leaf was cut out and placed in a 15 ml conical tube (covered with foil) containing 0.25% (w/v) Evans blue dye and the mixture was incubated for 20 minutes at room temperature. The leaves were then rinsed with distilled water in order to remove excess dye. The leaves were suspended in 1% (w/v) SDS and incubated at 55°C for 1 hour. Following centrifugation at 2000 X *g* for 5 minutes, absorbance of the supernatants (200 µl) was measured in triplicate at 600 nm wavelength using a FLUOstar Omega UV-visible

spectrophotometer (BMG LabTech GmbH, Ortenberg, Germany) in order to determine the level of Evans blue taken up by the dead cells.

2.8 Ascorbate peroxidase enzymatic activity assays

2.8.1 Native PAGE In-gel activity assay

The detection of APX activity in well-watered and water-deprived leaves and roots of maize followed a procedure previously described by Seckin *et al.,* (2010) on frozen material from four different plants for each of well-watered or water-deprived treatments. Before protein electrophoresis, non-denaturing PAGE gels were prepared and equilibrated with a running buffer containing 2 mM ascorbate for 30 minutes at 4°C. Thereafter, 40 µg of leaves and roots treatments were loaded onto the gels and electrophoresed at 4°C in 12% polyacrylamide mini gels in an electrophoresis buffer (192 mM Glycine, 25 mM Tris pH 7 and 2 mM ascorbate). After the electrophoresis, gels were incubated in 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM ascorbate for 20 min and then transferred to solutions containing 50 mM potassium phosphate buffer (pH 7.8), 4 mM ascorbate and 2 mM H_2O_2 for 20 min. The gels were then submerged in a solution of 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM N,N,N', N'-tetra methyl ethylene diamine (TEMED) and 2.5 mM nitroblue tetrazolium (NBT) for 10–20 min with gentle agitation in the presence of light. On visualization of the protein bands, the staining reaction was stopped by discarding the stain and suspending the gels in distilled water. Four independent gels were produced in this manner. The gel images were captured and analyzed by densitometry using AlphaEase FC imaging software (Alpha Innotech Corporation).

2.8.2 Spectrophotometric ascorbate peroxidase activity assay

For determination of ascorbate peroxidase (APX) activity in leaves and roots of well-watered and water-deprived maize, 50 µg of protein extracts was homogenized in 50 mM K_2HPO_4, pH 7.0; 0.1 mM EDTA; 2 mM ascorbate. The reaction was initiated by the addition of 10 mM H_2O_2 to a final reaction volume of 200 µl. APX activity was calculated using the extinction coefficient of 2.8 mM^{-1} cm^{-1} by following the change in absorbance at 290 nm as previously described by Nakano and Asada (1981). APX activity was expressed as $\mu mol.min^{-1}.mg^{-1}$ protein oxidized.

2.9 Catalase enzymatic activity assays

2.9.1 Native PAGE In-gel activity assay

Catalase in-gel activity detection was achieved by separation of leaves and roots (well-watered and water-deprived) proteins in a 12% native PAGE gel (1.5 M Tris-HCl pH 8.8) with 5% stacking gel (1 M Tris pH 6.8) at 4°C, 90 V for 7 hours. The activity staining followed the procedure previously described by Gara *et al.,* (1997) with minor changes. After electrophoresis, the gel was washed three times with distilled water, followed by soaking in 0.003% (v/v) H_2O_2 for 10 minutes. Hydrogen peroxide was discarded and the gel again washed twice with distilled water. For staining, in the presence of light, the gel was soaked in 2% (w/v) of both ferric chloride ($FeCl_3$) and potassium ferricyanide ($K_3[Fe(CN)_6]$ for 5-10 minutes. On visualisation of achromatic bands, the stains were discarded and the gel washed thoroughly with distilled water and photographed. This was done for four independent gels, which were subsequently analyzed by densitometry using AlphaEase FC imaging software (Alpha Innotech Corporation).

2.9.2 Spectrophotometric catalase activity assay

For quantitative catalase (CAT) activity assay, a spectrophotometric approach was used based on the method previously reported by Luck (1965), by measuring the rate of decrease in H_2O_2 absorbance at 240 nm at room temperature. Protein extracts (50 µg each) from well-watered and water-deprived leaves and roots (from four separate plants for each of well-watered or water-deprived plants) were mixed separately with 50 mM potassium phosphate buffer pH 8.0, 1.5 mM EDTA and 1mM H_2O_2. The rate of H_2O_2 decomposition by catalase was then calculated from the change in absorbance measured at 240 nm. The final units for the CAT activity assay were expressed as µmol H_2O_2 decomposed min^{-1}. mg^{-1} protein. The extinction coefficient was ε = 39.4 mM^{-1} cm^{-1} and the unit was defined as the amount of enzyme that liberates half the peroxide in 100 seconds at 25°C.

3. Superoxide Dismutase (SOD) enzymatic activity assays

3.1 Native PAGE In-gel activity assay

For the in-gel detection of SOD activity in leaves and roots of maize in response to drought stress, 200 µg of protein (from extracts described in 2.3 above) per treatment was loaded on native PAGE and electrophoresed at 90 V for 7 hours at 4°C in 12% polyacrylamide mini gel. Following electrophoresis, SOD activity was detected by staining. Firstly, this was done by soaking the gel in 50 mM potassium phosphate buffer pH 7.8, containing 2.5 mM nitro blue tetrazolium (NBT) for 15 minutes in the dark. The stain was then discarded and, in the presence of light, the gel was soaked in a solution of 50 mM potassium phosphate buffer pH 7.8, 28 mM riboflavin and 28 µl N,N,N,N-tetramethylethylenediamine (TEMED) until SOD isoforms were visible. For the identification of SOD isoforms based on their metallo-cofactors, selective inhibition with KCN and H_2O_2 was performed before staining for the activity bands. The gels

were soaked in 5 mM H_2O_2 (to inhibit both Cu/ZnSOD and FeSOD), or 3 mM KCN (to inhibit only Cu/ZnSOD) as previously described by Archibald and Fridovich, 1982. MnSOD is resistant to both inhibitors. The SOD activity staining procedure was previously described by Beauchamp and Fridovich, 1971. This was done for four independent gels, which were subsequently analyzed by densitometry using AlphaEase FC imaging software (Alpha Innotech Corporation).

3.2 Spetrophotometric activity assay

Superoxide dismutase (SOD) activity was assayed in extracts described in 2.3 above by following the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT) based on the procedure previously described by Beauchamp and Fridovich (1971). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM L-methionine, 2 µM riboflavin and 75 µM nitro blue tetrazolium (NBT) and 50 µg protein extracts from well-watered and water-deprived leaves and roots of maize. Blank assays were also prepared but without protein extracts. Riboflavin was added last and the reaction was initiated by exposing the solution to light for 10 minutes. The increase in absorbance at 560 nm was carefully monitored to follow the production of blue formazan. The unit of SOD activity was defined as the amount of enzyme that inhibits the nitro blue tetrazolium photo-reduction by 50%. SOD activity was expressed in units per mg of protein.

4. Densitometric analysis

Densitometric analysis was used to compare enzymatic activities for each isoform identified. For densitometric analysis of SOD, CAT and APX activities, four gels from four independent experiments of each enzyme were analysed. The gel images were photographed, uploaded on a computer and analysed by using AlphaEase FC imaging software (Alpha Innotech Corporation, USA).

5. Statistical analysis

All quantitative data were analysed using the one-way analysis of variance (ANOVA) and tested for significance by the Tukey-Kramer test at 5% level of significance.

6. Quantitative real-time PCR analysis of APX and CAT transcript accumulation in response to drought stress

6.1 Total RNA Extraction

Total RNA was extracted from leaves and roots (from three independent experiments) of well-watered and water-deprived maize plants (50 mg each) by using Direct-zol™ RNA Mini-prep kit (Zymo Research, USA) as described in the manufacturer's manual. To digest and remove any contaminating DNA from the RNA, the extracted RNA was treated with RNase-free DNase I (Zymo Research, USA) by following the manufacturer's instructions. Furthermore, RiboLock® RNase Inhibitor (Thermo Scientific, USA) was used according to manufacturer's instructions to inhibit RNase-mediated degradation of the extracted RNA.

6.2 Synthesis of the first strand cDNA

Total RNA (500 ng each) from leaves and roots of both well-watered and water-deprived maize plants was used to synthesize cDNA. The reaction was carried out by using the RevertAid Reverse Transcriptase kit (Thermo Scientific, USA), with the use of an Oligo (dT)18 primer (Thermo Scientific, USA) as specified by the manufacturer's instructions. The reaction was incubated at 42°C for 60 minutes followed by reaction termination at 70°C for 10 minutes.

6.3 Identification of maize ascorbate peroxidase (*APX*) and catalase (*CAT*) genes for RT-qPCR

Ascorbate peroxidase encoding genes were identified from Phytozome (http://phytozome.jgi.doe.gov/pz/portal) on the basis of data on maize *APX* genes and the homology of their corresponding encoded proteins to arabidobsis APX proteins that have been previously characterized. For catalase gene identification, a search on Phytozome maize database using the keyword 'catalase' was carried out. Unique (non-conserved) regions not exceeding 250 bp were selected for RT–qPCR.

6.4 Quantitative RT-PCR (RT-qPCR) analysis of *ZmAPX* and *ZmCAT* genes expression in response to drought stress

Variation of the expression of *APX1 – 4* and *CAT1* & *2* genes in treated (water deprived) and untreated maize (well-watered), both normalized to internal control maize genes elongation factor 1α, Actin2 and β-tubulin, in response to water deficit was measured independently using RT-qPCR. The experiments for well-watered and water-deprived maize leaves and roots were performed in triplicates, each in a total reaction volume of 10 µl by following manufacturer's recommendations (Thermo Scientific). Two microliters (2 µl) of first strand cDNA template was mixed with 1X Luminaris Colour HiGreenTM LowROXqPCR master mix and 0.3 µM gene-specific forward and reverse primers (Table 2). The primers for both *ZmAPX 1-4*, *ZmCAT1* & *2* and internal controls genes for the qPCR were designed using the Primer 3 software (Untergasser *et al*., 2012). For the APX genes, the RT-qPCRs were carried out using a three-step cycling protocol. An initial denaturation step at 95°C for 10 min was followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 56°C for 30 seconds and extension at 72°C for 30 sec. Data acquisition was set to occur during extension periods. A melting curve step was included after the PCR steps to verify primer specificity and identify the PCR products. For *CAT1* & *2* genes' RT-qPCR, conditions similar to the above were applied except that the annealing temperature was at 52.6°C. Similarly, RT-qPCR reactions were set up for three internal control maize genes:

elongation factor 1α, Actin2 and β-tubulin (primers sequences are provided in the table 2 below). Transcript accumulation levels are expressed relative to the untreated control, based on 2-ΔΔT method (Livak and Schmittgen, 2001). Both well-watered and water-deprived experiments were normalized to the average levels of expression of the three internal control genes (Actin2, elongation factor1α & β-tubulin). The delta-delta method requires the use of internal control which is uniformly expressed in all samples. Thus in this study, Elongation factor 1α, Actin2 and β-tubulin were used because their expression does not change in response to a variety of treatment conditions (Nicot *et al*., 2005). Statistical validity of all the data was tested by means of a one-way analysis of variance (ANOVA) and the Tukey-Kramer test at 5% level of significance was completed to compare the means using GraphPad Prism 6.01 software.

6.5 Primer efficiency determination

Primer efficiency was calculated using the following formula:

(X g/µl DNA/[gene size in base pairs x 660]) x 6.022 x 1023 = Y molecules/µl

Table 2: Primer sequences used in RT-qPCR for gene expression analysis.

Gene name (Loc name)	Forward primer (5' – 3')	Reverse primer (5' – 3')	Transcri length (b
ZmAPX1 (GRMZM2G120517	GGCAAGCAGATGGGTTTGA	CTCCACAAGAGGGCGGAAGA	211
ZmAPX2 (GRMZM2G137839	GAAACTTCCTGATGCTGGCCCAAG	TTCAACTGTCCATGATTGCCCACC	216
ZmAPX3 (GRMZM2G004211	GAAGCACCCCAAGATCACAT	CTCTTCTAGCATCCGGCAAG	216
ZmAPX4 (GRMZM2G054300	TGGCAAGCAGATGGGTTTGA	CTCCACAAGAGGGCGGAAGA	231
ZmCAT1 (GRMZM2G088212	TCAAGCCGAATCCAAAGACCA	TCGAGCAAGCATTTCACACCA	238
ZmCAT2 (GRMZM2G090568	GCACACGTACACGCTCGTCAG	GTCTTCCATCTCGGGGTCCAT	220
Actin 2	CTGAGGTTCTATTCCAGCCATCC	CCACCACTGAGGACAACATTACC	133
β-tubulin	CTACCTCACGGCATCTGCTATGT	GTCACACACACTCGACTTCACG	139
Elongation Factor 1	TGGGCCTACTGGTCTTACTACTGA	ACATACCCACGCTTCAGATCCT	135

CHAPTER 3

RESULTS

3.1 Drought causes biomass reduction in maize

Drought responses in plants are characterised by turgor loss, reduced water potential and decreased cell expansion and/or growth. During drought stress plant growth and development is reduced due to negative effects on many different biochemical and physiological processes such as respiration, growth promoters, photosynthesis, nutrient metabolism, carbohydrates metabolism, ion uptake and translocation of biomolecules (Jaleel *et al*., 2008; Farooq *et al*., 2008). Thus, plant biomass can be used as a marker to evaluate the response of plants to drought stress. The results of which can further be used to select or create new crop varieties with improved tolerance to drought-induced stress, resulting in better productivity. Thus, the impact of drought induced stress on the biomass of maize plants was determined after 28 days of exposure to drought. Water deprived maize showed a significant reduction in fresh weight (approximately 50%) when compared to the well-watered plants (figure 3.1).

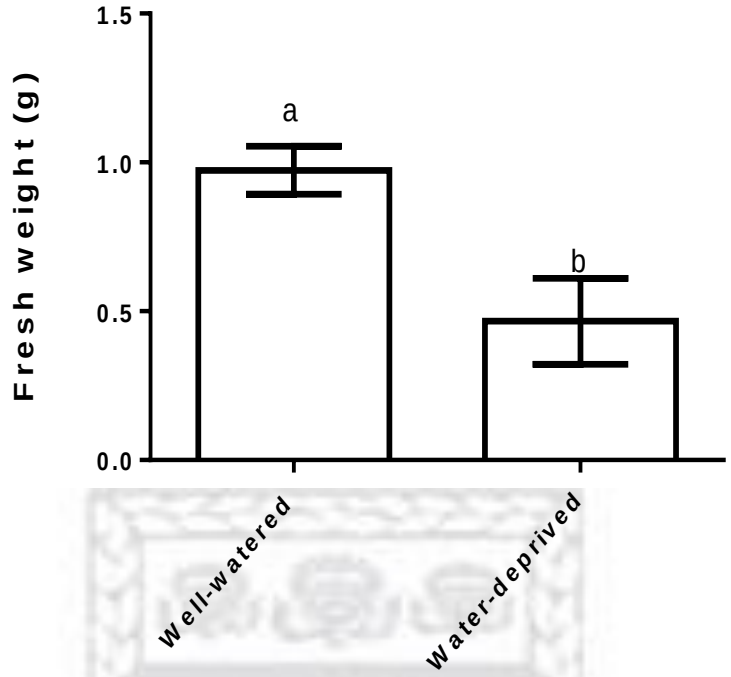

Figure 3.1: Effect of drought on maize shoot biomass. Immediately after 28 days of growth, the fresh weights of well-watered and water-deprived maize plants were measured. Data represent the mean (±SE) of 8 (n = 8) plants per treatment. The different letters (a and b) above the error bars indicates a significant difference between means ($P < 0.05$).

3.2 Drought stress increases hydrogen peroxide content in leaves and roots of maize

Hydrogen peroxide (H_2O_2) is one of the reactive oxygen species (ROS) that are produced as a secondary metabolic by-product under normal plant growth. During normal growth, where its concentrations are low, it is unable to cause damage as long as it is scavenged by different antioxidant mechanisms. However, when a plant is exposed to environmental stress such as drought, hydrogen peroxide levels can increase significantly and become toxic to the plant because of its high cellular concentration; resulting in damage to DNA, proteins and lipids which in turn negatively affects normal cellular function. The measurement of H_2O_2 content can therefore be used to evaluate the extent of damage to plant tissue due to drought-induced stress. In this study, hydrogen peroxide content was measured in leaves and roots of maize

(well-watered and water-deprived) after 28 days of treatment. Water-deprived leaves and roots contained more (60% and 25% increase, respectively) H_2O_2 when compared to well- watered plants (figure 3.2)

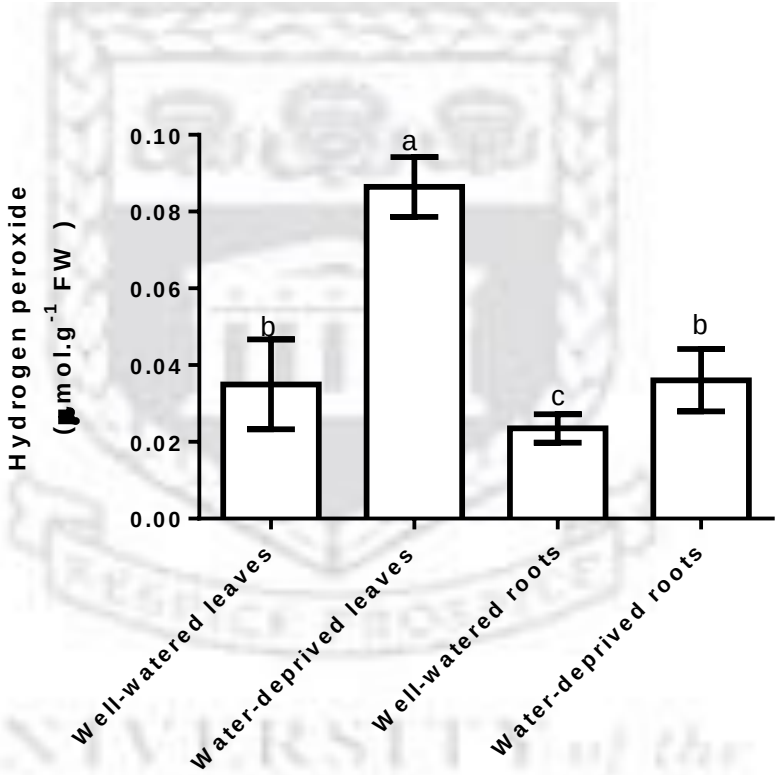

Figure 3.2: Hydrogen peroxide content measurement in leaves and roots of maize subjected to drought-induced stress for 28 days. The data represents the means ± standard error (SE) from four independent experiments measured in triplicate. The different letters (a and b) above the error bars indicate a significant difference between means ($P< 0.05$).

3.3 Lipid peroxidation as a biochemical marker for oxidative stress during drought stress in maize

Lipid peroxidation is the oxidative degradation of lipids in which ROS remove electrons from the lipids cell membrane. It occurs when a plant experiences stress, when ROS levels will far exceed the plant's ability to scavenge. One of the final products of lipid peroxidation is malondialdehyde and it causes detrimental changes to cell membrane such as ion transport, membrane fluidity, protein cross linking and loss of enzyme activity, leading to cell death. As a measure of drought-induced oxidative stress damage in maize, MDA content was measured in leaves and roots of maize (well-watered and water-deprived) after 28 days of treatment. The water-deprived leaves and roots contained higher levels of MDA (approximately 70% and 64% increase, respectively) when compared to the well-watered plants (figure 3.3).

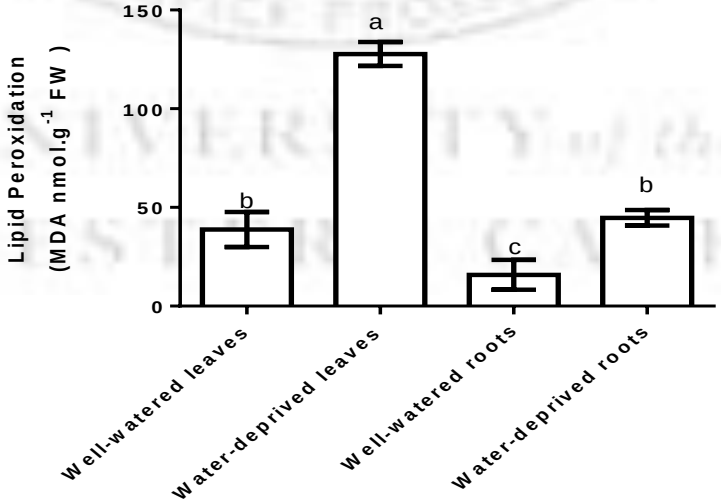

Figure 3.3: Lipid peroxidation (MDA) in leaves and roots of maize after 28 days of treatment. The bars represent the means ± standard error (SE) from four independent experiments measured in triplicate (n = 3). The different letters above the error bars indicates a significant difference between means ($P < 0.05$).

3.4 The effect of drought-induced stress on cell viability in maize leaves

When plants are subjected to abiotic stress such as drought, ROS are produced at higher levels and become toxic to the plant, leading to cell death. An increase in ROS, such as H_2O_2, induces lipid peroxidation causing the cellular membrane to rupture and leak. Therefore, to further evaluate the extent of the impact of drought-induced stress in maize, cell viability was measured in the leaves of well-watered and water-deprived maize. The assay is based on the ability of dead cells to take up Evans Blue stain while viable cells, with intact cell membranes, cannot. The results showed that water-deprived plants had significantly higher extent (± 60%) of dead cells when compared to well-watered plants (figure. 3.4).

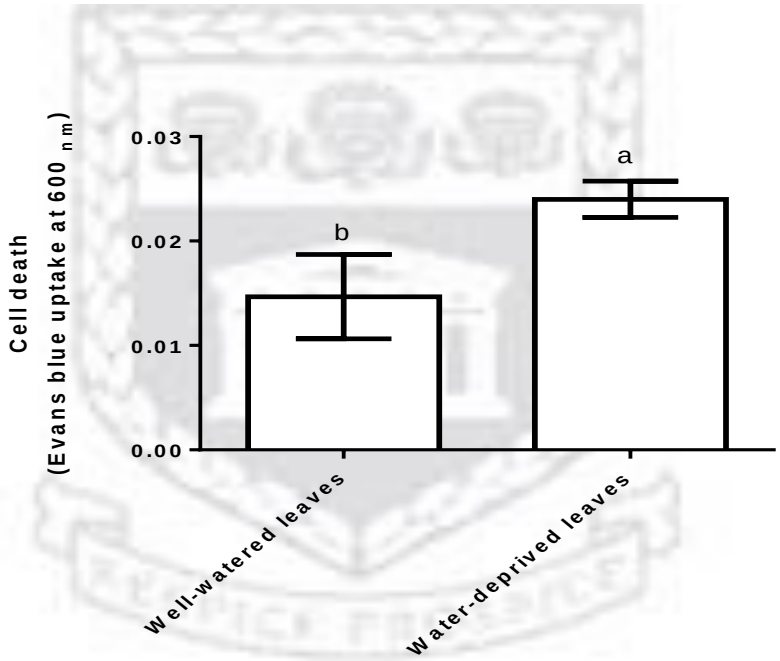

Fig. 3.4: Cell viability measurements in the leaves of well-watered and water-deprived maize, 28 days after treatment. The error bars are representative of the mean (±SE) of three independent experiments from 3 plants per treatment (n = 3). Means with different letters are significantly different from each other ($P < 0.05$).

3.5 The effects of drought-induced stress on APX activity in leaves and roots of maize

Ascorbate peroxidase is a major enzyme in the process of hydrogen peroxide detoxification in plant chloroplasts. This process occurs via an ascorbate-glutathione cycle where APX uses AsA as a specific electron donor for the reduction of H_2O_2 to water. Thus, APX activity of leaves and roots in response to drought stress was measured. Non-denaturing native PAGE was used for activity detection of each APX isoform and a spectrophotometric assay was used to measure total APX activity in all four treatments. Comparative analysis of individual isoforms activities that were detected in all four treatments was also determined by measuring their relative pixel intensity ratios. Seven active isoforms (ZmAPX 1 – 7) of APX were detected on the native PAGE, with the highest number of isoforms appearing in water-deprived roots (figure 3.5A). The contribution of individual isoforms to total activity correlated positively with the native PAGE activity results as shown by the significantly high total activity in water-deprived leaves and roots in comparison to well-watered treatments (figure 3.5B). The activity of isoforms 4 and 7 was detected in roots and leaves and revealed significantly high activity in water-deprived treatments when compared to well-watered treatments (figure. 3.5 D and G). Isoforms 3 and 6 were also detected in roots with pixel intensity ratios showing a high level of activity in water-deprived treatments in comparison to well-watered treatments (figure 3.5 C & F). It was interesting to note that isoform 6 activity (albeit very low) was also detected in water-deprived leaves but not in well-watered leaves (figure 3.5 A). Isoform 5 was also detected in well-watered and water-deprived roots and in water-deprived leaves but not in well-watered leaves. Isoform 5 activity (as a measure of pixel intensity) was much higher in water-deprived roots in comparison to well-watered roots (figure 3.5 E).

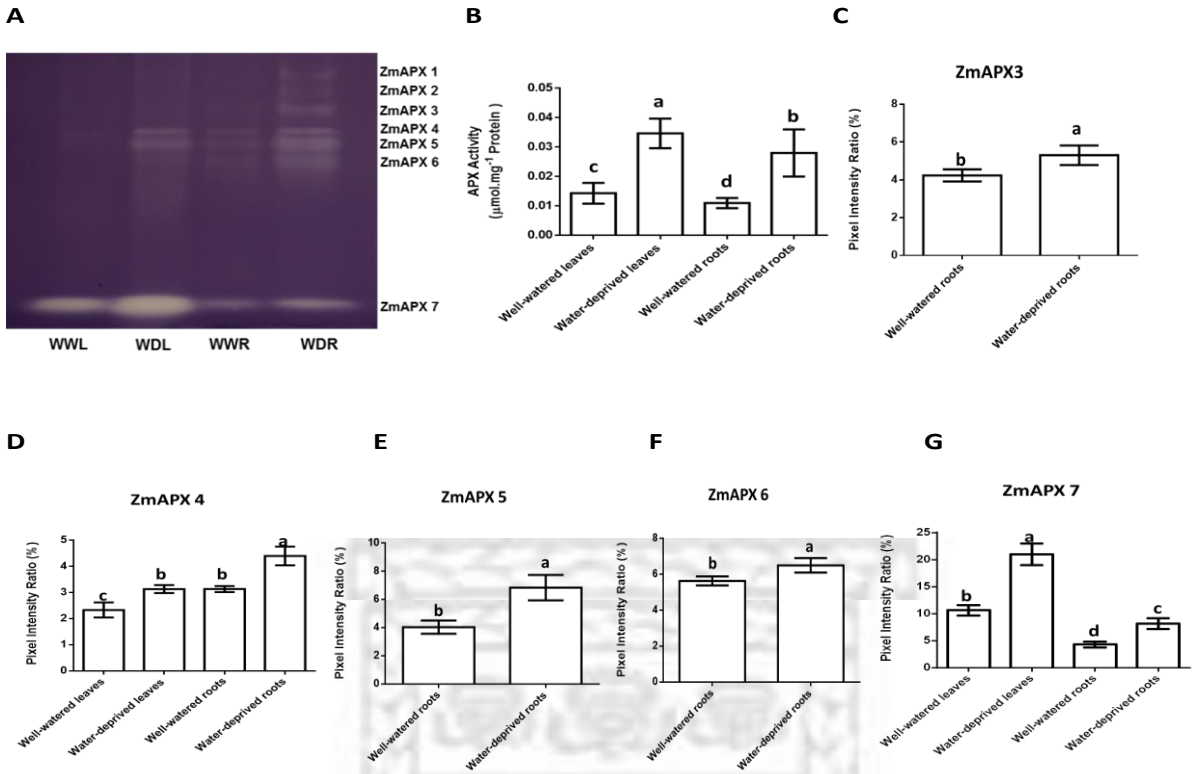

Figure 3.5: Ascorbate peroxidase activity in leaves and roots of maize plant after 28 days of treatment. In-gel native page activity assay (A), Spectrophotometric assay for APX total activity (B), Relative pixel intensity ratios of maize APX isoforms 3, 4, 5, 6 and 7 (C – G). The data represent the means ± standard error (SE) from four independent experiments measured in quadruple (n = 4). Means with different letters are significantly different from each other ($P < 0.05$). WWL = well-watered leaves, WWR = well-watered roots, WDL= water-deprived leaves and WDR = water-deprived roots.

3.6 Drought stress induces changes in catalase activity in maize

Catalase is an antioxidant enzyme that is found in all aerobic organisms and is responsible for catalysing the conversion of H_2O_2 into water and oxygen under stressful conditions. The activity of catalase in response to drought stress was therefore investigated using in-gel native PAGE, spectrophotometric assaying and pixel intensity analysis. The in-gel activity assay revealed five catalase isoforms with the highest activity displayed by isoform 4 in response to drought stress in the leaves (figure 3.6 A and D).

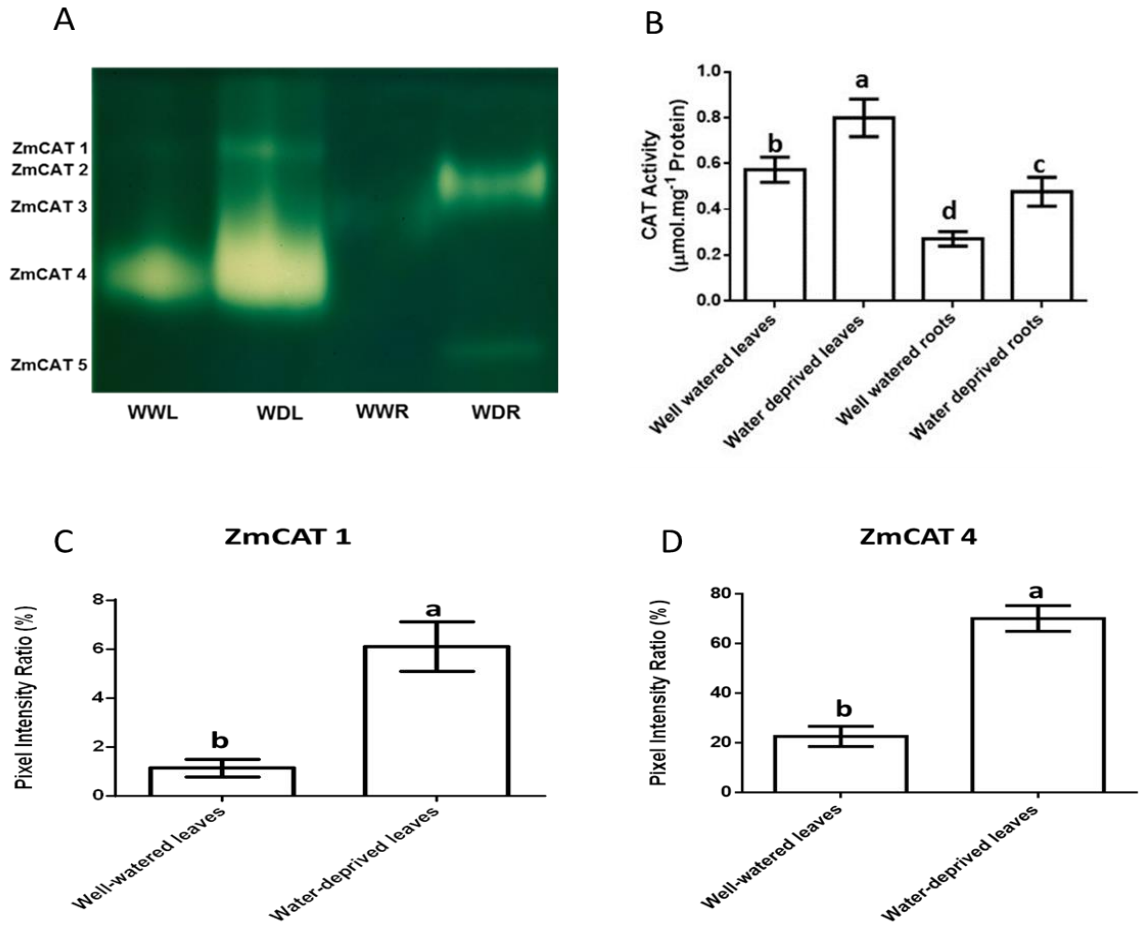

Figure. 3.6: Effects of drought treatment on catalase activity in maize leaves and roots, measured 28 days after treatment. After qualitative in-gel activity assay with native PAGE and staining, 5 catalase isoforms were detected (A). Total catalase activity in each treatment was quantified spectrophotometrically by measuring the rate of decrease in H_2O_2 absorbance at 240 nm at room temperature (B). Densitometric analysis of catalase activity in well-watered and water-deprived leaves (C). Error bars represent the means (±SE) of four densitometric values (n = 4). Error bars with different letters indicate mean values that are significantly different at ($P < 0.05$).

Total activity analysis results correlated positively with the in-gel activity assay in that CAT activity was significantly increased in response to drought stress, both in leaves and roots (figure 3.6 B). Isoforms 1 and 4 activity analysis (as a measure of pixel intensity) revealed a significant increase in activity in response to drought stress when compared to well-watered plants (figure 3.6 C and D).

3.7 Drought stress differentially alters SOD activity in maize

In order to prevent the oxidative damage that may be caused by these excessive ROS, SOD catalyses the decomposition of $O_2^{\cdot-}$ to H_2O_2. Therefore, it may be deduced that an increase in SOD activity is an indication of high levels of these ROS due to drought stress. Thus, SOD activity during drought stress was investigated and, as expected, its activity was enhanced in response to drought stress. The isoforms were identified and characterized by selective inhibition with KCN or H_2O_2. FeSODs were sensitive to H_2O_2 but resistant to KCN. CuZnSODs were sensitive to both KCN and H_2O_2. MnSODs were resistant to both inhibitors (data not shown). In total, one MnSOD, three CuZnSODs and one FeSOD were identified (figure 3.7 A). Spectrophotometric SOD total activity assay quantified the contribution of all isoforms to total activity in each treatment (figure 3.7 B). FeSOD isoform was detected in all four treatments and its activity measurement (as pixel intensity ratio) revealed the highest activity in water-deprived leaves.

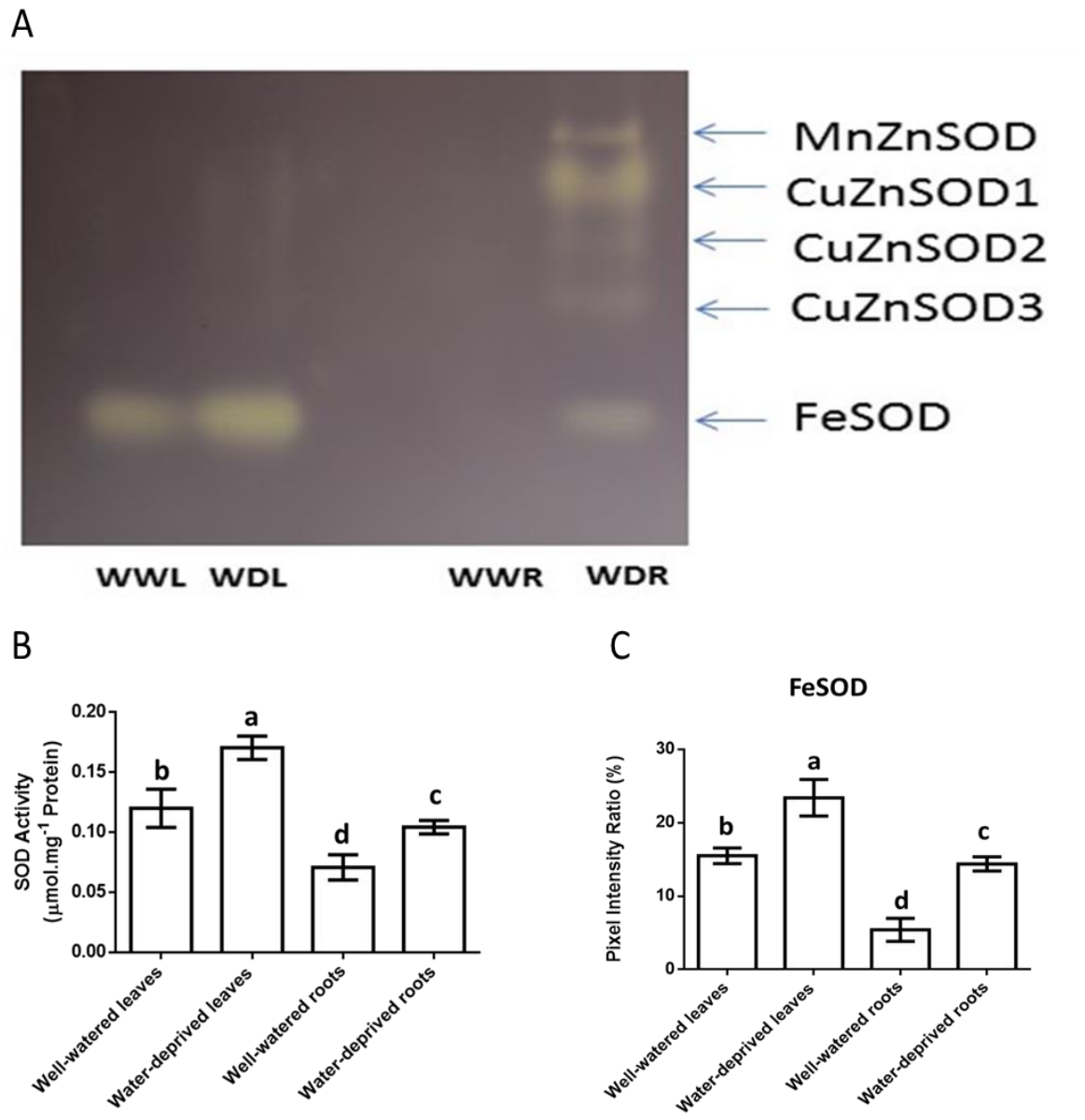

Figure 3.7: **SOD isoform identification and activity measurements in leaves and roots of maize measured after 28 of treatment**. Qualitative determination the responses of SOD isoforms after native PAGE on 12% (w/v) acrylamide gel from crude extracts was based on their response to inhibitors, H_2O_2 and KCN (figure.3.7 A). Quantitative total SOD activity (no inhibitors) was also measured spectrophotometrically from crude extracts of leaves and roots (figure 3.7 B). Relative pixel intensity ratio (as a measure of activity) of FeSOD was measured (figure 3.7 C). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of NBT reduction at 560nm wavelength. Data presented are means (±SE) of four independent experiments (n=4). Different letters above error bars denote mean values that are significantly different at ($P < 0.05$).

3.8 The effect of drought stress on the expression of *ascorbate peroxidase* (*APX*) and *catalase* (*CAT*) genes

Relative gene expression/transcript accumulation measurement, i.e. increase or decrease in transcript levels of *APX 1 – 4* and *CAT 1* & *2* in treated (water-deficient) versus untreated (well-watered) maize, both normalized to the average expression of internal control maize genes elongation factor 1α, Actin 2 and β-tubulin, was carried out using quantitative RT-qPCR. Relative gene expression results for APX are displayed in figure 3.8.1 and 3.8.2 while those of CAT are presented in figure 3.8.3. For *ZmAPX1* gene expression, an increase of 1.9 fold in response to drought was observed in the leaves (figure 3.8.1 A) while an increase of 5.1 fold in response to drought was recorded in the roots (figure 3.8.1 B). Figure 3.8 C and D represent *APX2* relative gene expression results in the leaves and roots, respectively. A 5.1 fold increase in *APX2* gene expression relative to the untreated control (WWL) is observed in the leaves (figure 3.8.1 C). There was no detectable expression of the *APX2* gene in well-watered roots but, a significant increase in expression in response to water deficit was observed (figure 3.8.1 D).

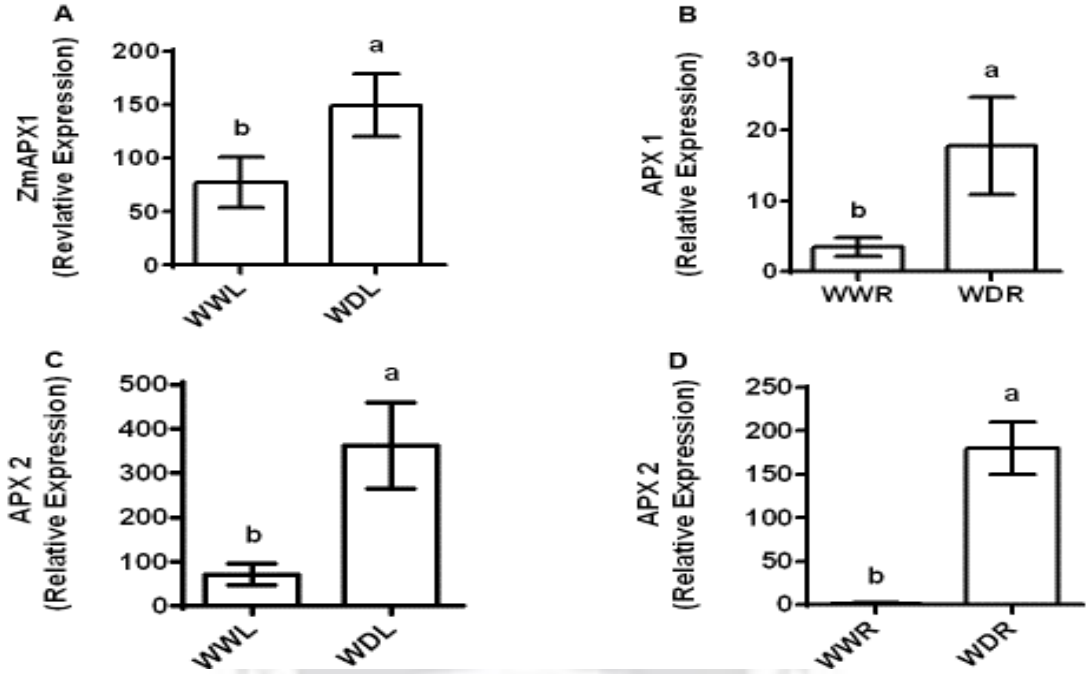

Figure 3.8.1: Expression of maize *ascorbate peroxidase* (*APX*) genes *ZmAPX*1 (A & B), *ZmAPX*2 (C & D) in leaves and roots respectfully, in response to water deficit. Transcript accumulation levels are expressed relative to the untreated control. Both well-watered and water-deprived experiments were normalised to the average levels of expression of the three internal control genes (Actin2, elongation factor1α & β-tubulin). Error bars represent the means (± SE; n= 3) of three independent experiments. The different letters indicate the difference between means at $P < 0.05$. WWL (well-watered leaves); WDL (water deprived leaves); WWR (well-watered roots) WDR (water-deprived roots).

Changes in transcript accummulation levels of *APX3* are presented in figure 3.8.2 A leaves) and B (roots). In the leaves, a 4.6 fold decrease in expression in response to drought was observed (figure 3.8.2 A). A similar (4.6 fold) increase was recorded in the roots in response to drought (figure 3.8.2. B). *APX3* results displayed an increase of 4.6 fold expression relative to untreated controls in both leaves and roots (figure 3.8.2 A and B, respectively). A significant increase of 11.3 fold for *APX4* gene expression in response to drought was recorded in the leaves (figure 3.8.2 C). Whilst there was no expression detected in well-watered roots, a significant increase in expression in response to drought was observed in water-deprived roots (WDR, figure 3.8.2 D).

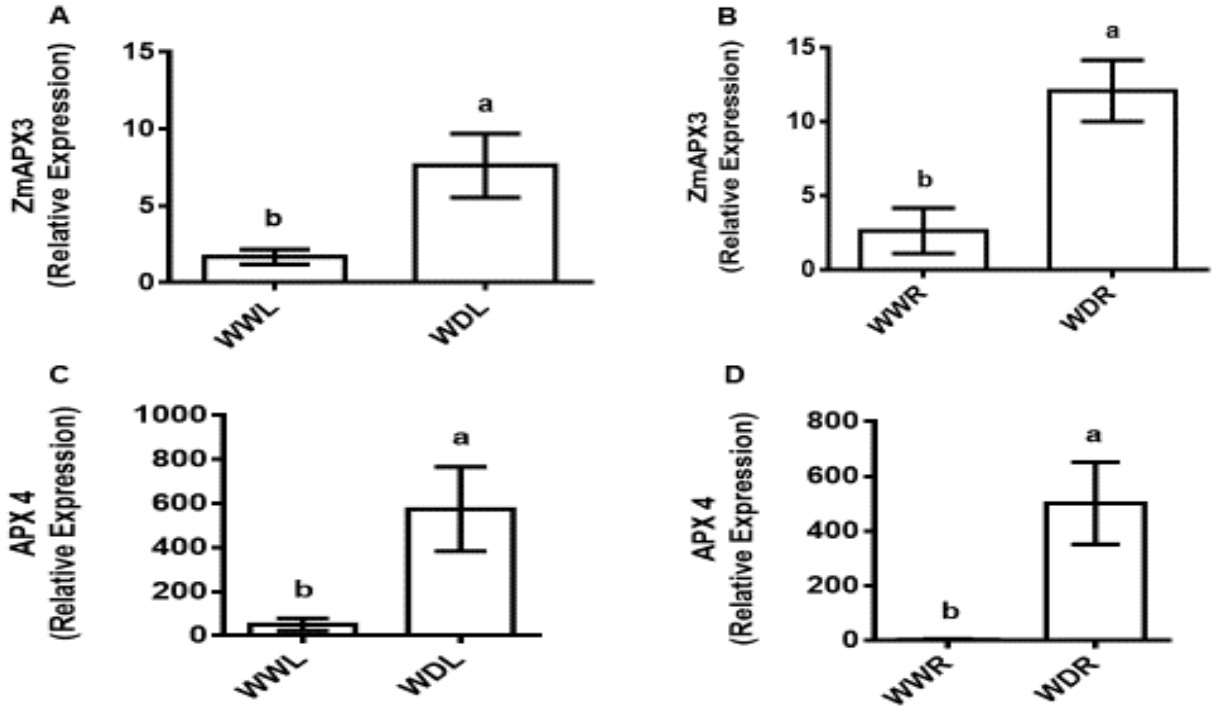

Figure 3.8.2: Expression of maize *ascorbate peroxidase* (A*PX*) genes *ZmAPX*3(A & B), *ZmAPX4* (C & D) in leaves and roots respectfully, in response to water deficit. Transcript accumulation levels are expresssed relative to the untreated control. Both well-watered and water-deprived experiments were normalised to the average level of expression of the three internal control genes (Actin2, elongation factor1α & β-tubulin). Error bars represent the means (± SE; n= 3) of three independent experiments. The different letters indicate the difference between means at $P < 0.05$. WWL (well-watered leaves); WDL (water deprived leaves); WWR (well-watered roots) WDR (water-deprived roots).

Changes in *CAT1* gene expression levels in leaves and roots are presented in figure 3.8.3 A and B, respectively. A significant 9.8 fold increase in *CAT1* gene expression levels was recorded in the leaves in response to drought (figure 3.8.3 A). In the roots (figure 3.8.3. B), a 4.3 fold increase in *CAT1* gene expression levels in response to drought was observed. *CAT2* gene expression levels recorded a 2.8 fold increase in the leaves in response to drought (figure 3.8.3 C). Similarly, relative expression levels of *CAT2* gene increased 3.9 fold in response to drought in the roots (figure 3.8.3 D).

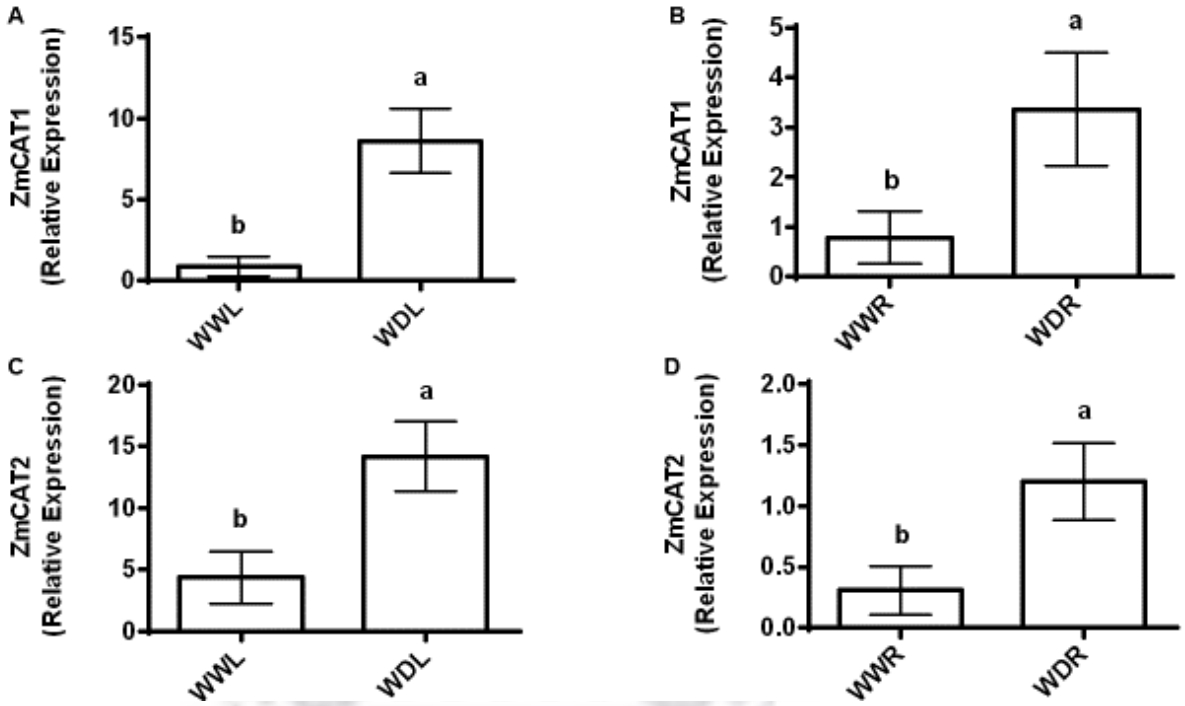

Figure 3.8.3: Expression of maize *catalase* (*CA*T) genes *CAT1* (A & B) and *CAT2* (C & D) in leaves and roots respectively, in response to water deficit. Transcript accumulation levels are expressed relative to the untreated control. Both well-watered and water-deprived experiments were normalized to the average level of expression of the three internal control genes (Actin2, elongation factor1α & β-tubulin). Error bars represent the means (± SE; n= 3) of three independent experiments. The different letters indicate the difference between means at $P < 0.05$. WWL (well-watered leaves); WDL (water deprived leaves); WWR (well-watered roots) and WDR (water-deprived roots).

CHAPTER 4

DISCUSSION

4.1 Drought stress reduces maize (*Zea mays L.*) biomass

Without water, plants cannot photosynthesise or maintain full metabolic activity required for plant functioning. In crop agriculture, water or scarcity availability can produce high yields or total crop failure. It has been reported that when enough water is available, the number of cultivated crops is doubled, leading to higher crop yield than when there are water limitations (FAO, 2011). In this study, the effects of long-term drought on the growth of maize were investigated on the basis of shoot fresh weight. The growth of plants is achieved through cell elongation, division and differentiation and can be measured by biomass and height (Aslam *et al*., 2015). The results showed that maize is sensitive to drought stress. A steep reduction in shoot biomass was observed in response to drought (figure 3.1), a clear indication of reduced growth. These results are not new and unique. Several other studies have also shown that maize is sensitive to drought stress (Li-Ping, 2006; Bai, 2006; Takele and Farrant, 2009; Benesova *et al*., 2012). The reduction in growth, leading to reduced biomass, can be attributed to physiological and biochemical processes that are negatively affected by drought, such as photosynthesis, respiration, translocation, ion uptake, carbohydrate metabolism and nutrient metabolisms (Jaleel *et al*., 2009; Farooq *et al*., 2008; Yang *et al*., 2015). The negative effect of drought stress is further characterized by reduction in water content, diminished water potential, turgor loss, impaired mitosis resulting in limited cell division and obstructed cell elongation that results in reduced growth (Farooq *et al*.,2009; Razmjoo *et al*., 2008). Impaired mitosis, cell elongation and expansion result in reduced plant height, leaf area and crop growth under drought conditions (Nonami, 1998; Kaya *et al*., 2006; Hussain *et al*., 2008). The results of this study showed that

drought stress caused morphological and physiological changes on the water-deprived maize compared to well-watered ones. Morphological responses to drought stress were characterized by visible loss of turgor with wilting and curling symptoms in leaves of drought-treated maize seedlings (data not shown). This resulted in growth inhibition and ultimately, biomass reduction.

4.2 Drought stress increases the concentration of H_2O_2 in leaves and roots of maize

When a plant is exposed to abiotic stress such as drought, H_2O_2 concentration is increased to toxic levels, resulting in oxidative stress (Yang *et al*., 2015; Li-ping *et al*., 2006). For example, this occurs when a plant attempts to conserve water by closing the stomata in response to water deficit. During stomatal closure, carbon dioxide cannot enter the leaves, resulting in decreased CO_2 assimilation, but simultaneously causes an increase in photo-respiratory oxygen assimilation (Luna *et al*., 2004). As a result, ROS, including H_2O_2, increase to levels that are harmful to the plant. Other recent studies have found that H_2O_2, in cross-talk with nitric oxide and calcium, can act as a signalling molecule in plant development and abiotic stress (such as drought) responses (Li *et al*., 2015; Zou *et al*., 2015; Hu *et al*., 2007; Tan *et al*., 2013). In this study, H_2O_2 levels increased in leaves and roots that were exposed to drought stress when compared to the well-watered leaves and roots of maize plant (figure 3.2). The increase was much more pronounced in water-deprived leaves than in water-deprived roots. This may be a consequence of increased photorespiration as a result of stomatal closure, possibly resulting from accumulation of ABA in the roots and leaves, since drought is known to induce ABA accumulation that leads to stomatal closure and an increase in H_2O_2 levels in plant tissue.

4.3 Drought stress differentially increases the production of MDA in leaves and roots of maize plant

It is well established that stressful growth conditions such as drought can induce cell membrane damage, increase membrane permeability and accumulation of ROS in plants, leading to oxidative stress (Ti-da *et al*., 2006; Li-ping *et al*., 2006). Cell membrane damage occurs through the peroxidation of lipids in which ROS remove electrons from the cell membrane lipids, leading to programmed cell death. A major product of lipid peroxidation is malondialdehyde (MDA) and is often used as a marker of lipid peroxidation. In this study, MDA content was significantly higher in roots and leaves of water-deprived maize than the roots and leaves of well-watered maize (figure 3.3). The results suggest that there was severe ROS damage in the leaves than in the roots, implying that the roots had a more efficient ROS scavenging system than the leaves. Indeed, APX (figure 3.5b), SOD (figure 3.7 B) and CAT (figure 3.6 B) total activity measurements revealed significantly higher activities in water-deprived leaves than in water-deprived roots. Furthermore, studies on maize which were conducted in our laboratory produced similar results to this study. In agreement with these results, a positive correlation between lipid peroxidation with other biochemical and physiological symptoms in response to drought stress have been reported in maize (Bai *et al*., 2006; Ali and Ashraf, 2011; Anjum *et al*., 2011a, 2011b and 2012).

4.4 Drought stress induces cell death in maize roots and leaves

As it has been stated before that MDA, the product of lipid peroxidation during abiotic stress is responsible for cell membrane damage, and can result in cell death (Sharma *et al*., 2012). In this study, this correlation between membrane damage and cell death was tested by measuring Evans blue uptake in leaves of maize under well-watered and water-deprived conditions. The results demonstrated that the leaves of water-deprived maize had less cell viability than the well-watered maize leaves (figure. 3.4). These results correlate well with the observed increase

in lipid peroxidation (figure 3.3) as a result of increased levels of H_2O_2 (figure 3.2) that eventually led to high levels of cell death. This high level of cell death is indicative of plants suffering from oxidative damage. Other similar study results are consistent with the findings of this study. For example, in a study conducted by Anjum *et al*., 2011 to assess the drought–induced oxidative damage in terms of ROS accumulation in maize, it was found that, when compared with well-watered conditions, drought stress caused oxidative stress through excessive production of ROS which led to reduced growth and yield, ultimately causing cell death in all maize hybrids (Anjum *et al*., 2011b & 2017).

4.5 Ascorbate peroxidase activity is differentially induced in response to drought stress in maize.

In total, seven APX isoforms activities (herein referred to as ZmAPX 1 – 7) were detected in leaves and roots of maize (figure 3.5 A). Native gel analyses showed that ZmAPX4 and ZmAPX7 represented the majority of the APX isoforms. The activity of these isoforms was highly enhanced in response to drought stress in water-deprived leaves and roots, with a highest response being displayed by ZmAPX7 in water-deprived leaves. Densitometry analyses of ZmAPX7 on both leaves and roots showed the highest activity levels in water-deprived leaves when compared to water-deprived roots in response to drought stress (figure 3.5 G). Indeed, total APX activity assay (the sum of individual isoforms' contribution) on both leaves and roots revealed that the highest response to drought stress was in the water-deprived leaves (figure 3.5 B). These results clearly suggest that ZmAPX7 may play a major role in response to drought stress in maize leaf tissue. The high activity of ZmAPX7 in response to drought stress correlate with the high levels of H_2O_2 measured in water-deprived leaves (figure 3.2). With high affinity for H_2O_2 than other antioxidant enzymes like CAT, APX breaks down H_2O_2 efficiently using ascorbate as the electron donor and may act as a regulator of ROS/H_2O_2 levels in cells (Harb *et*

al., 2015; Tan, M. *et al*., 2011). ZmAPX4 activity was also detected in all four treatments with a noticeable increased activity in water-deprived leaves and roots (figure 3.5 A). The increase in activity was also correlated with densitometry analyses that produced enhanced activities in leaves and roots in response to drought stress (figure 3.5 D). Taken together with ZmAPX7, ZmAPX4 activity detection in leaves and roots suggest that APX is a versatile enzyme and *ZmAPX* 4 & 7 gene expression is up-regulated during normal growth (in well-watered leaves and roots) with direct correlation to increased enzyme activity in response to drought stress (in water-deprived leaves and roots). Equally interesting results are those of ZmAPX5 activity in the leaves and roots. Under normal growth condition in the leaves (WWL), the activity of ZmAPX5 was not detected but, in response to drought stress (WDL) the activity was detected showing a very strong activity (figure 3.5 A). The absence of ZmAPX5 activity in well-watered leaves implies that ZmAPX5 encoding gene is silent or down regulated during growth under favourable conditions. However, well-watered roots displayed partial or low activity of ZmAPX5. However, when these roots were subjected to drought stress (WDR), ZmAPX5 activity was significantly enhanced relative to WWR (figure 3.5 A). The activity of ZmAPX5 in the roots was also analyzed by densitometry and the results showed a statistically significant enhanced activity in response to drought stress (figure 3.5 E). Similarly, ZmAPX4 and ZmAPX7 activity results indicate that these isoforms play a major role in the detoxification of ROS in leaves and roots of maize in response to drought stress. Taken collectively, ZmAPX4, ZmAPX5 and ZmAPX7, results showed that these isoforms contribute significantly to antioxidant activity in response to drought stress. ZmAPX7 displayed the highest contribution in the leaves and followed by the roots, respectively (figure 3.5 B). ZmAPX1 and ZmAPX2 activities were detected only in water-deprived roots while ZmAPX3 & 6 activity was significantly increased in water-deprived roots relative to well-watered roots (figure 3.5 A & C). The absence of ZmAPX1 & 2 activity in the leaves suggest that these

isoforms are localized in the roots only and that their activity is induced by abiotic stress such as drought.

4.6 Catalase is differentially expressed in response to drought stress

Catalase can either catalyze the direct dismutation of H_2O_2 into H_2O and O_2 (catalytic mode) or it can utilize H_2O_2 to oxidize substrates such as ethanol, methanol, formate or nitrite, formaldehyde (peroxidatic mode). In plants, catalase exists in multiple forms (or isoforms) that have been shown in maize to be coded for by distinct, unlinked genes (Scandalios, 1965 & 1968). Maize has a set of four discrete catalase isoforms of which 3 have been intricately analysed genetically and biochemically (Schmittgen and Livak, 2008). In this study, five active isoforms of CAT were detected (figure 3.6 A). On visualization of native-PAGE and densitometry analyses (figure 3.6 D), ZmCAT4 showed significantly enhanced activity in response to drought stress (water-deprived leaves) when compared to leaves from well-watered plants. In addition, the spectrophotometric total CAT activity assay revealed a significant increase in CAT activity in water-deprived leaves with a major contribution coming from ZmCAT4 in water-deprived leaves (figure 3.6 B). Densitometry analyses also revealed a statistically significant increase in ZmCAT4 activity in response to drought stress when compared to well-watered leaves (figure 3.6 D). Interestingly, ZmCAT4 activity was not detected in both well-watered and water-deprived roots. These results suggest that ZmCAT4 is localized in the leaves of maize but not in the roots. Two other active isoforms, ZmCAT2 and ZmCAT5 were detected in water-deprived roots in response to drought stress but not in well-watered roots nor leaves. This may suggest that *ZmCAT2* and *ZmCAT5* genes are stress responsive and that they are located in the roots. ZmCAT3 activity was detected in well-watered roots only, with no corresponding activity in leaves in response to drought. This might be an indication that ZmCAT3 isoform does not play any role in ROS scavenging in response to severe stressful conditions and that it is only found in the roots.

Alternatively, this may imply that the activity of ZmCAT3 is inhibited by drought stress, at least in the roots. Along with ZmCAT4, the activity of ZmCAT1 was significantly enhanced in response to drought stress in well-watered leaves in comparison to water-deprived leaves (figure 3.6 A). Pixel intensity analyses of ZmCAT1 revealed a statistically significant activity increase in response to drought stress (figure 3.6 C). These results reveal a variable response of CAT isoforms activity in response to drought stress, particularly in the roots. The presence of distinct isoforms in the leaves (ZmCAT1 and ZmCAT4, both expressed in well-watered and water-deprived leaves only) and in the roots (ZmCAT3, expressed in well-watered roots only; ZmCAT2 and ZmCAT5 expressed in water-deprived roots only) suggest that different CAT response mechanisms to drought stress exists between the leaves and roots of maize. In a similar study, distinct CAT isoform responses to severe drought stress in sugarcane were reported (Boaretto *et al*., 2014). In describing the CAT isoform profile in two sugar cane cultivars, the authors found no major changes in isoform activity in both control and water restricted treatments, but observed two main CAT isoform in the drought sensitive cultivar. The same isoforms were detected in the drought tolerant cultivar with only CAT1 being present in the control and only CAT2 was detected in the treated plants under mild drought stress conditions. However, when both cultivars were subjected to severe drought stress, the drought tolerant cultivar expressed CAT1 in both treatments. However, the drought sensitive cultivar expressed enhanced CAT2 activity in both treatments. Based on these results, the authors suggested that the drought tolerant cultivar responded differently to the level of stress. They concluded that this may be due to different production levels of H_2O_2 in different cell organelles leading to different CAT isoforms to respond differently (Boaretto *et al*., 2014). Further supporting evidence can also be found in another study by Tan *et al*., 2011. The authors investigated the effects of mild water stress induced by polyethylene glycol (PEG) on the activities of antioxidant enzymes and their isoforms, antioxidant content of different subcellular compartments in maize leaves. For the

results, the authors found that different kinds of antioxidant isoforms in different subcellular organelles had different responses to water stress. With CAT in particular, they found two isoforms in peroxisomes with CAT1 showing higher activity than CAT 11, and the priority of CAT1 increasing with time in response to drought stress. While on the other hand they found one CAT isoform in apoplasts in response to water stress. Indeed, depending on the type of stress, its intensity and duration, plant species and genotype, environmental stress has been shown to result in either elevated or depleted CAT activity (Moussa and Abdel-Aziz, 2008; Sharma and Dubey 2005; Devi *et al*., 2012). Of major interest in these results is the significant contribution made by CAT4 isoform in response to drought stress and the accompanying positive correlation from total CAT activity results (figure 3.6 A and B respectively). This may be due to apoplasts being the major source of H_2O_2 accumulation in plants' leaves that have been subjected to water stress (Bartoli *et al*., 2004; Hu *et al*., 2006; Jubany-Mari *et al*., 2009), leading to upregulation of antioxidant enzymes in maize leaves (Hu *et al*., 2005 & 2006) and that CAT is one of the major H_2O_2 scavenging enzymes in plants. In general, CAT activity in maize under drought stress conditions has been shown to increase (Koralovic *et al*., 2009; Tekele and Farrant, 2009; Chugh *et al*., 2011; Sofalian and Valizadeh, 2016).

4.7 Drought stress induces differential SOD isoform activity in maize

Different plants produce different numbers of each type of SOD isoforms (Gratao *et al.,* 2005). In total, one MnSOD, three CuZnSODs and one FeSOD activity bands were observed (figure. 3.7 A). In response to drought induced stress, FeSOD activity was significantly increased in leaves (WDL) and roots (WDR), with the highest activity displayed in the leaves (figure 3.7 A & C). This high activity of FeSOD implies that FeSOD may play a significant role in protecting maize against oxidative stress and may lead to tolerance under drought stress. It is also worth-noting that the highest activity of FeSOD, a chloroplast-located enzyme (Kliebenstein, *et al*., 1998), in response

to drought stress in the leaves may suggest that the photosynthetic apparatus, a vital component of plant growth and development, may well be protected. This suggestion is supported by a study in which an expression of transgenic Arabidopsis FeSOD in chloroplasts showed an improved oxidative stress tolerance in tobacco (*Nicotiana tabacum*) by protecting the plasma membranes and PSII (Van Camp *et al*., 1996). Indeed, increased FeSOD activity in response to drought-induced stress has been reported in maize (Avramova *et al*., 2015; Van Breusegem *et al*., 1999).

Three CuZnSOD isoforms were observed in both leaves and roots in response to drought-induced stress. Their response was more pronounced in WDR and less in WDL (figure 3.7 A.), suggesting that these isoforms play a greater role in the roots than in leaves in maize drought tolerance. To further support this argument, it is worth noting that these CuZnSOD isoforms were not detected in WWL but observed in WWR, albeit poorly expressed (figure 3.7 A). The activity of these CuZnSOD isoforms in WWR was detected through total SOD activity measurements (figure 3.7 B). In the absence of CuZnSOD isoforms in WWL and its poor expression in WWR in figure 3.7 A, it is important to explain that the higher total SOD activity observed in WWL, in comparison to a lower activity in WWR in figure 3.7 B, is likely derived from the high expression of FeSOD isoform. This is clearly supported by the densitometry analysis of FeSOD, where the pixel intensity of FeSOD in WWL is significantly greater to that in WWR (figure 3.7 C). The total SOD enzyme activity (figure 3.7 B) correlated positively with in-gel activity assay (figure 3.7 A), showing an induction by drought stress and highest activity from the FeSOD isoform (WDL & WDR in figure 3.7 B & C). The densitometry analysis of FeSOD (figure 3.7 C, WDL and WDR) suggest that FeSOD may play a key role in drought response of maize. Overall, these results are consistent with other studies reporting an increase in SOD activity in response to drought stress in sunflower (Gunes *et al*., 2008), poplar (Xiao *et al*., 2008), cowpea (Manivannan

et al., 2007b), liquorice (Pan *et al*., 2006) and wheat (Bakalova *et al*., 2004). Besides, SOD responses to stressful conditions have been shown to be substrate inducible (Tsang *et al.,* 1991). This implies that an increase in the SOD activity is indicative of elevated levels of reactive active oxygen species, serving as substrate that lead to increased expression of genes encoding SOD. Furthermore, the analysis of individual SOD Isoforms is important, because it can help to understand how drought-induced stress may affect the different subcellular compartments in maize.

4.8 Relative expression of *ZmAPX* and *ZmCAT* genes is differential in response to drought stress in leaves and roots.

Reverse transcription qPCR (RT-qPCR) was used to investigate changes in the transcript levels of *APX1-4* and *CAT1 & 2* genes in response to drought stress in maize and to establish if there is any correlation between the enzyme activity and transcript expression levels. Previous studies have shown that genes encoding antioxidant enzymes are frequently important in developing plants with enhanced drought tolerance. For example, increased tolerance to drought has been observed in *Nicotiana tabacum* that overexpress *APX* gene (Badawi *et al*., 2004). In transgenic rice, *OsMT1* gene overexpression indicated a higher level of CAT and APX activity and caused an increase in drought resistance (Yang *et al*., 2009). In this study, when one compares the expression of *APX* genes in leaves versus roots, it is noted that the *APX* genes (figure 3.8.1 A & B and A - D; figure 3.8.1 C & D) shows differential expression relative to untreated controls in response to drought stress. Further to the differential expression of *APX* genes between the leaves and roots, a pattern of expression emerges within the leaves and roots – all *APX* genes are significantly up-regulated in the leaves but down-regulated in the leaves and roots in response to drought stress. These results suggest that all the *APX* genes in this study are likely to play a major role in the fight against drought stress. Indeed, previous studies (Bian and Jiang, 2009) reported an increase in *APX* gene transcript level in roots of *P. pratensis* in response to

drought but, a significant increase was also observed in the leaves, which is consistent with the results of this study for *APX* genes. Pronounced down-regulation of *APX2* & *4* genes expression in untreated control was also evident in the roots (figure 3.8.1 D and figure 3.8.2 D, respectively), and that the expression of these genes may be induced by the lack of water in the roots and that their expression is induced in response to drought stress. Although the *APX* genes that are tested here for relative expression may not necessarily be the complete genes encoding for the APX isoforms tested for activity in the leaves (figure 3.5 A), the relative expression of the *APX* genes are consistent with the pattern of APX isoform activity in response to drought stress within the leaves (figure 3.5 A). But because of the complex regulatory mechanisms of gene expression, gene expression cannot be directly correlated with enzyme activity (Harb *et al.*, 2015). Indeed, many factors that may account for discrepancies between measured gene expression and enzyme activity levels have been reported (Grunberg-manago, 1999). *CAT1* gene expression was significantly up-regulated in response to drought stress, both in leaves and roots (figure 3.8.3 A). *CAT2* gene expression was also up-regulated in leaves and roots (figure 3.8.3 B). Finally, similar regulation of *CAT* and *APX* gene expression could be explained by the fact that proteins encoded by these genes are involved in the scavenging of H_2O_2 produced during oxidative stress, thus their gene expression is likely regulated in the same way.

4.9 Conclusion

In this study, the effects of drought-induced stress on the activities of antioxidant enzymes; namely SOD, APX and CAT; were investigated. Hydrogen peroxide, MDA, cell death and biomass were also explored as part of the investigation in order to appreciate the impact at of drought on these biochemical and physiological aspects. In conclusion, the results obtained showed that drought stress leads to oxidative stress, resulting in oxidative damage in maize, as evident in the increase in H_2O_2, MDA, cell death and a decrease in biomass. High levels of H_2O_2, and MDA in the leaves than in roots in response to drought

suggest that the leaves are more sensitive to drought stress than the roots. Drought stress caused an increase in SOD, APX and CAT activities. FeSOD displayed the highest enzymatic activity in leaves compared to other SOD isoforms. APX 4 and 5 contributed significantly to APX-dependent antioxidant activity, with APX7 displaying the greatest contribution in the leaves than in roots. CAT4 displayed the highest contribution to CAT-mediated antioxidant activity and was detected in leaves only, implying that it may be located exclusively in the leaves. This may be explained by the fact that most ROS is generated (via photorespiration) in the leaves in response to drought stress. In conclusion, this study demonstrated that antioxidant enzyme responses are influenced by drought and are determinants of the efficiency with which ROS are scavenged in response to drought. It further illustrated that transcriptional regulation of antioxidant enzyme genes is not sufficient to explain the responses of antioxidant enzyme activities to drought, suggesting that post-transcriptional and post-translational mechanisms may also play a role in regulating maize responses to drought. The positive correlation between gene expression and enzyme activity seen during drought stress may indicate that the increase in enzyme activity response levels were caused by high mRNA levels and were up-regulated at the posttranscriptional level, which in part might be enzyme activation or synthetic processes induced by drought. To elucidate these mechanisms with reference to response of maize to drought stress, proteomic profiling may be a useful tool to adopt as it will identify changes in protein abundance and may provide clues to post-translational modifications to the existing protein pools to effect changes in their biological activities.

CHAPTER 5

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