Involvement of Abscisic acid and H_2O_2 in antioxidant enzyme activities mediated by nitric oxide synthase-like activity in maize

By

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ABSTRACT

Involvement of Abscisic acid and H_2O_2 in antioxidant enzyme activities mediated by nitric oxide synthase-like activity in maize

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In recent years, nitric oxide (NO) has emerged as an important endogenous plant signalling molecule that mediates many developmental and physiological processes. NO regulates the activity of antioxidant enzymes in response to drought-induced stress by controlling the expression of the genes that encode these enzymes. Antioxidant enzymes function in scavenging reactive oxygen species like superoxide ion (O_2^-) and hydrogen peroxide (H_2O_2) that are generated in response to drought-induced stress and other abiotic stresses. Abscisic acid, a phytohormone that acts as a stress-related hormone in plants, also stimulates production of H_2O_2, thus further triggering the antioxidant enzyme activity in order to scavenge the excess H_2O_2. Accumulated data indicate that NO interacts with reactive oxygen species, notably hydrogen peroxide and superoxide. This study was aimed at clarifying the role of NO derived from nitric oxide synthase-like (NOS-like) enzymatic activity in scavenging of H_2O_2 and to establish if this is dependent or independent of ABA signaling. This was achieved by using Nω-Nitro-L-Arginine methyl ester (L-NAME), an inhibitor of NOS to control the amount of NO in maize tissue. The study investigated the effect of L-NAME on the accumulation of superoxide, which is scavenged by superoxide dismutase. Furthermore, the study determined the role of NOS-like activity in ABA-mediated production of H_2O_2. Lastly, the effect of L-NAME on H_2O_2 accumulation and antioxidant enzyme activity was also investigated. Application of L-NAME altered the enzymatic activity of superoxide dismutase, ascorbate peroxidase and catalase. These changes in enzymatic activity were coupled with altered levels of O_2^- and H_2O_2 in leaves and roots. Treatments with ABA in combination with L-NAME resulted in reversal

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of H_2O_2 content to basal levels. These results suggest that nitric oxide, produced by nitric oxide synthase-like activity, is important in regulation of antioxidant enzyme activity and cross-talks with ABA.

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DECLARATION

I declare that "*Involvement of Abscisic acid and H_2O_2 in antioxidant enzyme activities mediated by nitric oxide synthase-like activities in maize*" is my own work, that it has not been submitted for any degree or examination at any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full name: Siphiwe Gift Hlatshwayo ` 3569174

Signed at: The University of the Western Cape Signature:

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
ANOVA	Analysis of variance
APS	Ammonium persulfate
APX	Ascorbate peroxidase
AsA	Ascorbic acid
BSA	Bovine serum albumin
CAT	Catalase
Cu/Zn-SOD	Copper zinc superoxide dismutase ix
DNA	Deoxyribonucleic acid
DTNB	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
Fe-SOD	Iron superoxide dismutase
FW	Fresh weight
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulfide
KCN	Potassium cyanide
L	Liter
L-NAME	Nω-Nitro-L-Arginine methyl ester
MDA	Malondialdehyde

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mg	Milligrams
mL	Milliliter
mM	Millimolar
Mn-SOD	Manganese superoxide dismutase
MTT	(3-(4,5-diphenylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
MW	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitro blue tetrazolium
nM	Nanomolar
PAGE	Polyacrylamide gel electrophoresis
pM	Picomolar
PMS	Phenazine methosulfate
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TCA	Trichloroacetic acid
TEMED	N, N, N’, N’-Tetramethylethylenediaminese
Tris	Tris(hydroxymethyl)aminomethane
V	Volts
v/v	Volume by volume
w/v	Weight by volume

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Zm	*Zea Mays*

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CHAPTER 1 – Literature review

1.1 INTRODUCTION

Drought stress is one of the most agriculturally and economically detrimental factors that affect growth, development and yield of plants. Drought is characterized by a long period of absence of rainfall, leading to a deficit in soil water and reduction of ground water, which greatly affects farming (Ku *et al*., 2013). Due to climate change, drought has been worsening the water scarcity challenges experienced by living organisms, affecting crop growth as a result of cell damage (Farooq *et al*., 2009), harming plant development and further limiting plant production (Zhao *et al*., 2009). This has a negative impact on agricultural productivity, especially in developing countries that consume cereal crops as a staple diet. Water stress, as caused by drought, remains as one of the important environmental stresses known to interfere with many processes of the plants including photosynthesis, hormonal balance and plant nutrition (Farooq *et al*., 2009).

In response to water deprivation stress, plants demonstrate a variety of adaptive mechanisms. Part of the defence responses of plants to water deficit is the production of compatible solutes such as proline, which play a fundamental role in adjusting plant cell water status to prevent further dehydration (Ashraf and Foolad, 2007). Accumulation of this compatible solute occurs in the cytosol to prevent the detrimental effects caused by water deprivation and other abiotic stresses. (Moharramnejad *et al*., 2015). In many species, enhanced accumulation of proline has been found to act as osmoprotectant and improve the growth and development of plants exposed to a variety of abiotic stresses including drought, high temperature and salinity (Ashraf and Foolad, 2007). Many studies indicate that in many plant species, the accumulation of proline leads to significant increases in growth and final crop yield under environmental stresses (Ashraf and Foolad, 2007; Moharramnejad, 2015); depending on the water status, growth stage and the plant cultivar. Furthermore, the accumulation of these compatible osmolytes has been

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found to reduce oxidative damage occurring in the plants' cell under severe drought stress (Hasanuzzaman *et al*., 2014**;** Padmavathi and Rao 2013; Quan *et al*., 2008).

The production of reactive oxygen species (ROS) is the result of the partial reduction of atmospheric O_2 (Carvalho, 2008). There are four forms of cellular ROS, namely singlet oxygen (O_2), superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl (OH^-); all functioning as signaling molecules when produced under normal conditions (Anjum *et al*., 2011). Hydrogen peroxide acts as a secondary messenger in stress-response signal transduction and in development. It is the most stable reactive oxygen species that has the ability to easily diffuse from one cellular compartment to another. Due to its role in biochemical activities, H_2O_2 is known to regulate the expression of some genes in cells (Quan *et al*., 2008). Depending on its concentration, H_2O_2 can also be used as a plant growth promoting chemical by farmers in small-scale and large-scale farming, reported to have regulatory effects on growth, development and quality of fruit (Quan *et al*., 2008). The production and control of H_2O_2 as a reactive oxygen species during drought-induced stress is still a controversial subject that is yet to be resolved as H_2O_2 seem to have a dual effect under stress conditions, depending on its production level in the cells. If kept at relatively low levels, H_2O_2 was shown to function as a component of a stress-signalling pathways, triggering stress defence responses (Anjum *et al*., 2011). However, at high levels, it reaches a certain level of phytotoxicity, initiating uncontrolled oxidative damage to the cell, further triggering lipid peroxidation, enzyme inhibition and eventually leading to cell death (Sharma, 2012).

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Reactive oxygen species are kept under tight control by protective enzyme-catalyzed systems that function in balancing the production of reactive oxygen species with their scavenging and maintain normal cellular function (Anjum *et al*., 2011; de Carvalho, 2008). The balance between ROS production and activities of antioxidative enzymes can determine whether signalling and/or damage will occur (Anjum *et al*., 2011). These antioxidant defence system includes antioxidants such as glutathione and ascorbate, and ROS-scavenging enzymes such as superoxide dismutase, ascorbate peroxidase, catalase and glutathione reductase. Maintaining a high level of antioxidant enzyme activities may contribute to drought tolerance in plants by increasing the antioxidant capacity against oxidative damage (Sharma, 2012).

There seems to be a complex relationship between reactive oxygen species and the stress signal hormone abscisic acid (ABA). ABA is a plant phytohormone which has been shown to be involved in a wide range of plant physiological responses (Hancock *et al.*, 2011). Plants undergoing drought-induced stress accumulate ABA, which plays a key role in regulating plant responses to drought from a whole plant level to cellular level perspective (Reddy *et al.*, 2004). As a result of ABA accumulation in response to drought stress, evidence suggests a model of stress response showing functions mediated by ABA that can be used for improving drought stress tolerance in plants (Neil *et al.*, 2008). Plants require the coordinated functioning of hormones such as ABA along with the coordination of downstream signal transduction mechanisms involving, among others, hydrogen peroxide and nitric oxide.

In recent years, nitric oxide (NO) has emerged as an important endogenous plant signaling molecule that mediates many developmental and physiological processes (Neill *et al.*, 2008; Palavan-Unsal and Arisan, 2009; Siddiqui *et al.*, 2011; Scheler, 2013; Yu *et al.*, 2014). Nitrogen is one of the most important minerals needed by plants for normal growth. Plants absorb inorganic nitrate from the soil through its roots and convert it enzymatically into NO. Accumulated data indicate that NO interacts with reactive oxygen species, notably hydrogen peroxide and superoxide (Finkel, 2011; Scheler *et al.*, 2013). The mechanisms by which nitric oxide functions in driving signal transduction are partly shared between animals and plants. In plants, nitric oxide synthase-like proteins are important enzymes responsible for the production of NO from arginine, using O_2 and NADPH as co-substrates (Misra *et al.*, 2011). However, there are no homologues of the nitric oxide synthase (NOS) protein that have been identified in higher plants. However, NOS activity is detectable in plants, where use of NOS inhibitors has been reported to reduce nitric oxide content and influence superoxide dismutase activity (Qiao and Fan, 2008; Halliwell and Gutteridge, 2015).

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Despite the progresses made in studying the individual role of H_2O_2 and ABA in plant antioxidant activity in mediating plant tolerance against various abiotic stresses, the effects of nitric oxide synthase and how it is involved in ABA and H_2O_2 on the antioxidant enzymatic activity is still not fully understood. This study was aimed to clarify the role of NOS-like enzymatic activity in ROS scavenging by using Nω-Nitro-L-Arginine methyl ester (L-NAME). L-NAME will act as an inhibitor of nitric oxide synthesis. Furthermore, the study aimed to determine the role of NOS-like activity in ABA-mediated production of H_2O_2. Lastly, it aimed to investigate the effects of inhibition of NOS on antioxidant enzyme activity. To achieve these aims, measurement and comparison of proline content, reactive oxygen species including the hydrogen peroxide content and superoxide anion, lipid peroxidation and antioxidant enzyme activities of superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) were done across various treatments.

Investigating the involvement of NO signaling in the regulation of antioxidant activity responses in relation to ABA and H_2O_2 provides a new opportunity to understand plant responses to drought-induced stresses in order to increase agricultural productivity since NO, ABA and ROS are instrumental in regulating plant responses to drought stress. It is crucial to improve drought tolerance in maize in order so to maintain good yield even at low water availability so as to sustain the country's agricultural economy. Furthermore, sustained maize production will help to meet food demand in the face of steep population growth. This physiology and biochemistry study, combined with other advanced molecular studies, will contribute towards understanding the mechanisms by which plants perceive and transduce water deficit stress and the adaptive response they employ. It will also identify antioxidant enzymes that are influenced by nitric oxide synthase towards developing drought tolerant crops. This would contribute to food security.

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1.2 What is stress in plants?

The term stress is used in different instances, with various meanings to suit different circumstances. Stress is defined as the altered physiological condition leading to altered stability in response to particular factors (Shao *et al.*, 2008). Plants are exposed to a wide range of both abiotic and biotic stresses, including pathogens, harmful insects, light, drought, salinity and high temperatures. Drought stress, characterized by water scarcity and also referred to as water deficit stress, is the most devastating stress affecting growth, yield and quality of most plants (Patanè, and Cosentino, 2010). Water deficit stress occurs when plants are growing in relatively dry soil as the leaves lose more water than is taken up by the roots, resulting in cellular dehydration, damage, and ultimately death. Larcher (2003) further describes water deficit stress as a state in which water potential, the movement of water from one area to another due to osmosis in plants, is reduced (Ockham, 2012); thus interfering with its normal function, which leads to stomatal closure and disruption of gas exchange in leaves of plants.

A study by Shao *et al.* (2008) showed that water deficit stress affects water supply to roots, disrupting its ability to absorb nutrients from the soil. In the study, various physiological and biochemical processes were altered; these included nutrient metabolism and hormone levels. The physiological processes affected by water scarcity include growth, metabolism, yield and quality (Munns, 2002; Patanè, and Cosentino, 2010). A review by Bita and Gerats (2013) demonstrated how water scarcity can also disturb the energy balance in plants and disturb the normal redox state of cells, leading to a toxic state where the plant over-produces reactive oxygen species that damage all components of the cell.

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Water deprivation stress in plants trigger a wide variety of molecular responses, ranging from metabolic changes and altered gene expression to proteomic changes. These changes adopted by plants exist to withstand the potentially harmful effects caused by water stress (Shao *et al.*, 2008). The quantity and quality of crops depend

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on cell division and differentiation, all of which are drastically affected by water stress.

1.3 Abscisic acid and its biosynthesis

Abscisic acid (ABA) is a ubiquitous, sesquiterpenoid ($C_{15}H_{20}O_4$) containing a single asymmetric carbon atom at C-1’ as shown in figure 1 (a). ABA is a major phytohormone that regulates many physiological processes in plants (Shakirova *et al.*, 2003). ABA-deficient mutants of maize (*Zea mays*) and tomato (*Lycopersicon esculentum*) were instrumental in discovering the pathway for ABA biosynthesis. The studies suggested that in maize plants, ABA is indirectly synthesized through the cleavage of a C_{40} carotenoid precursor, followed by a two-step conversion of the intermediate xanthoxin to ABA via an ABA-aldehyde (Xiong *et al.*, 2003).

The first step in the ABA biosynthesis pathway is the epoxidation of zeaxanthin and antheraxanthin to violaxanthin in the plastids. The first step is catalyzed by a zeaxanthin epoxidase (ZEP) (Xiong *et al.*, 2003). Following a series of structural modifications, 9-*cis*-epoxycarotenoid dioxygenase (NCED) catalyzes the oxidative cleavage of 9-*cis*-neoxanthin to a C_{15} intermediate xanthoxin. The product xanthoxin is converted to ABA in the cytosol in a two-step reaction. The first step of this reaction is catalyzed by a short-chain alcohol dehydrogenase/reductase (SDR), producing ABA aldehyde. In the second step, ABA aldehyde oxidase (AAO) catalyzes ABA aldehyde to generate abscisic acid (Xiong *et al.*, 2002).

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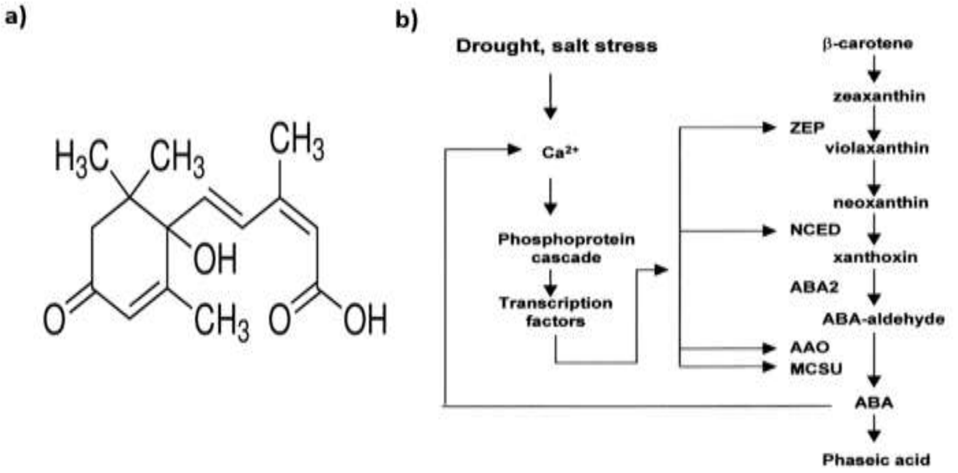

Figure 1.1: (a) The chemical structure of abscisic acid (Adapted from Zocchi *et al*., 2017). (b) Biosynthesis and regulation pathway of ABA induced by drought and salinity (Adapted from Xiong *et al*., 2002).

ABA serves as an important regulator that coordinates growth and development in plants in response to the environment. Under normal conditions, ABA in plant cells is maintained at low concentrations, a requirement for normal plant growth, as observed in a study of Finkelstein and Rock (2002).

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1.4 Abscisic acid as a stress-related hormone influencing root architecture

Originally studied to gain a greater understanding on bud dormancy in fruits, ABA is now known to be involved in drought-induced stress responses, including control of transpiration in leaves (Hancock *et al.*, 2011). The production of ABA has been shown to be triggered by drought-induced stresses (Zhang *et al.*, 2006). The reason behind the increased endogenous level of ABA is related to a survival mechanism plants have acquired as a way to perceive incoming stresses and regulate their physiology accordingly (Zhang *et al.*, 2006). The response takes place in such a way as to avoid any inhibition of plant growth /functions under normal conditions. Once the stress condition is relieved, ABA should be rapidly degraded and deactivated for normal plant growth and functions to resume. A study by

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Daszkowska-Golec (2016) has reported how ABA has an effect in controlling the major check points in root growth, including cell division and cell elongation.

Plant roots act as primary sensor of water deficiency in soil and transduce signals to the aerial parts to modulate the growth and development. The root system of maize comprises of axillary and lateral roots, all serving as primary sensors of drought-induced stress. Axillary roots are further comprised of primary, seminal and nodal roots (Aslam *et al.*, 2015; Feix *et al.*, 2006). Primary and seminal roots are collectively known as embryonic roots. Seminal roots are permanent and have functional role in growth and development of maize (Aslam *et al.*, 2015). Initially, ABA was thought to have a negative role in root architecture (De Smet *et al*., 2003). Roots of maize plant become elongated under mild drought-induced stress to explore the soil for nutrients and for more water uptake whereas, under severe drought-induced stress the root volume, density and number of roots are reduced (Obidiegwu *et al.*, 2015). Physiological analysis in *Medicago truncatula* in a study by Liang *et al.* (2007) showed a positive role of ABA in forming and maintaining root meristem function, further controlling root elongation. In the study, the cell length was regulated through the expression of respiratory burst oxidase homolog (RBOH) genes that encode an enzyme that generates superoxide. Furthermore, a review by Smith and De Smet, (2012) shows that ABA has an influential role in *Arabidopsis* root growth by regulating cell division at the tip of the root.

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Elevated ABA levels as a result of exposure of plants to environmental stress aids in maintaining the local water availability during water deprivation by modifying root architecture (Basu and Rabara, 2017), suggesting that alteration in root structure and function occurs as a result of ABA. Other researches have shown the direct influence of ABA on root growth, influencing the growth of primary roots (López-Bucio *et al.*, 2003; Anjum *et al*., 2011), further increasing the length of the elongation zone and diminishing the accumulation of ROS (Liszkay *et al*., 2004). A study by De Smet *et al.* (2003) showed how ABA significantly promotes the formation and growth of adventitious roots. However, in a study by Guo *et al*. (2009) low concentrations of exogenously applied ABA inhibited lateral root development, while high concentration were shown to inhibit growth. Furthermore,

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excessive ABA concentrations inhibited lateral root growth in rice seedlings (Belimov *et al*., 2014). The influence of ABA on plant roots in promoting growth and development under stressful conditions is dependent on the concentration. Low levels of ABA have a positive effect by increasing root hair development, while high concentrations inhibit growth (Niu *et al*., 2012). ABA also regulates signalling processes involving nitric oxide and H_2O_2 in the guard cells, thereby reducing transpiration (Bright *et al.*, 2006).

1.5 Nitric oxide synthase in plants

Mechanisms that underlie the functions of NO in plants are receiving a great deal of attention. Nitric oxide is a free radical that is often difficult to detect and to quantify due to its high reactivity and short half-life (Santolini *et al.*, 2017). However, at very low concentrations of pM to nM found in plants, NO is relatively stable (Mur *et al.*, 2013). The biosynthesis of nitric oxide was controversial in plants after it was acceptably clarified in the animal kingdom. A growing body of evidence indicates that NO is formed by mammalian-like nitric oxide synthase (NOS) activity (del Río *et al*., 2004), nitrate reductase (Yamasaki *et al*., 1999), or non-enzymatic sources (Bethke *et al*., 2004). Nitric oxide can be generated through non-enzymatic mechanisms where NO is supplied as a by-product of nitrous oxide oxidation in the atmosphere during nitrification/ denitrification cycles.

A study by Palavan-Unsal and Arisan (2009) has shown that plants release NO under normal growing conditions. However, the effects of NO on plant growth and development are highly dependent on the concentration. At high concentrations (>10 µM), NO can cause oxidative and nitrosative damage leading to cell death (Palavan-Unsal and Arisan, 2009). At a lower concentrations, it possess beneficial features that counteract the oxidative and nitrosative stresses during abiotic stresses (Siddiqui et al., 2011).

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1.6 Nitric oxide synthase as a source of NO

1.6.1 Removal of NO as a signaling molecule in plants

Nitric oxide synthase-like activity was first detected in plants by Ninnemann and Maier (1996) and its various processes could be inhibited by, Nω-Nitro-L-Arginine methyl ester (L-NAME), N-nitro-L-Arg (L-NNA) and NG-monomethyl-L-Arg, all of which are known inhibitors of mammalian NOS enzymes (Hao *et al.*, 2008). Furthermore, conclusive biochemical evidence was shown of plant enzymatic activity that uses L-arginine to generate NO (Neill et al., 2003). Although no gene has been found to code for nitric oxide synthase enzyme and no protein with NOS activity has been isolated in higher plants (Besson-Bard *et al.*, 2006), NOS-like activity has been detected widely in plants, and inhibitors of mammalian NOS have been shown to inhibit NO generation in plants (Corpas *et al*., 2006; Liu *et al*., 2007).

There are many reports in which L-NAME has been shown, in correlation with an inhibition of NO production (Neill *et al.*, 2003; Lamotte *et al.*, 2005; Jasid *et al.*, 2006; Foresi *et al.*, 2010) with an increase in arginine levels observed in plants treated with ABA. This confirms that NOS-like enzymes do exist in plants. However, clarification on the identity of these enzymes and how they utilize L-arginine is still unknown.

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1.6.2 Role of nitric oxide in regulation of reactive oxygen species

The development and survival of plants is influenced by many endogenous and exogenous factors. The growth and yield of plants is dependent on coordinated functioning of many hormones such as abscisic acid, along with the coordination of other downstream signal transduction mechanisms involving hydrogen peroxide as a reactive oxygen species and reactive nitrogen species such as nitric oxide (Hancock *et al*., 2011). In a similar manner to abscisic acid, not only are nitric oxide and hydrogen peroxide involved in drought-induced stress responses, but the interaction of both nitric oxide and reactive oxygen species is involved in a wide range of stress responses. These include drought, cold, heat and pathogen challenge responses (Farooq *et al*., 2009).

Different types of mechanical, chemical and environmental stressors in a variety of plant species trigger NO production to regulate plant responses to the abiotic stresses (Qiao and Fan, 2008). A study by Desikan *et al*. (2004) has observed that nitric oxide and H_2O_2 are generated in response to the same stimuli and with the same kinetics. Nitric oxide and hydrogen peroxide can interact chemically to form compounds such as peroxynitrite due to the reaction between NO and O_2^-, damaging a wide array of molecules in the plant cell (del Río, 2015).

A study by Wendehenne *et al*. (2004) suggested that high concentrations of H_2O_2 in the plant cell emerges due to the synthesis of NO. Additionally to their report, it was further proposed how NO collaborates with H_2O_2 and increase the chances of plant survival under drought stress.

Similar to H_2O_2, nitric oxide acts as a bioactive molecule, displays properties as both a prooxidant and an antioxidant (Groß, 2013) by either inducing or inhibiting oxidative stress. As an antioxidant, NO plays a role in scavenging excess H_2O_2 in the plant cell (Quan *et al*., 2008). A study by Gechev *et al*. (2006) reported that NO acts synergistically with H_2O_2 to induce the process of cell damage, depending on its relative timing and intensity.

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In maize, exogenously applied H_2O_2 increases salt tolerance by increasing the activities of antioxidants enzymes and increased proline accumulation, conferring tolerance to salt stress in rice (Roy *et al.*, 2016). Furthermore, an experiment was carried out to investigate the protective role of H_2O_2 against high temperature stress in maize at the early vegetative stage. Exogenous application of 1.2 mM of H_2O_2 improved heat tolerance by stimulating the antioxidant enzyme system and membrane injury (Hasanuzzaman et al., 2013).

1.7 Biochemical responses - Oxidative stress in plants

Oxidative stress is better defined as the imbalance in the production of reactive oxygen species, also referred to as free radicals, and antioxidant defence if this leads to cellular damage (Betteridge, 2000). The accumulation of reactive oxygen species (ROS) and changes in cellular redox state induced by ROS has

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been a central theme in many stress responses. It was considered of little or no importance until 16 years ago, when the predominant view on ROS was questioned and were believed to be toxic molecules that cause cell damage to the plant (Møller *et al*., 2007), and this has led to the perception that the tolerance to environmental stress conditions could be engineered in plants by further improving antioxidant capacity. High production of reactive oxygen species is one of the most striking and damaging results of abiotic stress in crop plants in different cellular and sub-cellular compartments (Rao and Chaitanya, 2016). They are generated due to the decreased content of intracellular CO_2, resulting in the transfer of electrons from the electron transport chain to oxygen at photosystem I. Production and synthesis of ROS such as superoxide (O_2^-), hydroxyl (OH^-), hydrogen peroxide (H_2O_2) and singlet oxygen (O_2) leads to the damage of lipids, nucleic acids and sugars (Vellosillo *et al*., 2010).

1.7.1 Generation of reactive oxygen species

In plants, chloroplasts and peroxisomes are the main sources of reactive oxygen species production as depicted in figure 2, through photorespiration during light and mitochondria in darkness phase (Anjum *et al*., 2011). Chloroplasts are a major producer of superoxide and hydrogen peroxide. Under water stress, photosynthesis decreases due to stomatal limitation when light energy absorption exceeds its capacity for it to be utilized (Moharramnejad *et al*., 2016). The excess light energy, which is not produced during photosynthesis and not dissipated as fluorescence or heat, will be improperly transferred to oxygen or neighbouring molecules, creating reactive oxygen species (Gill and Tuteja, 2010). An estimated 1–5% of the oxygen consumption of isolated mitochondria results in ROS production. The peroxisomes produce O_2 and H_2O_2 in several key metabolic reactions. NADPH oxidase in the plasma membrane produces O_2^-, which participates in several physiological processes (Anjum *et al*., 2009).

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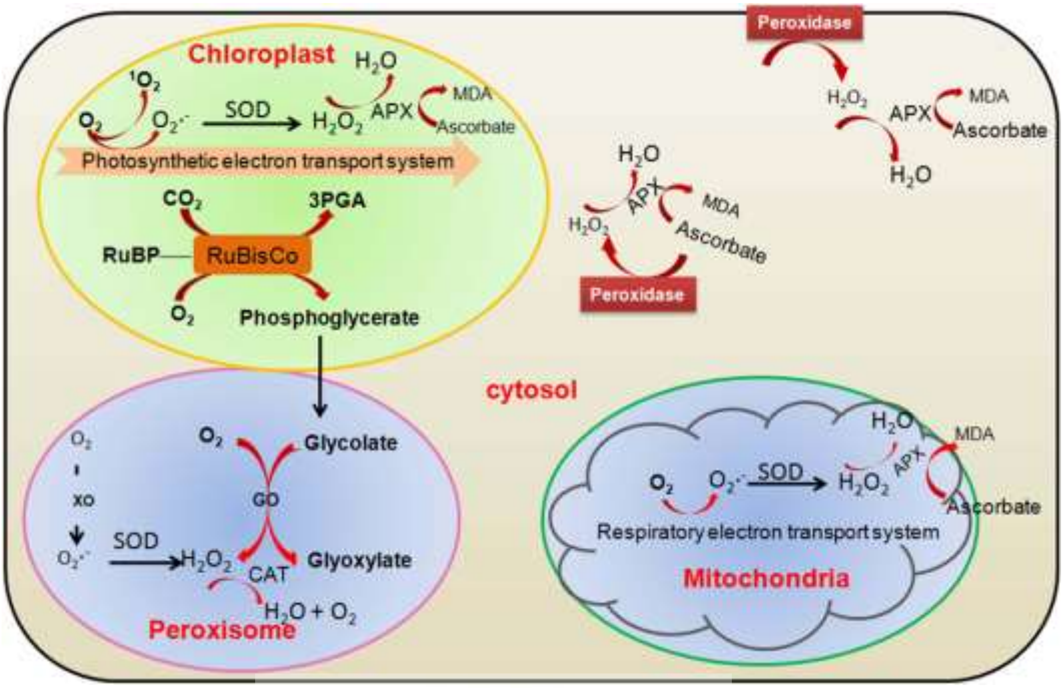

Figure 2.1: Production and scavenging of ROS in a plant cell. The major sites of reactive oxygen species production and involvement of antioxidant enzymes (Adapted from Jajic *et al.*, 2015).

1.7.2 The double impact of reactive oxygen species in plants

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As stated in studies by Fujita *et al.*, 2006; Halliwell and Gutteridge, 2007; and Miller *et al.*, 2010, high production of reactive oxygen species have a double impact under abiotic stress conditions as outlined in figure 3, that depends on their overall cellular concentrations. Under circumstances where the reactive oxygen species are kept at moderately low levels, they are liable to work as part of a stress signalling pathway. However, under abiotic stresses, the balance between the production and elimination of ROS gets disturbed in cellular components of plants (Karuppanapandian *et al.*, 2009 and 2011; Vellosillo *et al* 2010), resulting in oxidative stress and ultimately cell death (Karuppanapandian *et al.*, 2011).

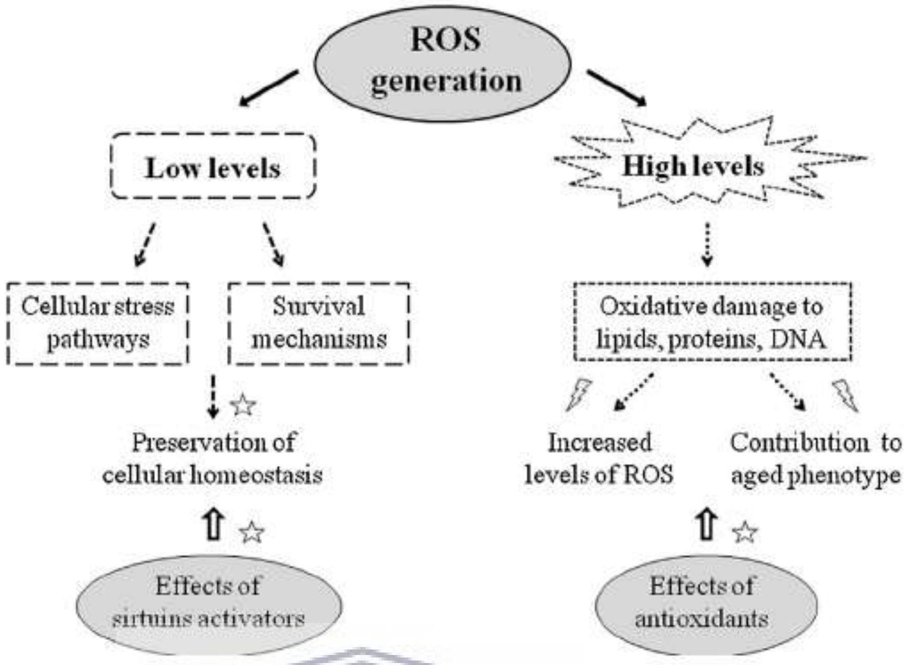

Figure 3.1: Dual role of Reactive Oxygen Species levels. Low levels of ROS in cellular stress responses result in survival mechanisms, while elevated levels of ROS can produce oxidative damage to key macromolecules and result in cellular damage (Adapted from R Ramis *et al*., 2015).

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1.8 Targets of ROS

Reactive oxygen species are known to cause damages in biomolecules such as lipids, proteins and DNA (Das and Roychoudhury, 2014). When the ROS are produced, their removal must be strictly controlled in order to avoid oxidative stress, a state where the level of ROS exceeds the defence mechanisms (Sharma, 2012). However, the equilibrium between production and scavenging of ROS is also disturbed under water stressful conditions. These reactions can alter intrinsic membrane properties like fluidity, loss of enzyme activity, inhibition of protein synthesis, ultimately resulting in cell death (Das and Roychoudhury, 2014).

1.8.1 DNA damage

Modification in the nucleotides of the DNA can result in the mismatches with the other nucleotides in the complementary strand, resulting in mutations (Sharma *et al.*, 2012). The damage suffered by DNA, also referred to as spontaneous DNA damage, results from the damage of DNA by OH^- and O_2^-. The DNA damage can be attributed to the reaction of O_2^- with amino acid guanine (Gill & Tuteja, 2010). Damage to DNA by ROS occurs through base deletion and modifications (alkylation and oxidation) as well as formation of pyrimidine dimers, cross-links and strand breaks (Tuteja *et al.*, 2001). Enhanced DNA degradation was observed in plants exposed to various environmental stresses (Sharma *et al.*, 2012).

1.8.2 Protein degradation

ROS can damage proteins (Anjum *et al.*, 2009). Protein degradation in this case occurs via a covalent modification in the protein (Ahmad *et al.*, 2009). Proteins can be modified directly or indirectly when the ROS attacks those proteins (Ahmad *et al.*, 2009). The attack by ROS on proteins may cause modification in a variety of ways, some are direct and others indirect. Direct modification is the modulation of a protein's activity through nitrosylation, carbonylation, disulphide bond formation and glutathionylation. Indirect modification involves conjugation of proteins via amino acid peroxidation (Das and Roychoudhury, 2014).

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1.8.3 Lipid peroxidation

ROS can react with lipids in cell membranes, causing lipid peroxidation (Comas *et al.*, 2013; Labudda, 2013). When ROS levels are above a given threshold, enhanced lipid peroxidation takes place in both cellular and organelle membranes, which, in turn, affect normal cellular functioning. Lipid peroxidation aggravates the oxidative stress through production of lipid-derived radicals that themselves can react with and damage proteins and DNA (Sharma *et al.*, 2012).

To measure the extent of lipid peroxidation in plants, the content of malondialdehyde (MDA) has been most frequently used as an indicator of oxidative

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damage, making it an appropriate marker for membrane lipid peroxidation for it is generated from the degradation of polyunsaturated lipids by ROS (Moller *et al.*, 2007). ROS-induced lipid peroxidation leads to changes to the membrane intrinsic properties like fluidity, transportation of ions, transmembrane protein stability and membrane integrity. These modifications lead to cell death (Sharma *et. al*., 2012). For this reason, cell membrane stability has widely been utilized to differentiate between drought tolerant and drought sensitive crop cultivars. In a study done by Yousfi *et al*. (2010), MDA concentration was significantly increased in all populations of maize under water deficit stress.

1.9 Antioxidative activity – Scavenging mechanisms

To ease and repair damage initiated by ROS under drought-induced stresses, plants have developed a defensive mechanism consisting of a complex antioxidant system, which is sufficiently intricate to maintain normal cellular function (Horváth *et al.*, 2007). The antioxidant defence system comprises of the co-ordinated action of enzymatic and non-enzymatic antioxidants that provide an efficient mechanism of controlling the toxicity rendered by ROS.

1.9.1 Non-enzymatic antioxidants

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1.9.1.1 Ascorbic Acid

Ascorbic acid is the most abundant and the most extensively studied antioxidant compound. It is considered powerful as it can readily donate electrons to forming a monodehydroascorbate (MDHA) radical, reacting with reactive oxygen species and regenerates α-tocopherol from tocopheroxyl radical to protect the plant's membranes from oxidative damage (Shao *et al.*, 2005). On further oxidation, MDHA yields an uncharged radical dehydroascorbate (DHA). The electron donating capability and formation of less reactive MDHA forms the basis for the antioxidant and scavenging activity of ascorbate (Rao and Chaitanya, 2016).

In the ascorbate–glutathione cycle, the plants' antioxidant enzyme ascorbate peroxidase, that has been shown to be upregulated when plants are undergoing

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stress, uses two molecules of ascorbic acid to reduce hydrogen peroxide to water, since ascorbic acid has been shown to be the most important reducing substrate for hydrogen peroxide (Jareel *et al*., 2009). A direct protective role of ascorbic acid has also been demonstrated in various plant species (Chen and Gallie, 2004; Shao *et al*., 2008).

1.9.2 Osmoprotective compounds

One form of stress response in plants is the overproduction of different types of compatible organic solutes. Compatible solutes are characterized as highly soluble, low molecular weight compounds that are reported to be nontoxic at high cellular concentrations (Ashraf and Foolad, 2007). Generally, they function in protecting plants from environmental stresses through different courses, including detoxification of reactive oxygen species and stabilization of enzymes. They are commonly referred to as osmoprotectants because some of these solutes also protect cellular components from dehydration injury.

1.9.2.1 Proline

Proline is an amino acid that serves as an osmolyte and an antioxidant (Das and Roychoudhury, 2014). It is widely reported to play an important role in safeguarding cells from damage caused during drought stress. Furthermore, it has been found to scavenge ROS and acts as molecular chaperone stabilizing the structure of proteins (Rao and Chaitanya, 2016; Szabados *et al.*, 2010). Proline is synthesized using glutamic acid as a substrate, via a pyrroline5-carboxylate intermediate. It serves as an efficient scavenger of OH^- and O_2^- and can inhibit the damages caused by these reactive oxygen species. During stress, characterized by enhanced production of ABA, large amounts of proline accumulate as a result of either enhanced synthesis or reduced degradation (Verbruggen and Hermans, 2008).

The biosynthesis of proline, shown in figure 4, originates from L-glutamic acid. The enzymes *pyrroline*-5-*carboxylate synthetase* (P5CS) and *pyrroline*-5-*carboxylate reductase* (P5CR), play major roles in the biosynythesis of proline. In *Brassica juncea* grown under drought conditions, activities of the proline

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biosynthetic enzymes P5CR are high in the tolerant lines (Gill and Tuteja, 2010). In light of the documented information available on the enhanced accumulation of proline in plants under abiotic stress, ABA-dependent and ABA-independent signalling pathways mediate the accumulation of proline in plants (Ashraf and Foolad, 2007). This was shown in a study by Xiong *et al*. (2001) after the expression of *P5CS* gene was regulated by ABA accumulation in plants in response to osmotic stress.

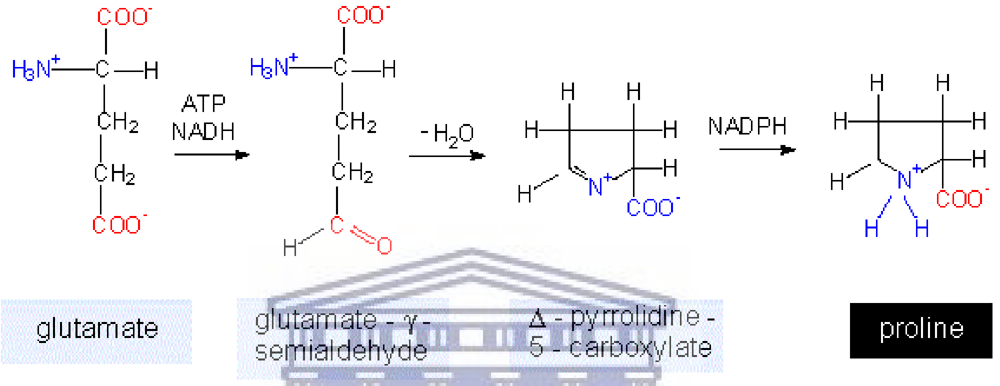

Figure 4.1: Biosynthesis of proline with the regulation of P5CS and P5CR in higher plants (Adapted from Ashraf and Foolad, 2005).

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Many studies have showed a positive relationship between accumulation of proline and water deficit stress tolerance (Ashraf and Foolad, 2007; Vendruscolo *et al*., 2007), while some have argued that the increase in proline concentration under stress is a product of, and not an adaptive response to, stress (Claussen, 2005). It has been stipulated that not all plant species are capable of natural production or accumulation of proline in response to stress. It was determined that, in response to water deficit stress, enhanced concentration of proline in maize roots was associated with increased concentration of abscisic acid (Ashraf and Foolad, 2007). Furthermore, the concentration of proline in rice was increased in the leaves subjected to water deficit (Hsu *et al*., 2003). A study by Nayyar and Walia (2003) revealed that the rate of proline accumulation and utilization was significantly higher in the drought-tolerant cultivar following assessment of the effects of

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drought stress on proline accumulation in a drought-tolerant and a drought-sensitive cultivar.

1.9.2.2 Glycine betaine

Glycine betaine (GB) is an amphoteric quaternary amine that acts as a compatible solute in most plants during drought stress. It is referred to as an osmoprotectant due to its occurrence most abundantly in response to dehydration stress, where it plays a vital role in adjustment and protection of the cell membrane (Ashraf and Foolad, 2005; Chen et al., 2008). As depicted in figure 5, GB is synthesized from serine via choline and betaine aldehyde, with betaine aldehyde dehydrogenase (BADH) acting as the vital enzyme in the final process of producing this amine. The production of glycine betaine is synthesized in the plant cell to protect against osmotic stress. Plants usually contain a low amount of glycine betaine, whereas *Arabidopsis* and few crop plants like rice and tomato do not naturally accumulate glycine betaine in the absence of water deficit (Fariduddin *et al.*, 2013).

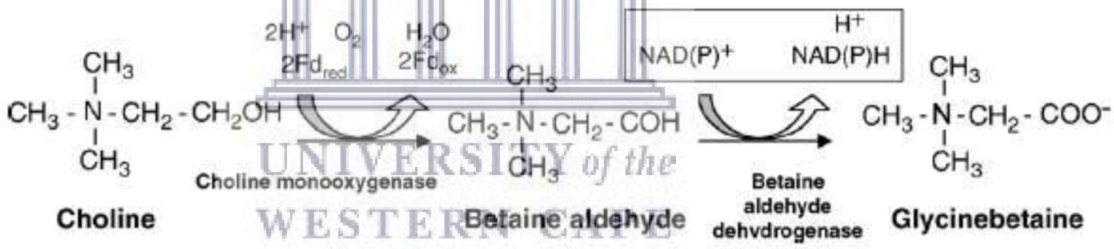

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Figure 5.1: Biosynthesis of glycine betaine. Plants convert choline to betaine aldehyde by the activity of choline monooxygenase (Adapted from Ashraf and Foolad, 2005).

Maize (*Zea mays*) has been shown to be amongst the plants that lack the ability to synthesize glycine betaine in the absence of stress but ROS elevation triggers its production in maize (Ullah *et al*., 2016). In many regions of Southern Africa, maize is one of the most important staple foods and an economical valuable crops available. A study by Hossain *et al* (2010) investigated the possible role of glycine betaine in regulating reactive oxygen species. The study found that glycine betaine provides a protective role against oxidative damage by reducing hydrogen peroxide and lipid peroxidation levels.

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1.10 Enzymatic antioxidant systems in plants

Enzymatic antioxidants such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) also detoxify reactive oxygen species that are produced during stress conditions (Vardharajula *et al*., 2011; Rahal *et al*., 2014). Additionally, other enzymes like dehydroascorbate reductase (DHAR), glutathione reductase (GR), and monodehydroascorbate reductase (MDHAR) function in the ascorbate–glutathione cycle to reduce the toxic oxygen derivatives (Gill and Tuteja 2010). There are numerous reports in the literature that stress the role of activities of antioxidants enzymes to ease and repair damage initiated by ROS under drought stress (Sharma *et al*., 2012; Noctor *et al*., 2014). The following antioxidants enzymes are instrumental in maintaining normal cellular redox status (Horváth *et al*., 2007).

1.01.1 Superoxide dismutase (SOD)

Superoxide dismutases (SODs) belong to a family of metallo-enzymes that catalyse the conversion of superoxide to oxygen (O2) and hydrogen peroxide (H_2O_2), as shown in the reaction scheme below (Sharma *et al*., 2012). Based on the metal co-factor, SODs are classified into three types, namely the FeSOD (localized in chloroplasts), MnSOD (localized in mitochondria), and Cu/ZnSOD (localized in chloroplasts, peroxisomes, and cytosol). These three isoenzymes have been shown to be responsive to H_2O_2, depending on the developmental stage of the plant and the state of environment (Van Camp *et al*., 1997; Abedi and Pakniyat, 2010; Arnholdt-Schmitt *et al*., 2006).

$$O_2^- + O_2^- + 2H+ \rightarrow 2H_2O_2 + O_2$$

The first plant SOD gene was cloned from maize (Cannon *et al.,* 1987). A study by Kumar *et al.* (2013) indicated that an increased expression of the *MnSOD* gene enhances tolerance to many environmental stresses, with the over-expression of Cu/ZnSOD enhancing the tolerance of potato to drought stress. The efficiency of scavenging of O_2^- by SOD depends on the distribution of the SOD enzyme within the cell and the physiological state of the cell as influenced by its environment

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(Karuppanapandian *et al.*, 2011). Studies by Mishra *et al.* (2011) and Sharma and Dubey (2005) reported increased SOD activity in plants exposed to various environmental stresses, including drought and metal toxicity, often correlated with increased tolerance of the plant against environmental stresses.

1.10.2 Catalase (CAT)

Since high concentration of H_2O_2 are also toxic to plants, excess H_2O_2 is scavenged by catalase and several other classes of enzymes (Vellosillo *et al.*, 2010). Catalase is a tetrameric heme-containing enzyme located in the peroxisomes that catalyses the conversion of H_2O_2 into H_2O and O_2, as depicted in the reaction equation below (Moloudi *et al.*, 2013). Catalases have a high enzyme turnover ratesbut lower affinity for H_2O_2 than APX, where approximately five million H_2O_2 molecules can be converted to H_2O and O_2 by one catalase molecule per minute (Goodsell, 2004).

$$H_2O_2 \rightarrow H_2O + (1/2)\ O_2$$

A study by Scandalios and colleagues characterized three catalase genes in maize, namely the *Catl*, *Cat2*, and *Cat3*, encoding the three biochemically distinct isozymes CAT-1, CAT-2, and CAT-3 (Scandalios *et al.*, 1997; Guan and Scandalios, 2002). Catalase is affected by many environmental conditions including drought, salinity and light. Catalase genes have also been shown to respond differently to exogenously applied abscisic acid (Fariduddin *et al.*, 2014).

1.10.3 Ascorbate peroxidase (APX)

Ascorbate peroxidase serves as the major plant peroxidase that is distributed throughout the cell. It uses ascorbic acid as an electron donor in the initial stage of the ascorbate–glutathione cycle to detoxify H_2O_2 to form H_2O and monodehydroascorbate (Karuppanapandian *et al.*, 2011). Ascorbate peroxidase appears to have a higher affinity for H_2O_2 (µM range) than catalase (mM range) as described in a study by Das and Roychoudhury, (2014). All these outcomes have prompted the speculation that APX, an enzyme situated in every cellular compartment that produces reactive oxygen species, may function in signalling purposes, whereas catalase, found exclusively in the peroxisomes, may function in

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removing the excess mass of reactive oxygen species generated under environmental stress conditions (Anjum *et al.*, 2015). This fact was observed in a study by Zhang *et al*. (2013) where the cytosolic APX were knocked out of rice resulting in inhibition of growth under drought, salt and cold stress.

1.10.4 Glutathione reductase (GR)

Glutathione reductase, the last enzyme of the ascorbate-glutathione cycle, has a major role in ensuring that the intracellular glutathione pool is maintained in the reduced state (GSH) involved in regenerating the ascorbate pool, resulting in the production of oxidized glutathione (GSSG). GSH and GR are involved in the detoxification of the hydrogen peroxide, and further as a reducing agent that recycles the oxidized ascorbic acid to its reduced form using the enzyme dehydroascorbate reductase (DHAR) (Gill and Tuteja, 2010). Firstly, ascorbate peroxidase reduces H_2O_2 to H_2O using ascorbate as an electron donor, resulting in the formation of monodehydroascorbate. Since monodehydroascorbate is a radical that is able to disproportionate into ascorbate and dehydroascorbate, the dehydroascorbate reductase further reduces dehydroascorbate to ascorbate yielding oxidized glutathione. Lastly, the oxidized glutathione (GSSG) is reduced by GR using NADPH to form GSH (figure 6).

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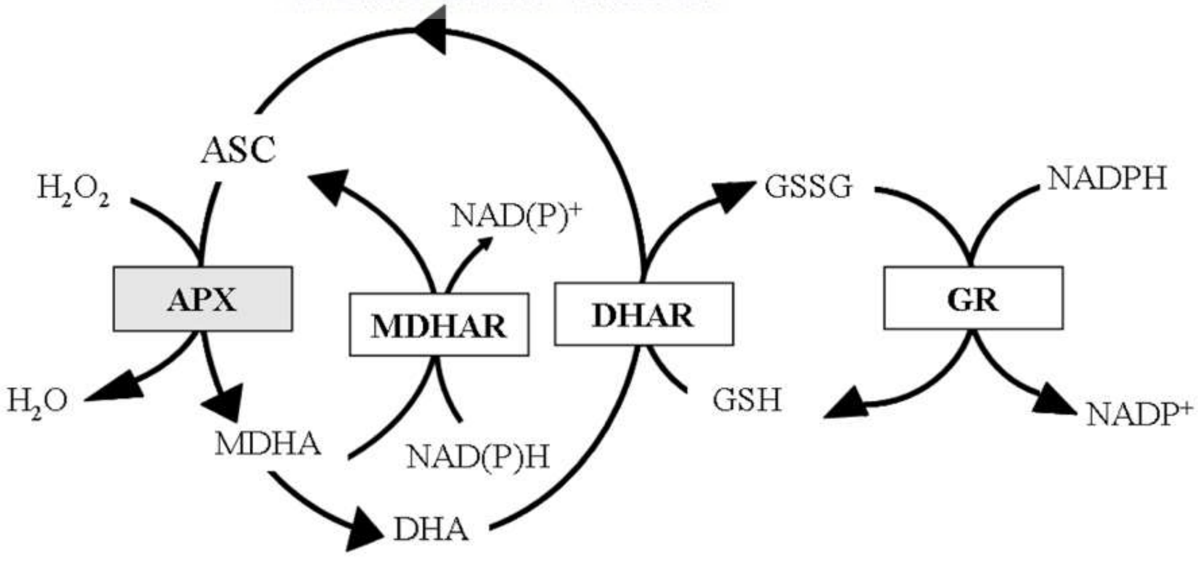

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Figure 6.1: The regulation of glutathione-ascorbate cycle. APX, ascorbate peroxidase, MDHAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase; GR, glutathione reductase; AsA, ascorbate; MDHA, monodehydroascorbate; DHA, dehydroascorbate; GSSG, oxidized form of glutathione; GSH, reduced form of glutathione. (Adapted from Locato *et al.*, 2013).

The implication of glutathione reductase in the defence against oxidative stress has been studied in drought-induced stresses. Increased GR activity has been widely observed in *Zea mays* and other species in response to drought (Ashraf, 2009). The dryness of the soil due to insufficient rainfall has been shown to enhance GR activity, resulting in the accumulation of GSH that acts as an antioxidant and radical scavenger (Lucato *et al.*, 2013).

1.11 Significance of maize as a cereal plant

Maize (*Zea mays* L.), also known as mealie in South Africa, is a warm-season cereal crop that is rich in starch in a form of carbohydrate, thereby providing dietary energy to both animals and humans (Kaur *et al.*, 2014). Maize is believed to have been domesticated initially in Mexico, after which it has since spread to the rest of the world due to its ability to grow in diverse climates in adequately rain-fed soils (Bates *et al.*, 2009). Maize consists of shallow roots, which makes it susceptible to drought since the roots cannot access water deep in the lower layers of the soil when the upper layers of the soil are dry.

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Maize has overtaken the other traditional cereals in terms of production, ranked first amongst cereal crops seconded by rice then wheat (Ranum *et al.*, 2014). Several studies report enormous losses of agricultural yields which are attributed either to lack of tolerance against nutrient-deficient soils or to susceptibility of maize to abiotic stress such as drought (Wassmann *et al.*, 2009; Devi *et al.*, 2017). Unlike in developed countries where it serves as a second-cycle produce in the form of meat, eggs and dairy products, maize serves as a major direct source of food for millions of people in Africa, also used as a breakfast cereal. In a processed form it is also found as starch which can further be converted to products such as sorbitol and lactic acid, and appears in countless household items such as beer, cosmetics, paint, and many others (Avérous, 2004). However, due to its higher necessity for sunlight, water, and its susceptibility to dry soil, maize tends to suffer a lot of environmental stress at the physiological and biochemical level, affecting its growth and further leading to reduced yield. Annual maize production fluctuates widely depending on the rainfall, and recently it has remained constant over a period of time, one contributing factor being the imbalance of nitrogen oxide molecules that further leads to plant hormonal imbalance (Corpas *et al*., 2011).

1.12 Motivation of the study

According to Grain SA (2016) South Africa is the major producer of agricultural products in Africa as it produces a wide range of food commodities. The South African landmass is considered to have 12% arable land and only 3% of the land is regarded as truly fertile. The only 1.5% of the land that is under irrigation produces 30% of the crops in the country (AgriSA, 2016; Drought Task Team, 2016). Approximately 60% of the country's water resources is used mainly in agriculture (Baleta and Pengram, 2014). The distinctive physiological traits that makes maize require more water makes it vital to understand the physiological and molecular responses associated with drought tolerance in maize to overcome the challenges of drought on maize production and yield. Focus on breeding technologies and genetic engineering approaches in developing drought-tolerant maize lines to increase maize yield under dry conditions is of importance. To achieve such desired tolerance, understanding the contribution of genetically determined metabolic components to the regulation of drought responses is crucial. For this reason, even though the thesis does not investigate the responses of maize to drought in itself, this study investigated the link between NOS-like enzymatic activity, ABA and H_2O_2 in the regulation of antioxidant enzyme activity because these metabolic components are associated with plant responses to drought.

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Aims and objectives

- To determine the contribution of NOS-like enzymatic activity (by treating maize with L-NAME, which inhibits NOS) in regulation of superoxide levels.
- To determine the contribution of NOS-like enzymatic activity in ABA-mediated production of H_2O_2.
- To investigate the effects of inhibition of NOS on antioxidant enzyme activity.

Hypothesis

Nitric Oxide Synthase-like activity is involved in ABA signaling and H_2O_2 homeostasis.

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CHAPTER 2

MATERIAL AND METHODS

All the chemicals were purchased from Sigma-Aldrich, unless otherwise stated. The spectrophotometry assays were analyzed using a FLUstar Omega UV-visible spectrophotometer (BMG LabTech GmbH, Orthenberg, Germany).

2.1 Seed germination

Maize (S1 22B 24R cultivar) seeds were surface sterilized with 0.35% (v/v) sodium hypochloride for 10 minutes and washed with distilled water five times. The seeds were then germinated in a sterile paper towel soaked with distilled water placed in a sterilized container in the dark. Germination occurred in the maize seeds and they were allowed develop a radicle of about 1-2 cm long before transplantation.

2.2 Plant growth

The germinated maize seeds were transplanted in 20 cm plastic pots containing moist Promix organic (consisting of 50% coco peat and 50% perlite), supplemented with 1% Vita organic fertilizer (Vita grow 2:2 (16)) purchased at Windell Hydroponics, Cape Town, South Africa. A total of 3 seeds were transplanted into each pot. Each treatment (described below) was done in triplicate.

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2.3 Treatments

The seedlings were grown in the greenhouse maintained at 23˚C under 16/8 hours light/dark cycle, at a photosynthetic photon flux density of 400 μmol.m^{-2}.s^{-1} during the light phase. The seedlings were supplied with 100 ml of water at two days intervals until they reached the V2 stage. The maize plants were separated into 6 treatments: (1) Untreated, (2) 1 mM Nω-nitro-L-arginine methyl ester (L-NAME), which is an inhibitor of nitric oxide synthesis, (3) 100 µM abcisic acid (ABA), (4) 100 µM ABA + 1 mM L-NAME, (5) 100 µM H_2O_2 and (6) 100 µM H_2O_2 + 1 mM L-NAME. The plants containing L-NAME in their treatments were pre-treated with 100 ml solution containing L-NAME in 10 mM TRIS base buffer at pH 7.3. Following the 24 hour pre-treatment with L-NAME, the plants were treated with

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their complete respective treatments. All the treatment solutions were prepared in 10 mM Tris-HCL at pH 7.3. After 48 hours, all the maize plants were harvested and plants at the same developmental stage were selected for further analysis.

2.4 Analysis of plant growth parameters

Maize plants were removed from the Promix, being careful to avoid any loss of shoots and roots during the up-rooting of the plants. Twelve plants from each treatment were divided into shoots and roots. Each of these plants was scored for length of the shoots and roots, fresh weight of the shoots and fresh weight of the roots. This was done to establish the physiological role of NOS-like activity in ABA-mediated production of H_2O_2 in leaves and roots of maize. The harvested plant material was ground into a fine powder in liquid nitrogen using a mortar and pestle and kept at -80°C for further biochemical analysis.

2.5 Measurement of reactive oxygen species

2.5.1 Superoxide (O_2^-) content

A modified method of Russo *et al.* (2008) was used to measure the superoxide content in the maize leaves. From the second youngest fresh leaf , 1 cm^2 was cut and carefully inserted into a centrifuge tube containing 10 mM KCN (to inhibit Cu/Zn SODs), 10 mM H_2O_2 (to inhibit Cu/Zn SODs), 2% SDS (to inhibit Mn and Fe SODs) and 80 µM NBT. The plant material was incubated for 20 minutes inside the solution. Once the incubation was completed, the leaf material was crushed using a miniature pestle. The tube was centrifuged at 13000 x *g* for 5 minutes to pellet the leaf material, and the formed supernatant was removed carefully and loaded onto a microtitre plate by adding 200 µl in triplicates into each well. The samples were read at 600 nm wavelength. A calculation taking into consideration the extinction coefficient of 12.8 mM cm^{-1} was used to determine the superoxide content. The intensity of the blue colour produced by the reaction indicted the level of superoxide, which is detected spectrophotometrically.

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2.5.2 Hydrogen peroxide (H_2O_2) assay

Hydrogen peroxide content was measured based on a modified method described by Velikova *et al*. (2000). A standard curve containing varying concentrations of H_2O_2, 500 mM KI, 5 mM K_2HPO_4 and varying amounts of sterile distilled water was first prepared in order to extrapolate the amount of hydrogen peroxide produced in the leaves and roots. Plant material (~100 mg) of leaves and roots grounded into fine powder was homogenized in a 1:5 ratio with 6% (v/v) ice-cold trichloroacetic acid. The homogenate was briefly mixed by vortexing for a minute and centrifuged at a speed of 13 000 x *g* for 30 minutes at 4°C. The supernatant was separated from the pellet and transferred to a sterile centrifuge tube. The TCA extract served as hydrogen peroxide extract, with the reaction mixture consisting of 50 µl TCA extract, 5 mM K_2HPO_4 at pH 5 and 200 mM KI to a final volume of 200 µl. Both standard curve and sample measurements were done in triplicates. The reaction was allowed to take place at 25° for 20 minutes and the absorbance was measured at 390 nm. The H_2O_2 content was calculated based on a standard curve constructed from the absorbance (390 nm) using the extinction coefficient 39.4 $mM^{-1}.cm^{-1}$.

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2.6 Testing for cell damage

2.6.1 Malondialdehyde (MDA) quantification

Malondialdehyde, a naturally formed product of lipid peroxidation, was used to measure the degree of lipid peroxidation using a modified method of Terzi and Kadioglu, (2006). A mass of 100 mg of grounded leaf or root material was added into different 1.5 ml centrifuge tubes. A 1:5 volume of 6% (w/v) Trichloroacetic acid (TCA) was added. The tubes were mixed by vortex action, followed by a 13000 x *g* centrifugation for 10 minutes to pellet the leaf and root material. A volume of 200 µl of the supernatant was removed from the tube and added to a clean centrifuge tube, and to this tube 400 µl of solution containing 20% (w/v) TCA and 0.5% (w/v) thiobarbituric acid (TBA) was added. To allow the reaction to take place, the reaction was mixed with a vortex and incubated in a heating block at 90°C for 20 minutes. The reaction was stopped by placing the tubes onto ice for 10 minutes.

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The reaction mixture was further centrifuged at 13 000 x *g* for 5 minutes, after which 200 µl of the reaction mixture was loaded in triplicates into a microtiter plate. The specific absorbance was measured at 532 nm and the non-specific absorbance at 600 nm. After subtracting the non-specific absorbance from each sample, the MDA concentration was calculated at a molar extinction coefficient of 155 mM^{-1} cm^{-1} and further expressed as $nmol.g^{-1}$ FW.

2.7 Estimation of osmolyte content

2.7.1 Proline contents

Proline content was measured according to a modified method of Bates *et al*. (1973). Purified proline was used to construct a standard curve from which the concentrations were determined. Fresh leaf or root material (0.5 g) were homogenized in 3% sulfosalicylic acid (w/v) and the homogenate was centrifuged at 10 000 x *g* for 5 minutes. An aliquot of 2 mL of the filtrate was added into a tube containing 2 mL of acid ninhydrin solution and 2 mL of glacial acetic acid. The test tubes containing samples were heated in a water bath at 100 °C for 1 h. Thereafter, the reaction mixture was ice-cooled and 4 ml of toluene were added into the reaction. The absorbance of the mixture was recorded at 520 nm using a UV–visible spectrophotometer.

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2.8 Protein extraction

Protein extraction was prepared from both leaves and roots of the various treatments. The frozen ground leaf and root tissue (200 mg each) was homogenized in 400 µl of extraction buffer [40 mM K_2HPO_4 at pH 7.4; 1 mM ethylenediaminetetra acetic acid (EDTA) and 5% (w/v) polyvinylpolypyrrolidone (PVPP) weight = 40 000]. The resulting homogenate was thoroughly mixed by a vortex. After the mixture had been adequately mixed, the plant material was pelleted in a centrifuge at 13 000 x *g* for 15 minutes at 4 °C, and the supernatant was transferred into a sterile tube. Protein concentrations were determined

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according to the Bradford (1976) method, using bovine serum albumin (BSA) as a standard. Thereafter, the protein samples were stored at -20 °C.

2.9 Measurement of the antioxidant enzyme activity

2.9.1 *Superoxide dismutase activity*

Superoxide dismutase (SOD) enzymatic activity was measured using native PAGE gel and spectrophotometric assay on the leaf and root material. For the detection of SOD isoforms, a native PAGE was performed at 4°C in 12 % polyacrylamide separating gels and 5 % stacking gel with a running buffer containing 192 mM glycine and 24 mM Tris base (pH 7). Total protein extract of 100 µg was loaded from extracts of each treatment and the native PAGE was run at 4°C for 4 hours at 80 V. To obtain the SOD isoform patterns, the gels were incubated in 50 mM KPO_4 (pH 7.8) containing 6 mM H_2O_2 (to inhibit both Cu/ZnSOD and FeSOD), and 5 mM KCN (to inhibit only Cu/ZnSOD) (Archibald and Fridovich 1982), as MnSOD is resistant to both treatments. No SOD inhibitors were added to the third gel, and thus it was incubated in 50 mM KPO_4 only. The gels were further incubated in 50 mM potassium phosphate (pH 7.8) buffer containing 2.5 mM NBT for 15 minutes in the dark. Finally, the gels were stained on a light box with 50 mM potassium phosphate (pH 7.8) buffer containing 28 mM riboflavin and 28 mM TEMED until SOD isoforms became visible.

For the spectrophotometric SOD assay, a method modified from Beauchamp and Fridovich (1971) was used. For this method, a reaction mixture containing 50 µg protein extract, 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM L-methionine, 2 µM riboflavin and 75 µM NBT was prepared. The mixture was incubated at room temperature for 20 minutes on a fluorescent light box and absorbance readings at 560 nm were recorded. The SOD activity was then calculated based on the amount of the SOD enzyme that was required to cause 50% decrease in the reduction of the NBT to blue formazan, and the specific activity of plant extract was expressed as units per mg of protein.

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2.9.2 *Ascorbate peroxidase activity*

The activity of APX isoforms was determined with native polyacrylamide gel electrophoresis (PAGE) as described by Rao *et al.* (1996). A 12% polyacrylamide separating gel and 5% stacking gel was loaded with 50 µg of the quantified protein per sample. The non-denaturing PAGE was run at 4°C in a buffer containing 2 mM ascorbate, 192 mM glycine and 24 mM Tris base (pH 7). To obtain the APX isoform patterns, the gel was equilibrated with 50 mM phosphate buffer (pH 7.0) containing 2 mM ascorbate for a total of 20 minutes. This was followed by incubating the gel in 50 mM phosphate buffer (pH 7.8) containing 2 mM H_2O_2 and 4 mM ascorbate for 20 minutes. The gel was subsequently washed with phosphate buffer (pH 7.8) containing 28 mM TEMED and 2.5 mM NBT, with gentle agitation for approximately 10 minutes in the presence of light, after which the reaction was stopped by a brief wash with distilled water.

For the spectrophotometric determination of the ascorbate peroxidase (APX) activity, the quantified protein samples were aliquoted in 1.5 ml tubes and incubated with 2 mM ascorbate for 5 minutes. The reaction was performed by mixing the protein sample with 50 mM K_2HPO_4 (pH 7.0), 0.1 mM EDTA, 0.36 mM ascorbate and 0.72 mM H_2O_2 was added last to start the reaction. The reaction mixture was made up to a total volume of 200 µl with deionised water. The APX activity was then calculated by following the change in absorbance at 290 nm as described by Nakano and Asada (1981) using the extinction coefficient of 2.8 mM^{-1} cm^{-1}.

2.9.3 *Catalase activity*

Catalase (CAT) enzymatic activity was measured using a native PAGE gel and spectrophotometric assay from the leaf and root protein extracts. For the detection of CAT isoforms, a native PAGE was performed at 4°C in 7.5% separating gels and 5% stacking gel, loading 60 µg of the quantified protein per sample. The native PAGE was performed at 4°C for 10 hours at 60 V. After electrophoresis, the gels were rinsed extensively with deionised H_2O_2 followed by incubation in 0.003% H_2O_2 for 20 minutes in the dark. The gels were stained on the light box with 2% ferric chloride and 2% potassium ferricyanide until the formation of catalase bands.

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The bands representing catalase activity were detected as clear bands on a blue background.

For the spectrophotometric determination of the catalase activity, a modified method of Aebi, (1984) was used. The principle of the assay hinged on the decrease in absorbance due to the dissociation of H_2O_2. A volume of 1 ml reaction mixture containing 100 mM K_2HPO_4 (pH 7.0), 0.5 mM EDTA, 1 mM H_2O_2 and 20 µg protein extract was prepared. The absorbance of the reaction mixture was read at 240 nm and an extinction coefficient of 39.4 mM^{-1} cm^{-1} was used to calculate the catalase activity.

2.10 Statistical analysis

One-way analysis of variance (ANOVA) test was used to validate the statistical analysis. The means (from three independent experiments) were compared according to the Turkey-Kramer test at 5% level of significance, using GraphPad Prism 5.03 software.

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CHAPTER 3: RESULTS

3.1 Physiological and morphological responses to inhibition of Nitric Oxide Synthase-like activity in maize

3.1.1 Exogenous application of L-NAME causes a reduction in growth in maize

The effect of NOS-like activity in ABA-mediated production of H_2O_2 and inhibition by L-NAME on maize growth was evaluated by assessing the physiological parameters including shoot length (figure 3.1 A), shoot fresh weights (figure 3.1 B) and root lengths (figure 3.1 C). The shoot fresh weights, shoot length and root length were measured at V2 stage of growth. Abscisic acid reduced the length of the shoots by approximately 20% when compared to untreated plants (figure 3.1 A). However, the combination of 100 µM ABA and 1mM L-NAME treatment showed an improvement in plant growth as compared to ABA treatment. There was a slight decline in growth in the L-NAME treatment when compared to untreated shoots. The addition of L-NAME in the ABA treatment caused a reversal effect, showing a 20% increase. The H_2O_2 treatment showed approximately 20% increase in shoot length. However, a 50% decline was observed when L-NAME was added in the H_2O_2 treatment when compared to the H_2O_2 treatment alone. The addition of L-NAME to the H_2O_2 treatment showed no significant difference when compared to L-NAME treatment.

The shoot weight (figure 3.1 B) showed a rather different trend as observed from shoot length. The application of ABA led to a 35% decline in shoot weight when compared to the control plants. However, the addition of L-NAME caused a slight reversal of the effect of ABA on shoot weight (figure 3.1 B), with a 20% decline in shoot weight when compared to the untreated maize. Furthermore, the application of L-NAME alone caused a 30% decline in shoot weight when compared to the respective control plants. Exogenously applying H_2O_2 caused 18% decline in shoot weight when compared to the untreated plants, a trend that remains similar when L-NAME is added in the treatment to form a combined H_2O_2 + L-NAME.

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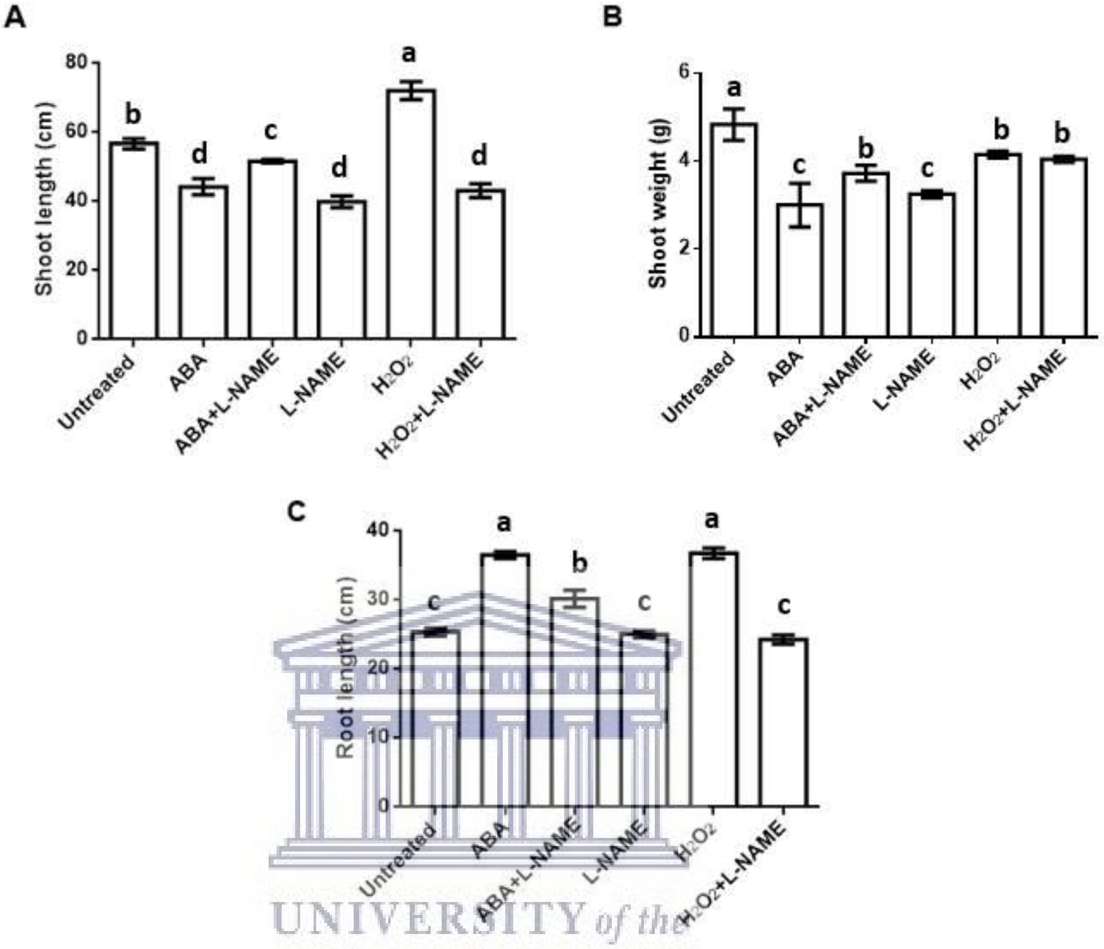

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Figure 3.1.1: Measurement of maize plant growth and biomass. The graphs of shoot length (A), shoot weight (B) and roots length (C). Harvesting occurred at vegetative stage 2 of growth after treatment with either nutrient solution only (Untreated), 100 µM ABA, 100 µM ABA + 1 mM L-NAME, 1 mM L-NAME, 100 µM H_2O_2 and 100 µM H_2O_2 + 1 mM L-NAME. Data represent the mean (±SE) of three independent experiments per treatment. The same letters above the error bars indicate that there was no significant difference between means ($P<0.05$).

Furthermore, the length of the roots subjected to various treatments was measured as shown in figure 3.1.1 and figure 3.1.2, and an interesting trend was observed too. ABA and ABA + L-NAME treatments followed a different trend observed in shoot weight. The plants that exogenously received abscisic acid were 20% longer in root length than the untreated plants (figure 3.1 C). Furthermore, ABA had developed proliferation of primary and nodal roots as shown in figure 3.1.2. However, the addition of L-NAME to ABA seemed to have a setback effect, with a slight decline in root length observed. L-NAME on its own appeared to not have an effect on root length as compared to its respective control. Also observed, was the thinning of both primary and nodal roots as seen in figure 3.1.2. The exogenous application of H_2O_2, just like ABA, increased the root length by 20% as compared to the untreated plants. The addition of L-NAME to the respective H_2O_2 treatment shortened the root length by approximately 20% as compared to the H_2O_2 treated roots.

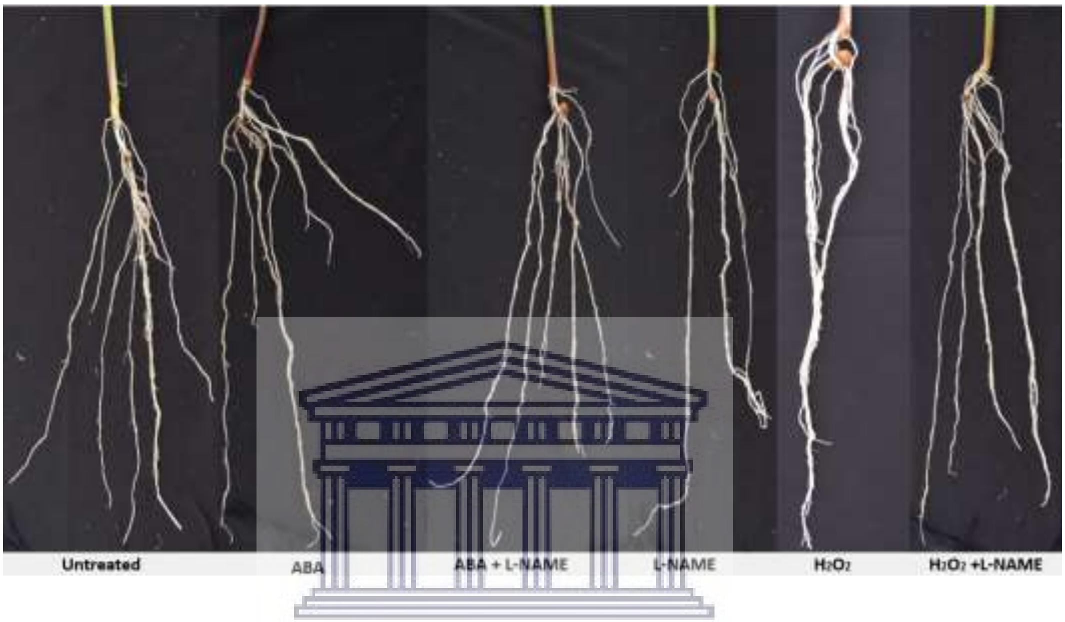

Figure 3.1.2: Proliferation of primary and nodal roots of maize. The plants were grown in solution containing 100 µM ABA, 100 µM ABA + 1 mM L-NAME, 1 mM L-NAME, 100 µM H_2O_2, 100 µM H_2O_2 + 1 mM L-NAME and the control.

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3.2 Inhibition of NOS-like enzymatic activity by L-NAME influences ROS accumulation and oxidative damage

In this part of study, the role of NOS-like activity in ABA-mediated scavenging of superoxide anion (O_2^-) to the production of H_2O_2 was investigated. The maize plants were grown and treated as described in chapter 2. Exogenously applied abscisic acid significantly reduced O_2^- content by approximately 50% relative to the untreated control (Figure 4.1 A), a trend that is not changed by the addition of L-NAME in the combined treatment. However, the application of L-NAME shows a 25% incline in O_2^- content in leaves as compared to the untreated plants. Furthermore, the application of L-NAME doubled the O_2^- content maize leaves as compared to the NOS inhibitor treatment when combined with ABA. In contrast,

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application of hydrogen peroxide showed a slight decline in superoxide content in both leaves and roots, with the addition of L-NAME reversing the effects observed. There was no significant difference in the O_2^- content observed in the H_2O_2 treatment as compared to the untreated leaves and roots.

Hydrogen peroxide content in leaves from the abscisic acid-treated plants was approximately 30% lower than the leaves of untreated plants, while the addition of L-NAME to ABA treatment shows a slight increase in H_2O_2, a reversal of what was observed in ABA treated plants as shown in figure 3.2 C and D. Application of L-NAME caused no significant difference compared to the untreated plants. High accumulation of H_2O_2 was observed when 100 µM H_2O_2 was added on the plant. Supplementing L-NAME in the H_2O_2 treatment resulted in an increase of approximately 35% compared to the H_2O_2 treatment in leaves. As observed thus far, hydrogen peroxide produced contrasting results in hydrogen peroxide levels as compared to superoxide content (3.2 A and B). The same trend of H_2O_2 content in leaves was observed in the roots, however at a lower concentration. Application of ABA in the roots caused a slight decrease in H_2O_2 content. However, the addition of L-NAME led to a reversal to the level of H_2O_2 in roots.

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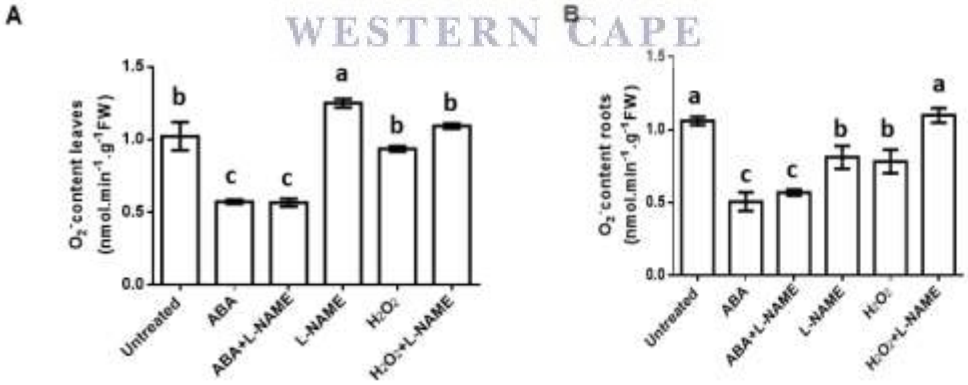

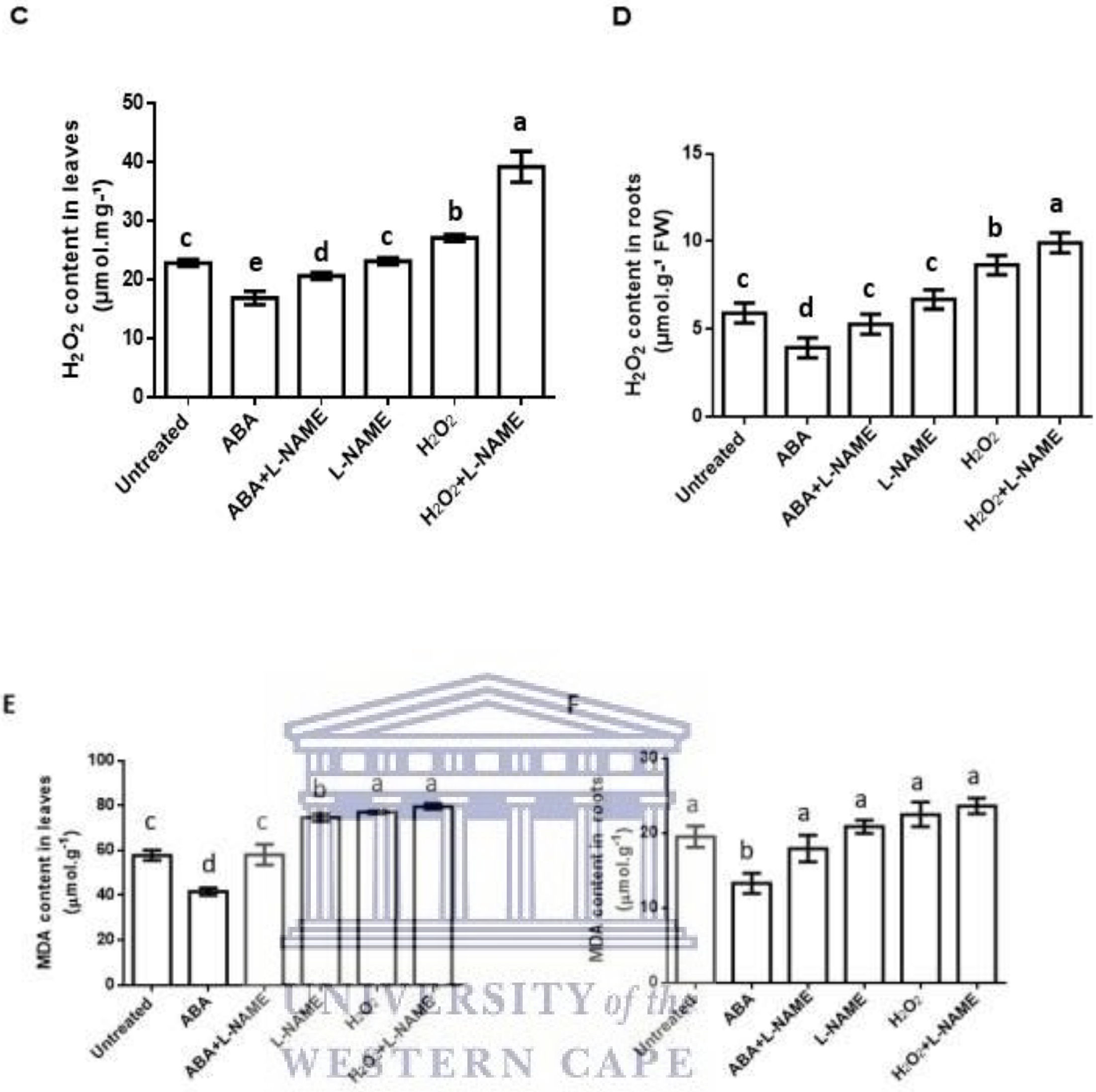

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Figure 3.2: The influence of L-NAME on ROS accumulation and oxidative damage. O_2^- content in leaves (A) and roots (B), H_2O_2 content in leaves (C) and roots (D), together with malondialdehyde (MDA) content in leaves (E) and roots (F) of *Zeamays*. Assays were measured at vegetative stage 2 of growth after treatment with either nutrient solution (HEPES) only (Untreated), 100 µM ABA, 100 µM ABA + 1 mM L-NAME, 1 mM L-NAME, 100 µM H_2O_2 and 100 µM H_2O_2 + 1 mM L-NAME. Data shown are the mean (±SE) of three independent experiments. Bars sharing a common letter are not significantly different at $p < 0.05$.

Malondialdehyde (MDA) content was measured as an estimate of the oxidative damage due to increases in O_2^- and H_2O_2 content. Exogenous application of 100 µM ABA treatment decreased the effect of lipid peroxidation in both leaves and roots as compared to the untreated (control) (Figure 3.2 E). The decrease in MDA

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content as a result of treatment with ABA was reversed in response to the addition of L-NAME to ABA and was in fact aligned with the untreated. Furthermore, the highest increase in the level of lipid peroxidation was observed for leaves in the L-NAME treatment (approximately 75%). Treatment with H_2O_2 enhanced lipid damage by approximately 20% in leaves when compared to the untreated plants. Supplementation of L-NAME had no significant effect in terms of lipid peroxidation as there was no change in the level of MDA content between the H_2O_2 and the combination of H_2O_2 and L-NAME in leaves. In the roots, the malondialdehyde content was lower than observed in leaves. Abscisic acid caused a decline in MDA content by approximately 30% as compared to the untreated. Supplementing L-NAME in both ABA and H_2O_2 showed no significant difference.

3.3 The effects of ABA and inhibition of NOS activity on proline content in maize

The effects of abscisic acid and H_2O_2, in the presence or absence of the NOS inhibitor (L-NAME) in maize leaves and roots are shown in figure 3.3. While the concentration of proline was 0.2 $\mu g.mg^{-1}$ FW under the condition of no stress, it increased by 25% when subjected to ABA treatment in leaves. Furthermore, the addition of the NOS inhibitor in ABA showed a slight increase as compared to the untreated leaves. However, endogenous proline concentration of samples decreased to approximately 50% as compared to the untreated when a 100 µM H_2O_2 was applied. Treatment with L-NAME in combination with H_2O_2 resulted in a decrease in proline content in maize leaves when compared to the untreated (Figure 3.3a).

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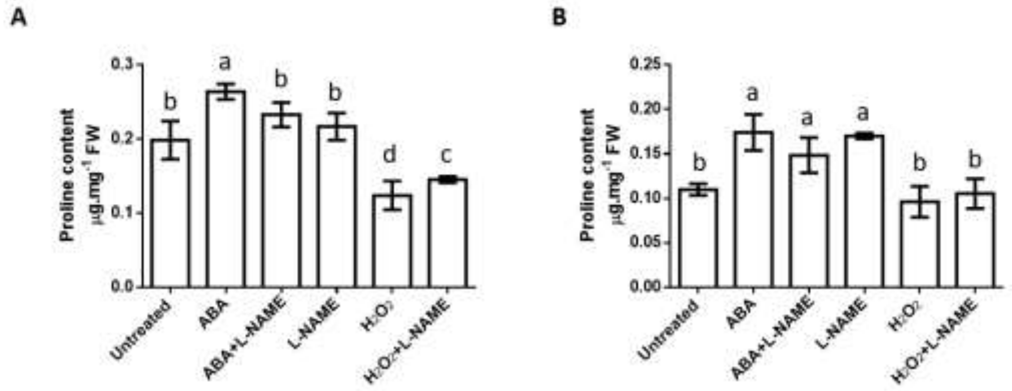

Figure 3.3: The effects of ABA and inhibition of NOS on Proline content in maize leaves (A) and roots (B). Data shown are the mean (±SE) of three independent experiments. Bars sharing a common letter are not significantly different at $p < 0.05$.

The proline content in the roots as shown in figure 3.3 showed a slight increase when subjected to ABA when compared to the untreated. There was no significant difference when L-NAME was added in the ABA treatment. However the proline content increased when L-NAME was applied in combination with ABA when compared to the untreated roots. In addition, proline content decreased when the roots were subjected to H_2O_2 treatment. Furthermore, a combination treatment with H_2O_2 and L-NAME effect when compared to the untreated roots.

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3.4 The effects of NOS-like inhibition on ABA- and H_2O_2-mediated antioxidant enzyme activity

3.4.1 The effects in the interaction of ABA and L-NAME on the activity of superoxide dismutase (SOD)

Analysis of the response of SOD enzymatic activity to treatment with L-NAME on abscisic acid and H_2O_2 signalling changes in maize leaves was carried out using native PAGE enzymatic and spectrophotometric assay for total enzymatic activity. Individual SOD isoforms were identified and characterized by incubating the native polyacrylamide gels with 6 mM H_2O_2 (to inhibit both Cu/ZnSOD and FeSOD)

(Figure 3.4.1 C), or with 5 mM KCN (to inhibit only Cu/ZnSOD) (Figure 3.4.1 B) (Fridovich, 1982). To better analyse the activity of the individual SOD isoforms in response to various treatments, the pixel intensity of the SOD bands was determined using the Alpha Ease FC Software. There were 4 SOD isozymes detected in the leaves of maize. Analysis of these results, based on resistance and sensitivity to KCN and H_2O_2, suggests the existence of three manganese SOD isoforms (MnSOD1, MnSOD2 and MnSOD3) and one copper/zinc SOD (Cu/ZnSOD) in leaves (Figure 3.4.1 A). Enzymatic activity detected for MnSOD1 and MnSOD3 increased in response to L-NAME treatment, with the highest increase in MnSODs enzymatic activity observed in H_2O_2 treatment. On the contrary, MnSOD1 and MnSOD3 activity was inhibited by L-NAME in the H_2O_2 + L-NAME combined treatment when compared to H_2O_2. Exogenously applying ABA showed a slight increase in the isoform intensity of MnSOD3 when compared to the untreated leaves. Based on the relative pixel intensity results, the CuSOD isoenzyme activities showed no significant difference to ABA when compared to the untreated leaves. Meanwhile, exogenously applying L-NAME and H_2O_2 decreased the CuSOD isozyme.

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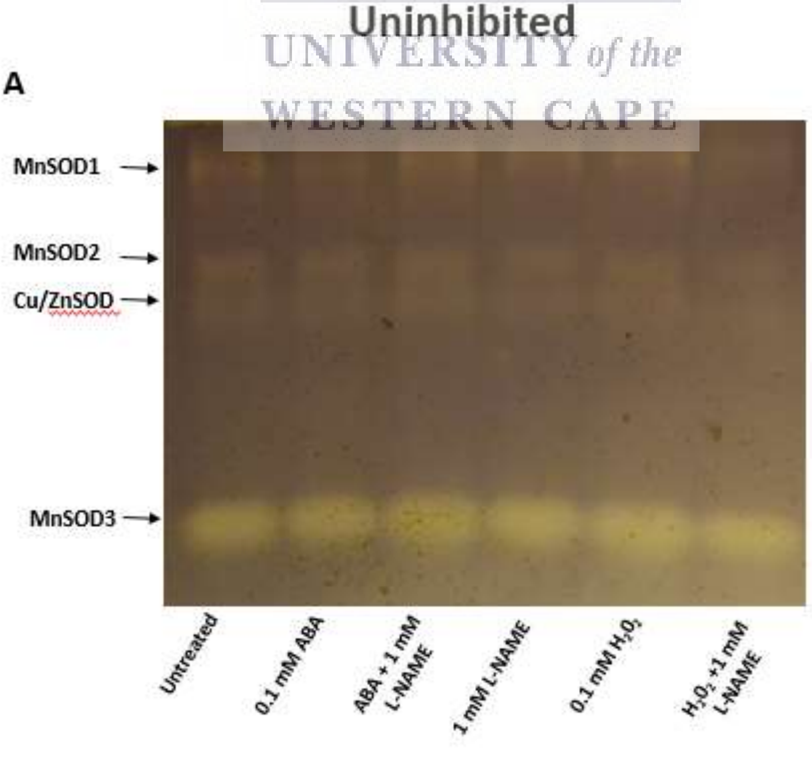

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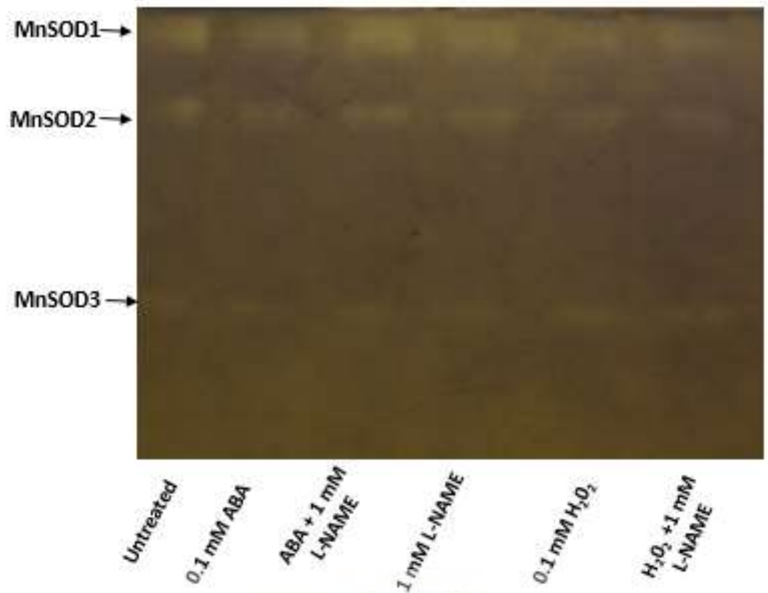
6 mM H_2O_2

B



5 mM KCN

C

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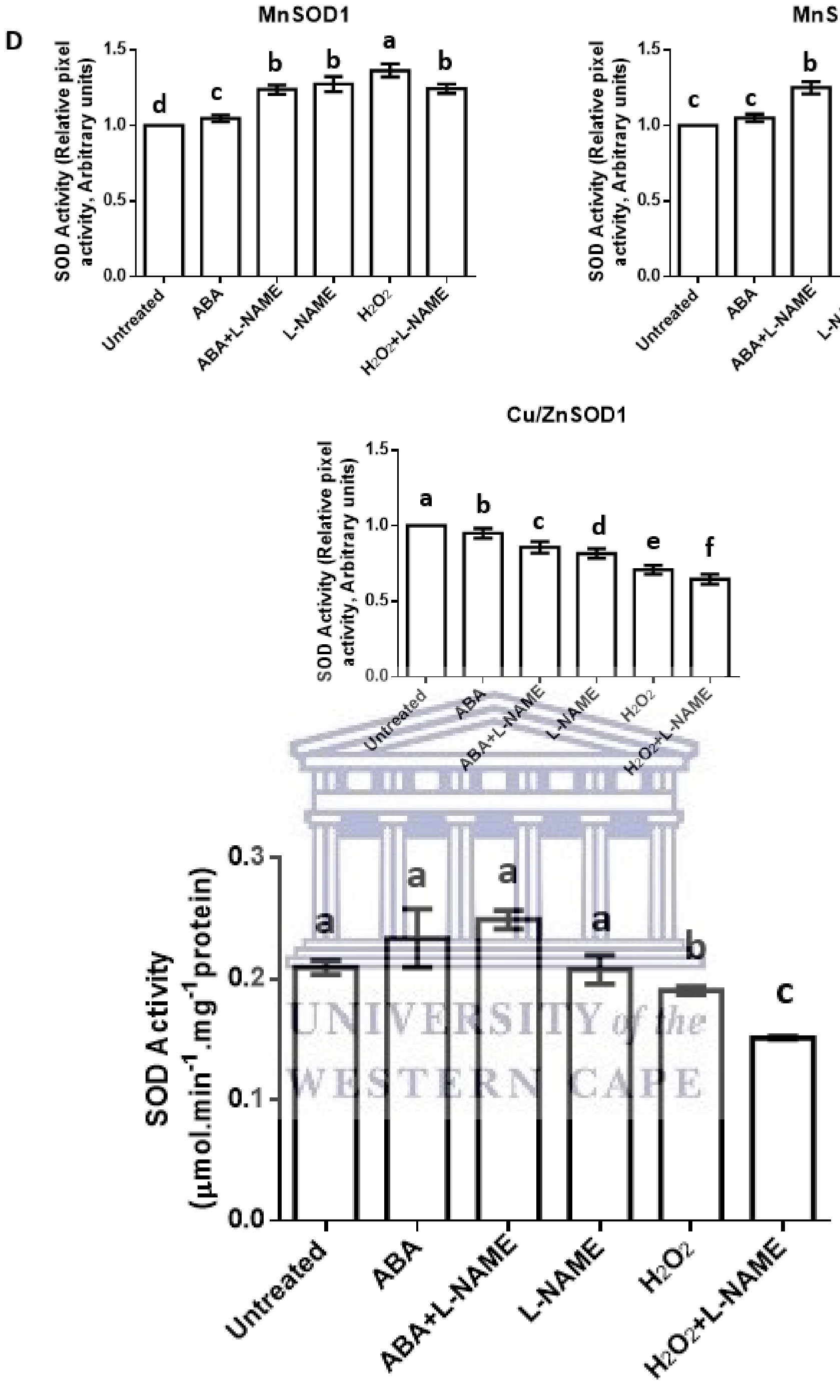

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Figure 3.4.1: Effect of inhibition of NOS on superoxide dismutase activity in leaves of *Zea mays*. The in-gel assays show the types of SOD isoforms present in leaves of maize (A) with no inhibitors, (B) in the presence of 6 mM H_2O_2 and (C) with 5 mM KCN total SOD activity was determined in leaves, from which (D) pixel intensities were determined in the uninhibited SOD. The three isoforms are referred to as MnSOD, FeSOD, and Cu/ZnSOD on the basis of their migration on the native PAGE gel. The total SOD activity in leaves (E) is represented. Data shown are the mean (±SE) of

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three independent experiments. Bars sharing a common letter are not significantly different at $p < 0.05$.

The measurement of the SOD enzymatic activity as shown by the spectrophotometric assay showed no significant differences in the levels of the total SOD activity in the L-NAME treatment as compared to the control (Figure 3.4.1 E). There were no changes observed in the ABA treatment. The addition of L-NAME onto ABA also did not show any significant change in SOD activity. However, when maize was supplemented with H_2O_2 there was an 8% decline observed with a further decline observed when L-NAME added into H_2O_2.

3.4.2 L-NAME alters the activity of ascorbate peroxidase (APX) isozymes in Maize leaves.

As SOD is responsible for scavenging O_2^-, yielding high levels of H_2O_2, ascorbate peroxidase (APX) is activated to detoxify H_2O_2 through the Halliwell-Asada pathway using ascorbate as a donor (Halliwell, 2006). Here the activity contributed by individual APX isoforms was evaluated (Figure 3.4.2 A and B). On the native PAGE activity gels, seven APX isozymes were detected in the maize leaves and seven in roots. Various treatments increased, while others decreased the activity of APX isozymes. APX 1 showed a slight increase in intensity in the ABA treated leaves when compared to the control in leaves, with densitometry analysis depicting no significance difference. The addition of L-NAME onto the ABA continued to increase the APX 1 activity in the leaves. There was no change in the intensity of APX 1 on the leaves when treated with L-NAME alone. Furthermore, no significant change in APX intensity was observed in H_2O_2 treatment and the combination treatment of H_2O_2 and L-NAME as compared to the control. Correspondingly, APX 7 isoform showed an enhanced intensity when L-NAME was supplemented in ABA. The pixel intensity verifies the result shown by the individual isoforms.

In the maize roots, 100 µM ABA enhanced the band intensity of APX 5 isozyme. The addition of the NOS inhibitor (L-NAME) slightly reduced the APX 5 band intensity. L-NAME alone caused a significant reduction in APX 5 band intensity as

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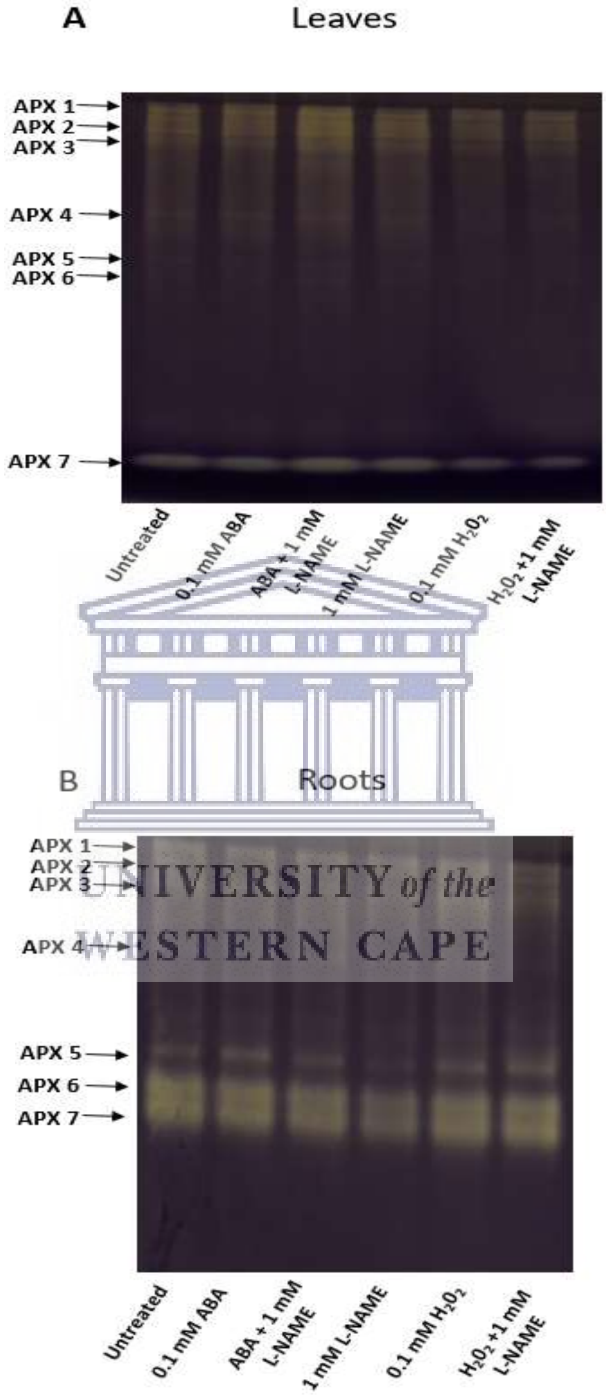
compared to the untreated. Furthermore, a reduction in APX 6 and 7 was observed when L-NAME alone was supplemented.



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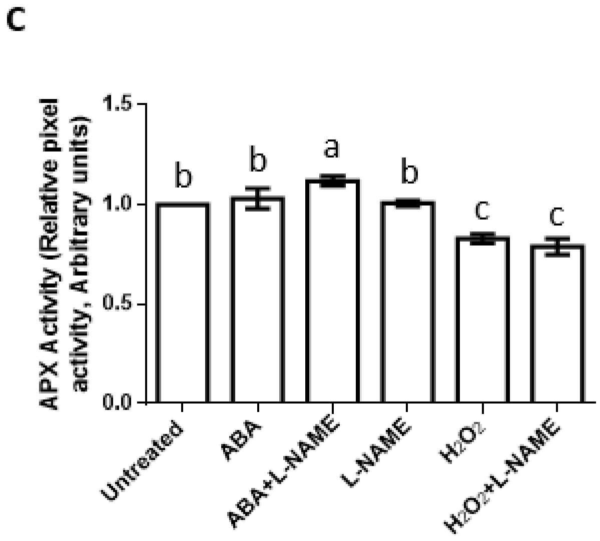

Figure 3.4.2: Changes of individual ascorbate peroxidase (APX) isoforms in response to nitric oxide synthase inhibitor (L-NAME). The pixel intensity of APX 7 in the maize leaves was determined. Data for ascorbate peroxidase content are mean ± standard error of three different plants, representing three independent experiments.

For the total APX activity, both control and ABA treatments showed no significance difference in the in the level of total APX activity (Figure 3.4.2). When L-NAME was combined with ABA, there was a slight increase (10%) observed in the total APX activity. The L-NAME treatment showed no significant change in the APX activity. Furthermore, the activity of APX declined by 12% when maize was subjected to H_2O_2. Supplementing L-NAME onto H_2O_2 also showed a slight decline as compared to the control.

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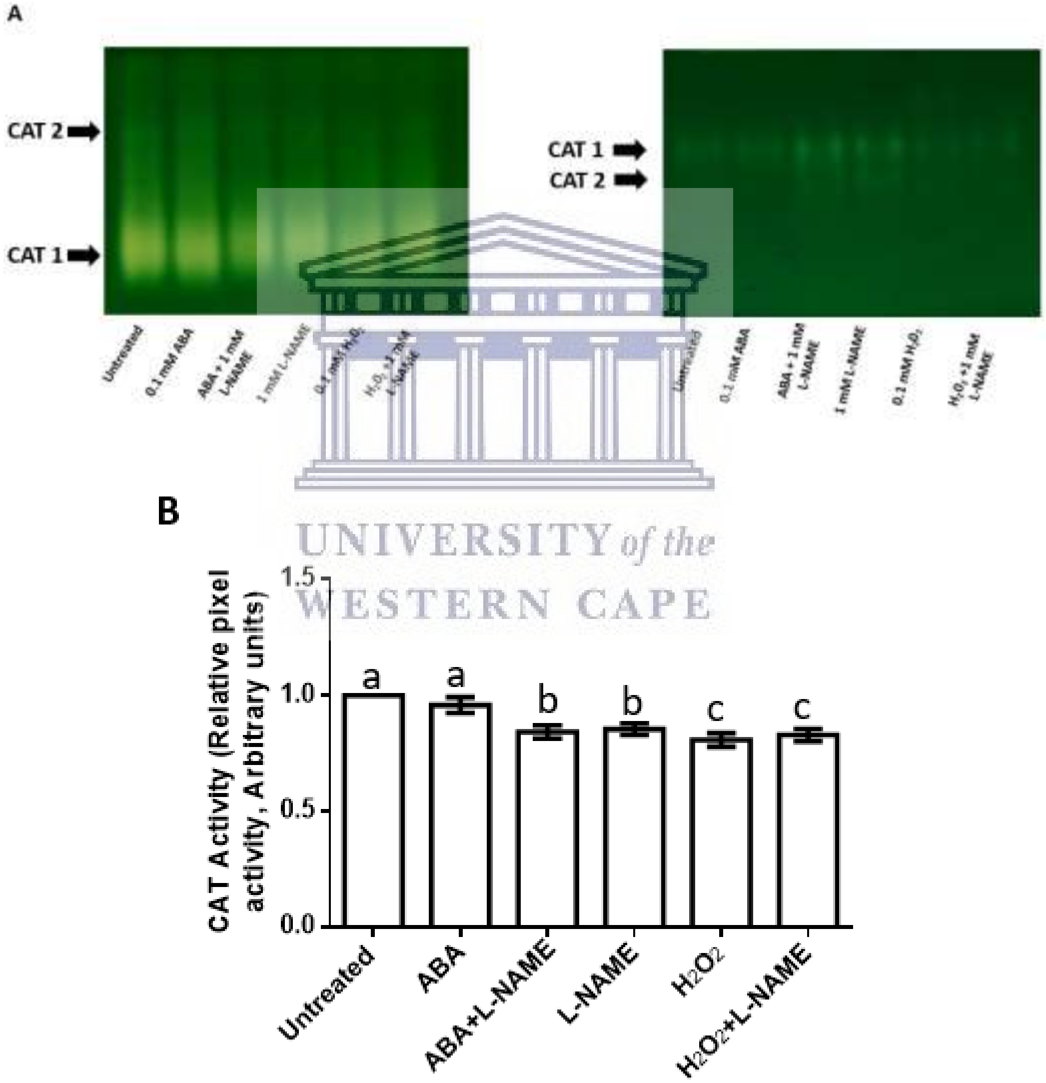
3.4.3 Catalase activity in maize roots is differentially regulated by NOS activity

Catalase enzyme decomposes H_2O_2 at an extremely rapid rate compared to APX, depending on the concentration of H_2O_2, to produce water and molecular O_2 (Vellosillo *et al*., 2010). Changes in catalase isozyme activities were determined and two catalase isozyme were detected in both maize leaves and roots. The catalase enzymatic activity observed in the isoform was reduced by the inhibitor of nitric oxide synthase. L-NAME decreased catalase activity by 20% when compared to untreated plants as depicted from the densitometry analysis (figure 3.4.3b).

Nonetheless, exogenously applying ABA on the maize plant caused no significant change in catalase enzymatic activity compared to the untreated plants. While L-NAME alone served to reduce the catalase isozyme activity, the same trend was observed when L-NAME was combined with ABA. The ABA + L-NAME treatment led to an inhibition in CAT1 activity when compared to the untreated leaves. Furthermore, H_2O_2 reduced the catalase activity in the leaves of maize, whereas the H_2O_2 + L-NAME combined treatment showed no reduction or induction effect on the catalase isozyme activity.



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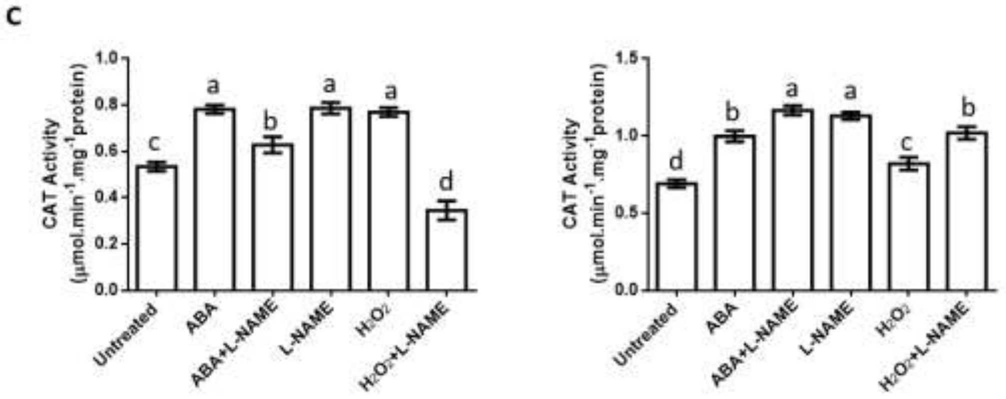

Figure 3.4.3: Changes of individual catalase (CAT) isoforms differentially regulated by NOS activity in A) leaves (left) and roots (right). The pixel intensities B) was determined in catalase 1 isoform in leaves. The total CAT activity C) in leaves (left) and roots (right) is represented. Data for catalase content are mean ± standard error of three different plants, representing three independent experiments.

The total catalase activity was analyzed in both leaves and roots of maize. The enzymatic activity of catalase was increased by 40 % in response to abscisic acid treatment in the leaves. L-NAME also increased catalase activity when combined with ABA by 10% when compared to control leaves. The treatment of H_2O_2 led to an increase in catalase activity of 40 % as compared to the control in the maize leaves. However, supplementing L-NAME in H_2O_2 treatment decreased the activity of catalase by 35 % when compared to control leaves.

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In the maize roots, the catalase total activity increased 35% as compared to the untreated. Supplementing L-NAME with ABA further increased the catalase activity by 85%. A slight effect of H_2O_2 is shown with a 20% increase in catalase activity in the maize roots, with the addition of L-NAME onto H_2O_2 further increasing the catalase total activity

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CHAPTER 4 – DISCUSSION

4.1 Morphological effects of the interaction of ABA and H_2O_2 in NO-mediated changes in maize

In this study, the results showed that after exogenous ABA was applied; the roots growth increased. This study is consistent with that of Tian and Li (2018), who showed that exogenously applying ABA effectively enhanced the root volume, root length and root surface area. According to Comas *et al.* (2013), water stress, characterized by enhanced ABA, stimulates the growth of roots into deeper soil. The roots can continue to grow deeper even though vegetative growth appears to stop, as shown in figure 3.1 (A and C).The inhibitory effect of L-NAME on plant growth and development has been well documented in this study. However, the combined treatment of ABA and L-NAME did not improve root length in comparison to the ABA treatment. ABA controls root growth responses through regulation of gene expression that favours maintenance of root growth, which optimizes water uptake (Harrison, 2012). This explains the length and volume of roots in the ABA treatment.

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Nonetheless, exogenous application of H_2O_2 led to a significant increase in shoot length. These findings are in agreement with the study of Wahid *et al*. (2008) showing that treating maize with H_2O_2 in early vegetative stage increases the shoot weight. Contradictory results were observed from a study by Roy *et al*. (2016) when rice (*Oryza sativa L.*), exposed to NaCl stress, exhibited a significant reduction in growth following H_2O_2 application.

The decline in shoot length in response to ABA might be due to a decrease in cell elongation or cell division, which impacts cell growth (Banon *et al*., 2006). Increased ABA concentrations induce stomatal closure via intracellular signalling in the guard cells, further accounting for the reduced growth of maize. Consequently, the concentration used for ABA could have accounted for the reduction in growth. Furthermore, the plants that were treated with 100 µM H_2O_2 grew better and had long roots. These findings are in agreement with the results of

Roy *et al*. (2016) where H_2O_2 application significantly increased root length. The study further showed how H_2O_2 significantly increased root and shoot growth in rice. H_2O_2 could be operating as a plant growth promoting signalling molecule as it was shown in a study by Quan *et al.* (2008) by farmers in small-scale and large-scale farming, in which H_2O_2 was reported to have regulatory effects on growth, development and quality of fruit.

4.2 Accumulation of ROS and its response in oxidative degradation of lipids

In this part, changes in the production of reactive oxygen species and cell damage were examined in response to NOS-like activity in ABA-mediated production of H_2O_2. The functioning of abscisic acid, along with the coordination of other downstream signal transduction mechanisms involving hydrogen peroxide and nitric oxide synthase play a major role in regulation of oxidative stress. Changes in environmental conditions lead to oxidative stress characterized by high accumulation of reactive oxygen species O_2^- and H_2O_2 (Hasanuzzaman *et al.*, 2012). Thus, the observed reduction on plant growth in Figure 3.1 A and B in ABA treatment could also be attributed to oxidative stress. Furthermore, it is shown in a study by Ullah *et al*. (2016) that inhibition of NOS activity affects O_2^- and H_2O_2 content in maize.

As shown in figure 3.2 in both leaves and roots, the application of ABA reduced the content of O_2^-, which is not changed by the NOS inhibitor. The suppression of NO production may have reduced the activity of enzymes that function in the activity of O_2^-. This will have to be investigated further to confirm if it is the case. One speculation of the mechanism with which ABA leads to the generation of O_2^- could be that the ABA-induced stomatal closure can lead to the reduction in the accessibility of CO_2 for photosynthesis, which may lead to the generation of O_2^- and more oxidative damage from the misdirecting of electrons in the photosystems (Jiang and Zhang, 2001).

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Moreover, the concentration of 100 µM of H_2O_2 was exogenously applied during the development stage. A slight increase was observed in H_2O_2 content, which corresponded to an increase in growth. Based on a study by Slesak *et al*. (2007), plants show resistance to fairly high concentrations of H_2O_2. It is proposed that the reason for the tolerance of plants to high levels of H_2O_2 is due to the fact that plants antioxidant response systems are intended more to control the cellular redox state than to completely eliminate the H_2O_2 (Ślesak *et al*., 2007).

The increase in H_2O_2 content observed in figure 3.2 B when the NOS inhibitor was added to ABA and H_2O_2 treatments resulted in membrane disruption as indicated by high levels of lipid peroxidation (figure 3.2 C). These findings are in agreement with the study by Ullah *et al*. (2016) where L-NAME was shown to increase endogenous H_2O_2 and MDA content. Both H_2O_2 and O_2^- are responsible for oxidation of polyunsaturated fatty acids, thus producing secondary products such as malondialdehyde (MDA), an indicator of lipid peroxidation (Repetto, 2012). Hydrogen peroxide mediated changes trigger the increases in the levels of lipid peroxidation, which is manifested as increased levels of MDA (Cho and Sohn, 2004). As shown in (Figure 3.2 E and F), the levels of MDA increased in the H_2O_2 mediated treatment. Moreover, the application of ABA led to a slight reduction in lipid peroxidation. Lower levels of lipid peroxidation is associated with higher ascorbate peroxidase activity in drought- or salt-tolerant tomato (Shalata and Tal, 1998) and rice (Demiral and Turkan, 2005). Compared with the control treatment, the decrease in MDA level as a result of application of 100 µM ABA might be an indication that exogenous ABA promoted metabolites that take part during normal growth conditions which led to a relief in membrane damage.

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4.3 Osmoprotectant proline accumulation in maize

Proline serves as one of the most important compatible solutes in plants, synthesized by L-glutamic acid (Gill and Tuteja, 2010). Proline is thought to play an adaptive role in inducing osmotic adjustment in stressed plants and protecting subcellular structures (Ashraf & Foolad, 2007). In response to water stress, characterized by enhanced ABA phytohormone, proline accumulation generally occurs in the cytosol where it plays significant role in cytoplasmic osmotic adjustment (Anjum *et al*., 2011). In this study, proline content of both leaves and roots increased in response to ABA. The increase in proline concentration in ABA-treated plants shows that ABA-induced changes in proline are influenced by ABA. The fact that L-NAME did not affect proline content in both the absence and presence is evidence that ABA-induced proline accumulation is independent of NO. The reduction on proline content upon H_2O_2 treatment shows that H_2O_2 has a negative effect on protective processes (such as osmoregulation) in plants and thus can be detrimental to drought responses.

4.4 Nitric oxide synthase increases the activity of superoxide dismutase enzyme activity

The link between the increase in the levels of ROS and lipid peroxidation, which leads to a reduction in plant growth, has been established.

In this study's native PAGE assay, four SOD isoforms were identified in maize leaves, and only MnSOD and Cu/ZnSOD activities were detectable, while FeSOD activity could not be detected. A similar outcome was observed in creeping bentgrass exposed to waterlogging stress (Wang and Jiang, 2007) and in the study by Fink and Scandalios, (2002) when investigating the effect of salt stress on enzymatic activity of superoxide dismutase (SOD) isozymes in shoots and roots ues of salt tolerant and salt sensitive wheat. In a study by Cannon *et al*., (1987) numerous SOD isoenzymes were reported in maize. As shown in figure 3.3.1A, MnSOD had the lowest electrophoretic mobility and was more abundant, while only one Cu/ZnSOD isoform was detected. The findings here showing induction of SOD under ROS-inducing conditions are in agreement with those obtained by Vuleta *et al.* (2006) in the adaptive responses of enzymatic antioxidants SOD, APX and CAT to high light stress. It has been shown that enzyme activity of Cu/ZnSOD is linked to changes in both xylem and phloem water and ion status in their functioning in transporting water and minerals (Walz *et al*., 2002). Karpinska *et al.* (2001) also proposed that Cu/Zn-SOD isoforms in vascular tissues might function in regulating H_2O_2 pulses, which are involved in the transmission of systemic

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signals in wounding or pathogen responses during plant growth. It was further demonstrated that diminished extracellular Cu/ZnSOD expression in poplar trees (like demonstrated in figure 3.4.1) regulates extracellular H_2O_2 level and plant development (Srivastava *et al*., 2006).

As shown in figure 3.4.1, there was high accumulation of H_2O_2 observed in the NOS inhibitor treatment. As a result, superoxide dismutase was activated. This observation was encountered in a study by Hao *et al.* (2008), where H_2O_2 was reported to be produced by SOD-mediated catalysis.

4.5 Influence of ROS accumulation on ascorbate peroxidase and catalase activity

It is well established that the scavenging capacity of H_2O_2 through the Halliwell-Asada pathway is mediated by the ascorbate peroxidase (APX) and ascorbate. The fact that exogenous application of abscisic acid to *Zea mays* plants led to reduced levels of H_2O_2 and improved the plant growth in NOS-inhibited *Zea mays* suggests that NO mobilized the enzymatic antioxidant defence system. Meanwhile, the combination of ABA and the NOS inhibitor up-regulated the expression of both SOD and APX isozymes in maize leaves. The activity of individual APX isoforms decreased in H_2O_2 treated maize leaves. The decrease in APX activity accounts for the observed accumulation of H_2O_2.

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In this study, there were two CAT isoforms detected in both leaves and roots. A study by Guan and Scandalios showed that native PAGE can separate three CAT isozymes (Guan and Scandalios, 2002). The CAT enzymatic activity showed a decrease in CAT isoform intensity of the treatments as compared to the untreated maize leaves. The decrease in CAT activity might be due to the generation of high level of oxidative stress that possibly inactivate/inhibit CAT. This would allow for excessive accumulation of H_2O_2.

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CHAPTER 5 - CONCLUSION AND FUTURE PROSPECTS

Drought tolerance is another area in plant biotechnology and molecular biology that needs more information to understand the morphological changes and the enzymatic basis for reactive oxygen species generation and oxidative stress. Drought tolerance has been reported to be directly correlated with increases in antioxidant enzymes and inversely correlated with levels of lipid peroxidation and H_2O_2 accumulation (Avramova, 2015). A major problem in agriculture is the effects caused by biotic and abiotic stress responses on plant growth and development. This highlights the importance of the ROS signalling network and antioxidant activity in plants. In particular the biochemical approaches undertaken in the last decade might substantially contribute to understand the underlying mechanisms regulating ROS-mediated responses such as drought, which are regulated by ABA and NO.

The data in this study showed that APX activity was increased by NOS-mediated changes linked to application of ABA in *Zea mays*. The enzyme activity results clearly showed that *Zea mays* plants supplemented with ABA in the presence of the NOS inhibitor exhibited an increased SOD and APX activity as a result of reversal of NOS inhibition by the supplemented ABA, which explains the reduction of ROS in these treatments. This is in accordance with de Souza *et al.* (2014) who demonstrated that ABA improves drought tolerance and grain increase by regulating reactive oxygen species scavenging through antioxidant enzyme activity. The study also shows that the ABA mediates the accumulation of proline, which has the capacity of scavenging reactive oxygen species under unfavourable conditions. It is thus concluded that ABA participates in the induction of proline accumulation and induction of antioxidant enzymes in maize and this is influenced by NOS-like enzymatic activity, which explains why plants in which ABA accumulation occurs (e.g. plants exposed to drought) would have high proline content and enhanced NO content that leads to altered antioxidant activity to scavenge the elevated ROS.

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