The effects of nitric oxide on soybean superoxide dismutase activity during osmotic stress

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Abstract

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Nitric oxide (NO) is a signaling molecule involved in mediating plant responses to various biotic and abiotic stresses. Major abiotic stresses (drought, salinity, cold) induce common cellular responses, causing osmotic stress in plants. This results in oxidative stress due to increased production of reactive oxygen species (ROS). The increased ROS levels simultaneously induce the antioxidative system (including antioxidant enzymes such as superoxide dismutase) that regulates ROS toxicity and enhance stress tolerance in plants. It is suggested that the scavenging of ROS by antioxidant enzymes can be controlled by NO. The aim of this study was to evaluate the role of exogenously applied NO on soybean (*Glycine max* L. Merr.) during osmotic stress, with the purpose of determining the effects of NO on the superoxide dismutase (SOD) activity in response to osmotic stress. This study also aimed at identifying and characterising SOD isoforms induced in soybean in response to osmotic stress and exogenous NO. To achieve these aims, soybean plants were treated with sorbitol (to induce osmotic stress), an NO donor [2,2'-(hydroxynitrosohydrazono)bis-ethanimine, DETA/NO] and its respective control (Diethylenetriamine, DETA). The results showed that exogenous NO alleviated osmotic stress-induced damage by reducing the superoxide radical content, lipid peroxidation levels and also maintaining cell viability in soybean leaves,

nodules and roots. Only two SOD isoforms i.e. manganese SOD (MnSOD) and copper/zinc SOD (CuZnSOD) were identified and characterised in soybean leaves and roots, iron SOD (FeSOD) was not induced. The isoforms identified exhibited low SOD activity in response to osmotic stress, with the exception of a few isoforms that had increased activity. The SOD activity was regulated by exogenously applied NO. The enzymatic activity of SOD isoforms was up-regulated by exogenous NO, except for a few SOD isoforms that were not responsive to NO. The results also showed that the increased SOD activity was associated with reduced lipid peroxidation levels. The results obtained from this study suggest that exogenous NO improves osmotic stress tolerance in soybean by regulating and increasing the SOD activity of only specific isoforms. The increased SOD activity maintains the redox homeostasis balance by detoxifying and controlling the superoxide radical levels, subsequently reducing lipid peroxidation and maintaining cell viability.

Declaration

I declare that *"The effects of nitric oxide on soybean superoxide dismutase activity during osmotic stress"* is my own work, that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

Babalwa Unice Jack **November 2012**

Signed: ……  **…….**

Acknowledgements

*To the almighty heavenly **God**, I thank you for the blessings in my life and for giving me strength for all my accomplishments.*

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List of Abbreviations

µl	Microliter
µg	Microgram
µM	Micromolar
µmol	Micromole
ABA	Abscisic acid
ANOVA	One-way analysis of variance
APS	Ammonium persulfate
APX	Ascorbate peroxidase
BSA	Bovine serum albumin
CAT	Catalase
cGMP	Guanosine 3,5-cyclic monophosphate
cm	Centimetre
CuZnSOD	Copper-zinc superoxide dismutase
DETA	Diethylenetriamine
DETA/NO	2,2'-(hydroxynitrosohydrazono)bis-ethanimine
EDTA	Ethylene di-amine tetra-acetic acid
FeSOD	Iron superoxide dismutase
FW	Fresh weight
GPX	Glutathione peroxidase
G	Gram
GR	Glutathione reductase
GSH	Glutathione

H_2O_2	Hydrogen peroxide
KCN	Potassium cyanide
LEA	Late embryogenesis abundant
LO	Lipid alcoxyl
LOO^-	Lipid peroxyl
L-NAME	Nω-Nitro-L-arginine methyl ester
L-NNA	N^G-nitro-L-arginine
min	Minute
MDA	Malondialdehyde
mg	Milligram
ml	Millilitre
mM	Millimolar
MnSOD	Manganese superoxide dismutase
NADPH	Nicotinamide adenine dinucleotide phosphate
nmol	Nanomole
NBT	Nitrotetrazolium blue chloride
NO	Nitric oxide
NOS	Nitric oxide synthase
NR	Nitrate reductase
$OH^{\bullet}$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
O_2	Oxygen
$O_2^{\bullet -}$	Superoxide radical
PAGE	Polyacrylamide gel electrophoresis
PCD	Programmed cell death

PUFA	Polyunsaturated fatty acids
PVP	Polyvinylpyrrolidone
ROS	Reactive oxygen species
sGC	Soluble guanylyl cyclase
SDS	Sodium dodecyl sulfate
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TEMED	N,N,N,'N'-Tetramethylethylenediamine
UV	Ultraviolet
V	Volt
v/v	Volume per volume
w/v	Weight per volume
XTT	3-bis (2-methoxy-4-nitro-5-sulfophenyl) -2H-tetrazolium-5-carboxyanilide
^{0} C	Degrees celsius
$^{1}O_2$	Singlet oxygen

Chapter 1

Literature Review

1.1 Introduction

Abiotic stresses cause major limitations in crop productivity worldwide (Siddiqui *et al.*, 2010). Exposure of plants to abiotic stress results in secondary stresses such as osmotic stress and oxidative stress. Oxidative stress occurs due to increased production of reactive oxygen species (ROS). Examples of ROS include the superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) (Sharma *et al.*, 2012). During normal plant growth, ROS are generated but their accumulation increases during abiotic stress (Møllar, 2001). ROS cause damage to proteins, lipids, DNA and thus lead to cellular mechanisms such as lipid peroxidation, protein oxidation and nucleic acid damage (Gill and Tuteja, 2010). This causes oxidative damage and subsequently programmed cell death in plants (Gill and Tuteja, 2010). On the other hand, ROS play an essential role in plant intracellular redox signaling (Siddiqui *et al.*, 2010). Therefore it is vital to control the concentration of ROS in plants for a survival response.

To maintain this survival response, plants activate an antioxidant defence system that reduces oxidative damage either by detoxifying ROS or preventing their excessive formation (Sharma *et al.*, 2012). This defence system particularly includes the enzymatic antioxidants such as the superoxide dismutase (SOD) which catalyses the dismutation of $O_2^{\bullet-}$ to produce H_2O_2 (Gupta *et al.*, 1993), H_2O_2 is further scavenged to water and oxygen by enzymes such as catalase, glutathione peroxidase and the antioxidant enzymes of the ascorbate-glutathione cycle (Sharma *et al.*, 2012). Plants also possess non-enzymatic antioxidants such as ascorbate or

glutathione that are important for protection against oxidative damage (Foyer and Noctor, 2005).

In addition plants also possess a signaling molecule nitric oxide (NO), which plays a crucial role in regulating diverse physiological processes in plants; such as growth and development and also in mediating plant responses to abiotic stresses (Qiao and Fan, 2008). The use of exogenously applied NO donors has been shown to enhance plant tolerance to several abiotic stress including drought (Garcia-Mata and Lamattina, 2001), salinity (Zhao *et al.,* 2004) and heat (Uchida *et al.*, 2002). NO has antioxidant properties that maintain cellular redox homeostasis and also regulate ROS toxicity, protecting plants from oxidative damage (Qiao and Fan, 2008). As a signaling molecule, NO regulates the expression of antioxidant genes (Qiao and Fan, 2008) that detoxify ROS and thus enhance stress tolerance.

The signaling interactions between nitric oxide and the antioxidant defence system is the important approach used to enhance abiotic stress tolerance in plants. This chapter reviews in details the effects of abiotic stresses and ROS molecules in plants, including the signaling pathways involving them. Nitric oxide as a signaling molecule which is involved in many physiological and molecular processes in plants is also discussed. Lastly the roles of the antioxidant defence mechanisms in plants are described; particularly the SOD enzyme is explained in detail.

1.2 Abiotic stress

Abiotic stresses are major environmental stress conditions which include drought, salinity, high/low temperatures, UV radiation and heavy metals, etc. caused by natural processes (Mahajan and Tuteja, 2005). These stressors affect plant mechanisms, plant physiology and

they lead to other stressors within the plant such as osmotic stress and oxidative stress. Abiotic stressors shape the evolution of plants (Zhu, 2002). The decline in crop yield and crop failure due to abiotic stress caused hundreds of million dollar losses in the agricultural sector (Mahajan and Tuteja, 2005).

1.2.1 Drought stress

Drought is regarded as one of the major environmental stresses that lead to limitations in agricultural productivity (Hao *et al.,* 2008). Drought or water-deficient stress occurs when crops are grown in soils with limited water content, where the water lost from the leaves (due to transpiration) exceeds the water taken up by the roots (Neill *et al.,* 2008). Drought is characterised by high transpiration rate, low root water uptake and ion leakage. Drought stress is more persistent and economically damaging (Zhu, 2002) and affects plant physiology and metabolism.

When plants are exposed to water stress, the leaf stomatal closure mechanism leads to reduced carbon dioxide (CO_2) uptake and therefore lower Calvin cycle activity resulting in a build up of NADPH. The net effect is over-reduction of the electron transport chain which leads to increased ROS production (Hsu and Kao, 2003). It has been demonstrated in a number of studies that water stress leads to oxidative stress, due to high ROS production (Hao *et al.,* 2008). Drought inhibits plant growth and photosynthesis, causes cellular dehydration, cell damage and plant programmed cell death. Drought imposes osmotic stress which can be artificially induced by growing plants in the presence of compounds such as sorbitol and polyethylene glycol for experimental purposes. Osmotic stress can be triggered by other abiotic stresses such as salinity and cold temperature (Neill *et al.,* 2008).

1.2.2 Plant responses to drought stress

When plants are facing water shortage they adapt by a number of morphological, physiological, biochemical and molecular changes (Hao *et al.,* 2008). Drought activates several defence responses that help plants to survive and adapt to drought stress. One of the most important defences is the stomatal closure (which closes during water shortage to prevent water loss) induced by abscisic acid (ABA). ABA is a plant hormone that regulates plant adaptative responses to abiotic stresses and helps plants by increasing their resistance to water stress (Zeevart and Creelman, 1988; Davies and Zhang, 1991; Lu *et al.,* 2009).

ABA induces stomatal closure by increasing the levels of cytosolic calcium ion (Ca^{2+}) concentration in the guard cells (Garcia-Mata and Lamattina, 2001). Important signal molecules like H_2O_2 and NO are involved in ABA-induced stomatal closure and they induce the activation of the antioxidant enzymes by the mitogen activated protein kinase (MAPK), which up-regulates gene expression of these enzymes (Zhang *et al.,* 2006; Lu *et al.,* 2009). Figure 1.1, shows the signaling interactions between ABA, H_2O_2 and NO during drought stress, mediating plant survival (Neill *et al.,* 2008).

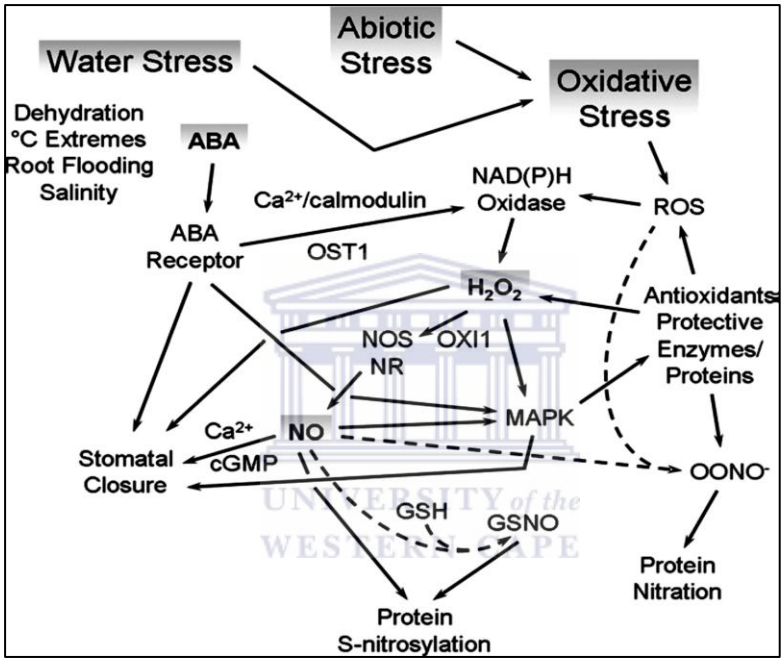

Figure 1.1: Illustration of water, abiotic and oxidative stress, and the signaling interactions between ABA, H_2O_2 and NO that occur in order to mediate plant survival under adverse conditions (Neill *et al.*, 2008).

Plants can induce a large set of genes that synthesize new protein accumulations in the vegetative tissues. These proteins are termed Late Embryogenesis Abundant (LEA) proteins; they are hydrophilic and highly expressed in seeds that are dehydrated. LEA proteins are abundant during embryo maturation and they have been shown to induce water stress tolerance, enabling plants to adapt during drought stress (Garcia-Mata and Lamattina, 2001). Another mechanism where plants can overcome drought is by increasing the metabolism of soluble carbohydrates that may act as compatible solutes or antioxidants (Shehab *et al.*, 2010) and also cellular solutes such as prolines (Al-khayri and Al-bahrany, 2002) that act as osmoprotectants and antioxidants. These compounds help plants to maintain their hydrated conditions and offer plant tolerance against drought and cellular dehydration (Mahajan and Tuteja, 2005).

1.2.3 Abiotic stress signaling

Plants have many signaling molecules that help them to survive abiotic stress. Such molecules include the stress responsive transcription factors. Transcription factors are essential in the activation of a number of genes that encode proteins involved in abiotic stress tolerance (Amudha and Balasubramani, 2011). They are trans-acting elements and they bind to the cis-acting promoter elements of the genes that code for stress tolerance and activate them. Examples of these transcription factors include DREB1 which is responsible for the expression of cold responsive genes and DREB2 which is responsible for the expression of drought responsive genes (Amudha and Balasubramani, 2011).

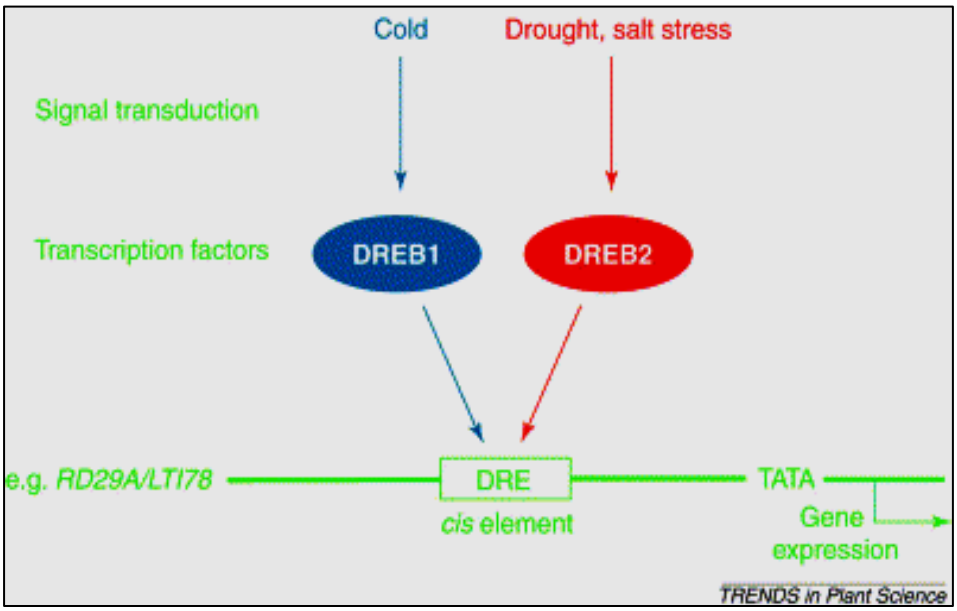

Figure 1.2: DREB1 and DREB2 transcription factors, key components in cross-talk between cold and drought signaling in *Arabidopsis* (Knight and Knight, 2001).

Ca^{2+} is also an important signaling molecule that serves as a second messenger during abiotic stress. Ca^{2+} plays an important role in ABA-induced stomatal closure by preventing drought stress (Chinnusamy *et al.*, 2004). Other protein kinases such as the calcium-dependant protein

kinases (CDPKs) have also been implicated to play a role in abiotic stress responses such as drought and cold (Chinnusamy *et al.,* 2004). CDPKs are serine/threonine protein kinases containing the C-terminal calmodulin like domain that binds Ca^{2+} (Xiong *et al.,* 2002). Examples of these kinases i.e. AtCDPK1 and AtCDPK2 are found in *Arabidopsis thaliana* and they can respond to drought and salinity stress (Knight and Knight, 2001).

Plants also use other phospho-proteins such as the mitogen activated protein kinase (MAPK) for abiotic stress signaling response. MAPK are also serine/threonine protein kinases and they are activated through the phosphorylation of MAPK kinase (MAPKK) by MAPK kinase kinase (MAPKKK) which in turn activates MAPK (Knight and Knight, 2001). Several MAPKs have been induced in response to hyper-osmotic stress (Mahajan and Tuteja, 2005). The plant's survival response during abiotic stress signaling is illustrated in figure 1.1.

1.3 Reactive Oxygen Species

Abiotic stresses can disrupt plant cellular processes such as photosynthesis and photorespiration, leading to changes in normal cell homeostasis (Miller *et al.,* 2010) and increase the levels of reactive oxygen species (ROS). When photosynthesis is disrupted, the electrons bind to the molecular oxygen in the cell and form ROS (Mittler *et al.,* 2004). ROS are more reactive compared to oxygen (Hancock *et al.,* 2001) as they can be reduced or activated derivatives of oxygen (Mittler *et al.,* 2004). The major cellular compartments for ROS generation include the mitochondria, chloroplast and the peroxisomes (Apel and Hirt, 2004).

During normal cell conditions ROS are produced at low levels, under stressful conditions the production of ROS increases dramatically (Miller *et al.,* 2010). ROS-associated cell injuries

in plants can be induced by osmotic stress and salinity (Serrato *et al.,* 2004; Borsani *et al.,* 2005; Miao *et al.,* 2006; Abbasi *et al,.* 2007; Zhu *et al.,* 2007; Giraud *et al.,* 2008). ROS are toxic and highly reactive molecules that can lead to oxidative damage in cells (Mittler *et al.,* 2004; Liu *et al.,* 2010). They induce damage to proteins, nucleic acids and lipids (Apel and Hirt, 2004; Miller *et al.,* 2010) and this can cause plant cell death (Gill and Tuteja, 2010). ROS also affect several cellular functions causing protein oxidation, nucleic acid damage and also lipid peroxidation (Gill and Tuteja, 2010). Although ROS enhance oxidative damage in plants, it has been shown in recent studies that they play an important role in plant signaling (Miller *et al.,* 2010) and they are key regulators of plant growth and development, programmed cell death, and also abiotic stress responses (Mittler *et al.,* 2004).

Major ROS molecules include superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\bullet}$). $O_2^{\bullet-}$ is the primary ROS molecule formed by reduction of O_2 electrons in a reaction catalysed by NADPH oxidase (Hancock *et al.,* 2001). However, $O_2^{\bullet-}$ can be dismutated at low pH conditions to produce H_2O_2 (Gill and Tuteja, 2010) and the enzyme superoxide dismutase (SOD) can also catalyse the dismutation of $O_2^{\bullet-}$ to form H_2O_2 (Hancock *et al.,* 2001). $OH^{\bullet}$ radicals are produced by Fenton or Harber-Weiss reactions in the presence of metal ions such as copper and iron (Hancock *et al.,* 2001; Gill and Tuteja, 2010); and they are the most reactive species with a relatively short half-life (Gill and Tuteja, 2010). $O_2^{\bullet-}$ can also react with other reactive molecules such as nitric oxide to form peroxynitrite ($ONOO^-$) (Hancock *et al.,* 2001). Singlet oxygen $^1O_2$ is another form of ROS which can be formed by photoexcitation of chlorophyll and its reaction with oxygen (Gill and Tuteja, 2010). Figure 1.3, shows different ROS species produced in different reactions.

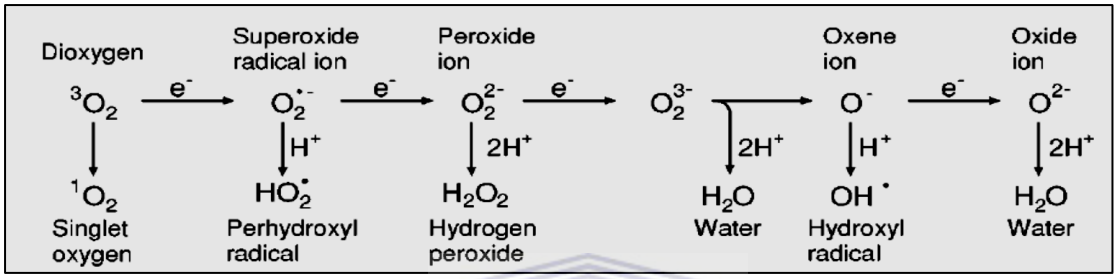

Figure 1.3: Generation of different ROS molecules (Apel and Hirt, 2004).

1.3.1 ROS generation

A number of enzymes have been implicated in ROS production and the most important one is NADPH oxidase (NOX) which catalyses the transfer of electrons from NADP to electron acceptors (Foreman *et al.,* 2003). NOX catalyses the production of superoxide by transferring electrons from NADP to molecular oxygen (Sagi and Fluhr, 2006). NOX is similar to the NADPH-dependant oxidase that is found in the mammalian phagocytes and B lymphocytes which produces ROS in response to pathogen attack (Apel and Hirt, 2004; Liu *et al.,* 2010). Plant NOX also produces ROS during pathogen attack (Liu *et al.,* 2010). Diphenylene iodinium, a chemical inhibitor of NOX, has been shown to inhibit ROS production in plants during stress (Mittler *et al.,* 2004). The plasma-membrane associated NOX is encoded by the respiratory burst oxidase homolog (*rboh*) gene (Miller *et al.,* 2010). An increase of ROS concentration is termed oxidative burst (Apel and Hirt, 2004).

In addition to NOX, other ROS producing enzymes include germin-like oxalate oxidase which produces H_2O_2 from O_2 and oxalic acid, amine oxidase which oxidises several forms of amines to release H_2O_2 (Liu *et al.,* 2010). Other oxidases playing a role in ROS production include NOX-like alternative oxidases and the glycolate oxidases. The pH-dependant cell wall peroxidises generate H_2O_2 in alkaline pH (Gill and Tuteja, 2010).

Xanthine oxidoreductase is also a source of ROS generation. This enzyme catalyses the oxidation of hypoxanthine to xanthine and then to uric acid and produces ROS such as $O_2^{\bullet-}$ and H_2O_2 (Hancock *et al.,* 2001). Cell organelles such as the chloroplast, mitochondria and the peroxisomes have mechanisms that can produce ROS (Mittler *et al.,* 2004). Other ROS producing sources that are not well known include the detoxification reactions that are catalysed by cytochrome P450 in the cytoplasm and the endoplasmic reticulum (Gill and Tuteja, 2010).

1.3.2 ROS signaling mechanisms

ROS are not only toxic molecules; they are also important signal molecules controlling stress response, growth and development. ROS signaling depends on a balance between ROS production and scavenging (Bailey-Serres and Mittler, 2006) since high levels of ROS are toxic whereas the presence of antioxidants and antioxidant enzymes can scavenge them. ROS molecules such as H_2O_2 play important roles in signaling whereas ROS such as $OH^{\bullet}$ are highly toxic (Hancock *et al.,* 2001). ROS are suitable signaling molecules because they are small and diffusible; several mechanisms induce their production and scavenging (Hancock *et al.,* 2001). In *Arabidopsis*, the lack of APX1 or thylAPX increased ROS production, which in turn increased tolerance against osmotic and salt stress (Miller *et al.,* 2007). ROS activate or inhibit a number of signaling pathways that are important for cell growth and development, cell death, cell cycle as well as cell response to environmental stresses.

1.3.3 The role of hydrogen peroxide (H_2O_2) in plant signaling

H_2O_2 is a reactive molecule that plays an important role in plant signaling. A number of plant cellular processes have been associated with H_2O_2 and these include: regulation of physiological processes such as photosynthesis, senescence and also plant growth and development (Quan *et al.*, 2008). The maintenance of H_2O_2 at low levels is important for cell tolerance to environmental stress (Quan *et al.*, 2008). The accumulation of high concentrations of H_2O_2 causes oxidative stress which can trigger plant programmed cell death (Neill *et al.*, 2002b). H_2O_2 induce signaling responses as well as signal molecules such as calcium (Ca^{2+}), salicylic acid (SA), abscisic acid (ABA), jasmonic acid (JA), ethylene and nitric oxide (NO), functioning together in signal transduction pathways to mediate responses to environmental resistance and also mediate plant growth and development. During signaling, H_2O_2 act as a second messenger molecule (Quan *et al.*, 2008).

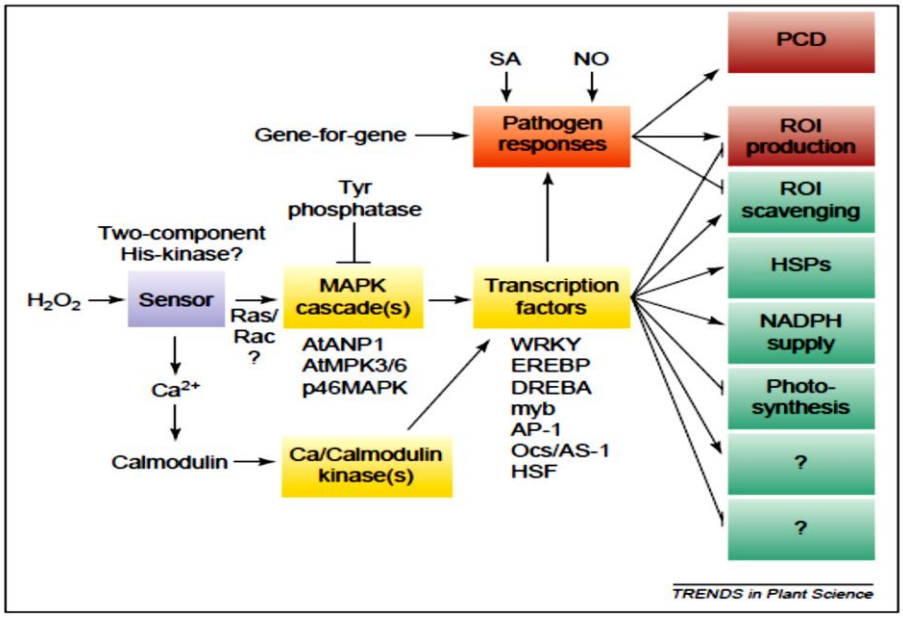

Figure 1.4: A suggested model for the activation of signal transduction events by H_2O_2 during oxidative stress (Mittler *et al.*, 2002).

Hypothetically in a signaling pathway, H_2O_2 is sensed by a sensor that might be histidine kinase which is a two component molecule that senses signaling as in yeast (Desikan *et al.,* 2001), illustrated in figure 1.4 (Mittler *et al.,* 2002). This leads to the activation of two signaling molecules; that is calmodulin which is a calcium binding protein and also mitogen activated protein kinase (MAPK). The role of H_2O_2 in MAPK was illustrated by Suzuki *et al.,* (1999) where tobacco was treated with a fungal elicitor and in response produced H_2O_2 and also activated the MAPK activity. The activation of these signal molecules results in the activation or suppression of the transcription factors. This regulates a number of signaling processes and responses in plants including programmed cell death, ROS production and scavenging and also cellular processes such as photosynthesis (Mittler *et al.,* 2002).

H_2O_2 inhibits the phosphatase pathways such as the tyrosine phosphatase pathway. H_2O_2 also interacts with other signaling molecules such as nitric oxide (NO) and salicylic acid (SA) to induce pathogen response during pathogen attack in plants (Mittler *et al.,* 2002). In addition it also plays an important signaling role in senescence; H_2O_2 was more induced in old leaves than in young leaves (Quan *et al.,* 2008). Furthermore it also plays an important role in ABA-induced stomatal opening and closing (Neill *et al.,* 2002b).

1.4 Plant programmed cell death

In plants, programmed cell death (PCD) is a genetically regulated process whereby the cells die. The mechanism of how plants die is not known but it is believed that plants and animals have similar morphological and biochemical apoptotic pathways (Solomon *et al.,* 1999). Particularly, the morphological characteristics of apoptosis (such as nuclear condensation, cytoplasmic shrinkage, membrane blebbing) and the biochemical characteristics of apoptosis

(such as the activation of proteases and DNA fragmentation) have been shown to occur during plant PCD (Solomon *et al.,* 1999).

PCD in plants is essential for cellular processes such as growth and development and also cell homeostasis control (Van Breusegem and Dat, 2006). Plant PCD has been implicated in a number of developmental processes such as seed development and germination (Van Breusegem and Dat, 2006) and also processes such as xylogenesis and senescence (Solomon *et al.,* 1999). Cell death in plants can occur in response to pathogen attack and also a number of environmental stressors such as high temperatures, ozone and UV radiation (Woltering *et al.,* 2002). PCD is essential for eliminating cells that are infected by pathogens or damaged by environmental stresses (Woltering *et al.,* 2002).

In animals PCD is initiated by a group of cysteine proteases termed caspases. However these caspases have not been shown to occur in plants but several studies have shown that caspase inhibitors also inhibited PCD in plants, therefore plant PCD is initiated by caspase-like activities (Sanmartín *et al.,* 2005). Caspase-like activities inducing PCD in plants include the vacuolar processing enzymes (VPEs) and the metacaspases (Woltering *et al.,* 2002). These proteases are similar to animal caspases in terms of their sequence and tertiary structure (Aravind and Koonin, 2002). Plant PCD is induced via ROS activation and depends on ROS concentration, meaning that low concentrations of ROS induce plant antioxidant enzymes and high concentrations can activate PCD (Solomon *et al.,* 1999). Levine *et al.,* (1996) showed that the use of protease or kinase inhibitors in cultured soybean cells inhibited PCD caused by oxidative stress or infection by virulent pathogens.

1.5 Nitric Oxide in plants

Nitric oxide (NO) is a small free radical gaseous molecule that is soluble in water and lipids (Durner *et al.,* 1999) and it has a relatively short half-life of about 3-30 seconds (Jagetia and Baliga, 2004). NO is highly reactive because of its unpaired electrons and can exist in three forms either as the radical ($NO^{\bullet}$); the nitrosonium cation (NO^{+}) or the nitroxyl anion (NO^{-}) (Neill *et al.,* 2003). NO is an important signaling molecule in mammals and acts as a second messenger during processes such as vasorelaxation, neurotransmission, cytotoxicity and immunoregulation (Neill *et al.,* 2003; Zhao *et al.,* 2004; Qiao and Fan, 2008).

NO also plays important signaling roles in plants (Neill *et al.,* 2003) and it is essential for plant physiological processes such as the induction of seed germination and reduction of seed dormancy (Beligni and Lamattina, 2000); induction of PCD (Siddiqui and Al-Whaibi, 2011); controlling stomatal movement (Garcia-Mata and Lamattina, 2001); regulating plant maturation, photosynthesis and senescence (Leshem *et al.,* 1998). NO also plays an important role in controlling multiple responses of plants to biotic and abiotic stresses (Qiao and Fan, 2008). In plants NO is either toxic or protective depending on its concentration, the tissue it acts upon and the environmental status (Garcia-Mata and Lamattina, 2001; Qiao and Fan, 2008). High dose of NO damage membrane proteins and cause DNA fragmentation (Qiao and Fan, 2008; Siddiqui and Al-Whaibi, 2011). Concentrations of NO that are above 10 µM have been shown to impair the leaf expansion, inhibit shoot and root growth and also cause cell death (Leshem *et al.,* 1998). However, at low levels NO induce normal growth and development in plants (Beligni and Lamattina, 2001).

1.5.1 NO generation in plants

NO can be synthesized endogenously in plants via enzymatic pathways such as the arginine or nitrate dependant pathway. The arginine dependant pathway is catalysed by the mammalian like nitric oxide synthase enzyme (NOS). NOS (EC 1.14.13.39) convert L-arginine to L-citrulline and NO (Qiao and Fan, 2008). This is a two-step reaction that first converts arginine to hydroxyarginine before L-citrulline and NO (Mur *et al.,* 2006) and requires cofactors such as NADPH and oxygen. The mammalian NOS enzyme is encoded by three isoforms: the neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) (Jagetia and Baliga, 2004). Both eNOS and nNOS are constitutively expressed in neuronal, endothelial and various cells, whereas the iNOS expression increases in the presence of lipopolysaccharides or interferon (Neill *et al.,* 2003).

The NOS enzyme has only been detected in animals; however a NOS-like activity has been illustrated in plants. To show the presence of NOS-like activity in plants, Ninnemann and Maier (1996) used mammalian arginine analogue inhibitors such as N^G-nitro-L-arginine (LNNA) and Nω-nitro-L-arginine methyl ester (L-NAME) to confirm that they inhibit NO in plants. Ribeiro *et al.,* (1990) used immunological assays to show that the mammalian mouse (anti-NOS) antibodies were able to recognize NOS or NOS-like molecules in maize. The radiolabelled L-citrulline was used to detect NOS-like activity in roots and nodules of *Lipinus albus* (Cueto *et al.,* 1996).

NOS-like activity was shown in several plants including soybean, pea and tobacco (Qiao and Fan, 2008) implying that the NOS activity does exist in plants, but the genes encoding NOS have not been identified in higher plants. However the AtNOS1 (*Arabidopsis thaliana* Nitric Oxide Synthase 1) gene isolated from *Arabidopsis thaliana*, which is similar to NOS from a snail (*Helix pomata*), was shown to code for a protein claimed to have NOS-like activity

(Guo *et al.*, 2003) but it was later revealed not to have the NOS activity but to be a GTPase (Zamojtel *et al.*, 2006) and it was also suggested to interact with the other proteins that form a complex to synthesize NO (Nigel and Crawford, 2006). Then it was named AtNOA1, which stands for *Arabidopsis thaliana* Nitric Oxide Associated 1 (Zamojtel *et al.*, 2006). The NOS gene from *Osterococcus tauri* (which is a eukaryotic, unicellular green alga species) is 45% similar to the human NOS in terms of the amino acid sequence and has been proved to have NOS activity (Foresi *et al.*, 2010). This *O. tauri* NOS can serve as a step forward for research of this enzyme in higher plant kingdom.

NO can also be synthesized by Nitrate Reductase (NR, EC 1.6.6.1) which is an NADPH-dependant enzyme (Rockel *et al.*, 2002) that is involved in nitrate assimilation. NR converts nitrite to NO and its derivative peroxynitrite ($ONOO^-$) *in vitro* (Yamasaki and Sakihama, 2000). The NR activity for NO biosynthesis was shown by Rockel *et al.*, (2002) where the supply of nitrate increased NO synthesis in anoxia conditions. The NR activity was also shown in *A. thaliana*, where the NR deficient mutants (*nia1* and *nia2*) could not produce NO in the guard cells and the stomata could not close in response to ABA treatment (Desikan *et al.*, 2002).

Other enzymes involved in NO synthesis include xanthine oxidoreductase (XOR) which produces NO and the $O_2^{\bullet-}$ radical in the presence of molecular oxygen (Hayat *et al.*, 2010). This enzyme has been found in plant peroxisomes (Neill *et al.*, 2003). The plasma membrane bound enzyme, nitrite: NO-reductase (Ni-NOR) also produces NO (Hayat *et al.*, 2010). NO can also be synthesized in non-enzymatic pathways. During nitrification/denitrification cycles, plants produce NO from nitrogen dioxide (NO_2) oxidation as illustrated in Figure 1.5. Carotenoids catalyse the light-mediated conversion of NO_2 to NO (Cooney *et al.*, 1994; Wojtaszek, 2000). This has been shown to occur at acidic pH conditions and at selected cell

compartments (Neill *et al.*, 2003). Ascorbate also reacts with nitrous acid in acidic or reducing environments to yield dehydroascobic acid and NO (Neill *et al.*, 2003). The reduction of nitrite by ascorbate at acidic pH conditions to synthesise NO was shown in the barley aleurone layer cells (Bethke *et al.*, 2004b).

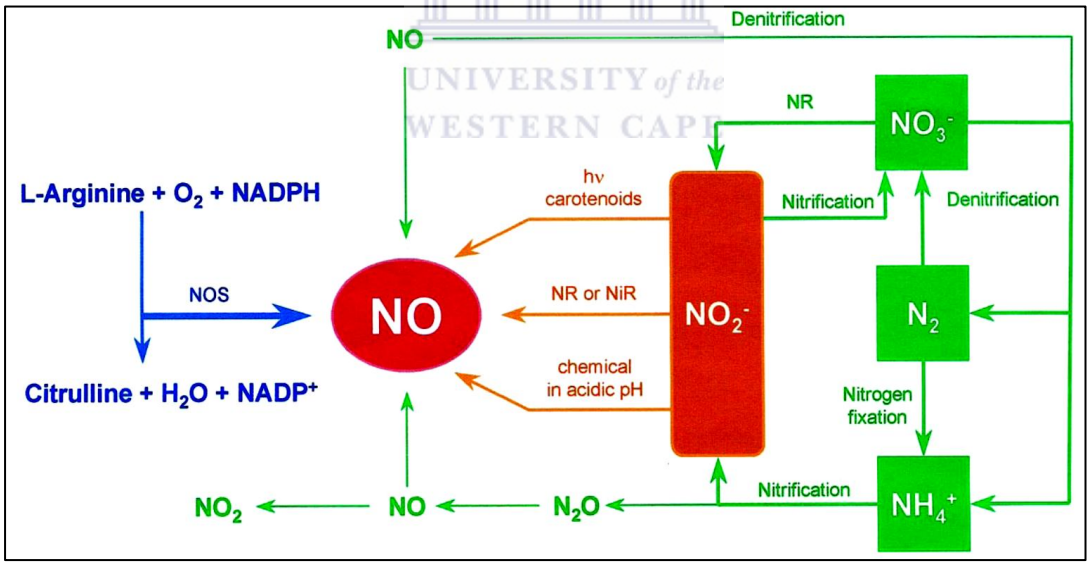

Figure 1.5: Enzymatic and non-enzymatic pathways for NO generation in plants (Wojtaszek, 2000).

1.5.2 NO signaling in plants

Nitric oxide (NO) is a second messenger molecule and its signaling can be mediated through a number of pathways. NO signaling involves the direct activation of proteins regulating gene expression or ion channel proteins or it can involve the indirect regulation of signal cascade proteins (Neill *et al.*, 2003). NO can be mediated through cGMP (cyclic guanosine monophosphate)-dependant pathways (Neill *et al.*, 2003) which is a second messenger molecule. In mammals, NO activates the soluble guanylate cyclase (sGC) enzyme by binding to the iron in the heme moiety of sGC (Hancock, 1997) and catalyses the synthesis of cGMP

from GTP. However the *Arabidopsis* GC enzyme (AtGC1) cannot be activated by nitric oxide (Ludidi and Gehring, 2003). NO signaling requires, in part, cGMP synthesis and it was shown that the inhibition of GC prevented NO-induced PCD and such effects were reversed by using 8-Bromo-cGMP, which is a cGMP analogue (Clarke *et al.,* 2000).

NO also influences the biosynthesis of cADP ribose (cADPR) via a cGMP-dependant pathway and cADPR increases the levels of cytosolic calcium (Cevahir *et al.,* 2007). Calcium is an important component in the stomatal ABA signaling pathways (Neill *et al.,* 2003) and cADPR regulates calcium levels in guard cells in response to ABA (Leckie *et al.,* 1998). The cGMP can also activate protein kinases known as cGMP-activated protein kinases. NO effects can also be mediated in a cGMP-independent pathway, where NO directly interacts with proteins containing thiol groups as well as metals such as iron, copper and zinc (Wendehenne *et al.,* 2001). NO can interact with proteins containing cysteine residues to form S-NO through S-nitrosylation and also with glutathione (GSH) to form S-nitrosoglutathione (GSNO). A number of S-nitrosylated proteins, including stress-related proteins, signaling proteins, redox-related proteins as well as proteins involved in plant photosynthesis and metabolism have been identified (Lindemayr *et al.,* 2005).

NO can interact with ROS such as $O_2^{\bullet -}$ to produce $OONO^-$ (Neill *et al.,* 2008). NO can also interact with signaling molecules such as H_2O_2 in processes that involve ABA-induced stomatal closure in the guard cells (Lu *et al.,* 2009). Garcia-Mata and Lamattina (2002) showed that NO interacts with ABA in fava bean (*Vicia faba*) during stomatal closure. NO also activates MAP kinase and increases antioxidant enzyme activities (Lu *et al.,* 2009).

1.5.3 The role of NO in plant growth and development

NO promotes plant growth including growth in the roots, leaves and shoots (Krasylenko *et al.,* 2010). NO is essential in stimulating leaf enlargement, seed germination and de-etiolation as well as inhibiting hypocotyls and internode growth (Cevahir *et al.,* 2007). The role of NO in plant growth and development is concentration dependant (Anderson and Mansfield, 1979). High concentrations of NO (40-80 pphm) have been shown to inhibit the growth of tomato, lettuce and pea plants (Cevahir *et al*., 2007) whereas low concentrations of NO (0-20 pphm) enhanced plant growth (Neill *et al.,* 2003; Hayat *et al.,* 2010). NO generated via nitrate reductase also inhibits ATP synthesis and the transport of electrons in the chloroplast, thus inhibiting photosynthesis (Takahashi and Yamasaki, 2002). NOS enzyme activity was shown to be essential for nodule functioning and development in soybean (Leach *et al.,* 2010).

The effect of NO on plant growth and development also depends on the type of plant tissue. The exogenous application of 0.1 mM sodium nitroprusside (SNP), which is a nitric oxide donor, promoted root development in cucumber whereas it inhibited the growth of hypocotyls in potato, lettuce and *Arabidopsis* (Hayat *et al.,* 2010). NO elevated chlorophyll content in guard cells of pea leaves. Graziano *et al.,* (2002) showed that NO inhibited chlorosis which is otherwise usually caused by the lack of iron nutrients in plants. NO also plays an important role in seed germination. Exogenous application of SNP reduced seed dormancy in lettuce, *Arabidopsis* and barley (Hayat *et al.,* 2010). NO also plays a role in plant senescence. Senescence is a form of cell death that results in loss of water and also desiccation of plant tissues (Hayat *et al.,* 2010). Senescence is induced by ethylene. The increased levels of ethylene increases senescence. NO has anti-senescence properties (Leshem and Haramaty, 1996).

1.5.4 The role of NO in programmed cell death (PCD)

A number of studies have contradictory reports about the role of nitric oxide on PCD. NO has been shown to induce PCD but some studies show that NO inhibits PCD. The effect of NO on cell death depends on its interaction with ROS (Delledonne *et al.,* 2001). The role of NO on PCD depends on the NO: $O_2^{\bullet-}$ ratio. When the $O_2^{\bullet-}$ levels are greater than the NO levels, NO will react with $O_2^{\bullet-}$ to form peroxynitrite ($ONOO^-$) and PCD cannot be induced. When the NO levels are higher than the $O_2^{\bullet-}$ levels, NO will react with H_2O_2 (formed by dismutation of $O_2^{\bullet-}$) and promote cell death (Neill *et al.,* 2003). High levels of both NO and H_2O_2 induced cell death in tobacco BY-2 cells (de Pinto *et al.,* 2002). NO was not able to induce PCD in soybean cell cultures (Neill *et al.,* 2003) whereas the elevated levels of NO in *Arabidopsis* cell suspensions were able to induce cell death (Hayat *et al.,* 2010). NO has antioxidant properties and it was shown to delay cell death in the barley aleurone layers that were treated with gibberellins (GA) (Beligni *et al.,* 2002).

1.5.5 The role of NO during abiotic stress

The role of NO during abiotic stress conditions has been extensively studied (Beligni and Lamattina, 2001) and it was reported that NO is rapidly induced by several stressors such as drought or salinity (Cevahir *et al.,* 2007) to regulate plant responses to abiotic stress (Qiao and Fan, 2008). The production of NO was shown to increase in response to abiotic stress (Qiao and Fan, 2008). NO increases antioxidant enzyme activity, which suppresses the levels of ROS, reducing plant damage following abiotic stress (Siddiqui and Al-Whaibi, 2011). During drought, exogenous supply of NO increased drought tolerance in cut leaves and seedlings of wheat (Garcia-Mata and Lamattina, 2001). The use of NOS inhibitors blocked the accumulation of ABA during drought stress whereas the use of NO donors increased the

synthesis of ABA in wheat roots (Arasimowicz and Floryszak-Wielzorek, 2007). NO also plays an important role during oxidative stress by increasing the up-regulation of antioxidants enzymes as well as the expression of antioxidant genes (Misra *et al.*, 2011). NO can reduce oxidative stress by directly scavenging ROS and that can prevent plant damage caused by lipid peroxidation (Qiao and Fan, 2008). NO also increases salt tolerance by inhibiting oxidative membrane damage and the translocation of Na^+ from roots to shoots (Guo *et al.*, 2009, Misra *et al.*, 2011).

1.6 Plant Antioxidant systems for ROS scavenging

During abiotic stress, excess numbers of reactive oxygen molecules are synthesized giving rise to oxidative damage. For survival against oxidative damage, plants have evolved ROS scavenging systems, known as the antioxidant defence mechanism that can be up-regulated in response to abiotic stress. These antioxidant defence mechanisms are found in almost all the cellular compartments where ROS are generated, including chloroplasts, mitochondria and the peroxisomes (Gill and Tuteja, 2010; Miller *et al.*, 2010). This mechanism comprises both enzymatic and non-enzymatic mechanisms.

1.6.1 Non-enzymatic antioxidants

Antioxidants are low molecular weight compounds that can remove or scavenge ROS (Noctor and Foyer, 1998). They are also known as redox buffers that can influence the expression of genes that play a role in biotic and abiotic stress tolerance (Foyer and Noctor, 2005). Ascorbic acid (ascorbate, vitamin C) is the most abundant antioxidant in plants that reduces ROS damage and occurs in all plant tissues especially in leaves (Smirnoff, 2005; Gill

and Tuteja, 2010). Ascorbate can scavenge ROS such as $O_2^{\bullet-}$ and $OH^{\bullet}$ (Gill and Tuteja, 2010) and it also plays an important role in the ascorbate-glutathione (ASH-GSH) cycle. Other important functions of ascorbate include photoprotection, cell cycle regulation, and regeneration of other antioxidants such as tocopherols (Noctor and Foyer, 1998). Ascorbate was shown to play an important role in *Arabidopsis* during salt stress by enhancing photosynthesis (Miller *et al.,* 2010). Another important antioxidant in plants is glutathione (GSH) which is a tripeptide abundantly occurring in a reduced form in plant tissues. It is essential in reducing ROS molecules, improving plant growth and development and also regenerating ascorbate in the ASH-GSH cycle (Foyer and Halliwell, 1976).

Plant pigments such as carotenoids also serve as antioxidants; they are lipid soluble antioxidants that remove ROS formed during photosynthesis in the photosynthetic apparatus (Gill and Tuteja, 2010). Davison *et al.,* (2002) showed that the overexpression of β-carotene hydroxylase in *Arabidopsis* increased oxidative stress tolerance induced by high light. Other important antioxidants in plants include: tocopherols (vitamin E) which have the ability to scavenge lipid radicals (Hollander-Czytko *et al.,* 2005), the bioactive secondary metabolites such as flavonoids which scavenge ROS molecules by neutralising the reactive radicals before oxidative damage occurs and the prolines molecules which protect plants against osmotic stress (Shehab, 2010), can also inhibit PCD induced by ROS (Gill and Tuteja, 2010).

1.6.2 Enzymatic antioxidants

These antioxidant enzymes catalyse the reactions that breakdown ROS molecules and they may also be involved in the formation of some ROS molecules (Noctor and Foyer, 1998). During oxidative stress, $O_2^{\bullet-}$ radicals are produced in different cell compartments by reduction of molecular oxygen. To protect cells against this reactive molecule, superoxide

dismutase (SOD, EC 1.15.1.1) provides the first line of defence by catalysing the dismutation of $O_2^{\bullet-}$ (Polle, 2001). SOD enzymes are found in almost all the cellular compartments. The reaction products of the SOD activity are H_2O_2 and O_2. However excess levels of H_2O_2 are not allowed in cells (Noctor and Foyer, 1998), therefore H_2O_2 can be further catalysed by catalase (CAT, EC 1.11.1.6) to water and O_2. Catalase is found in peroxisomes, although a *CAT3* isoform was shown in maize mitochondria (Scandalios *et al.,* 1980).

The alternative mode for H_2O_2 detoxification in the chloroplast is the use of ascorbate peroxidase (APX, EC 1.11.1.11), which uses the ASH-GSH cycle (Foyer and Halliwell, 1976) as the source of ROS scavenging in the chloroplast. In this cycle, APX is the most important enzyme as it scavenges H_2O_2 to form H_2O and O_2 using ascorbate as an electron donor. This also produces monodehydroascorbate (MDHA). MDHA can be reduced to ascorbate in an NAD(P)H-dependant manner by MDHA reductase (MDHAR, EC 1.6.5.4) or it can be converted to dehydroascorbate (DHA) non-enzymatically. DHA reductase (DHAR, EC 1.8.5.1) can reduce DHA using glutathione (GSH) to regenerate ascorbate, this causes GSH to become oxidised (GSSG). Then, glutathione reductase (GR, EC 1.6.4.2) regenerates GSSG to GSH using NAD(P)H (Foyer and Shigeoka, 2011). The balance and maintenance of the ASH-GSH cycle is essential for APX to scavenge H_2O_2.

Another important antioxidant enzyme is glutathione peroxidase (GPX, EC 1.11.1.9), which uses GSH to scavenge H_2O_2 to H_2O and also detoxifies lipid hydroperoxides and other hydroperoxides (Møllar, 2001). The mechanism of action for the ASH-GSH cycle and other antioxidant enzymes is shown in Figure 1.6. The roles of antioxidant enzymes in plants have been studied using the transgenic approaches by overexpressing these enzymes. These studies help to understand the role of the antioxidant system during abiotic stress tolerance. However, this study is only focused on SOD enzymatic activity.

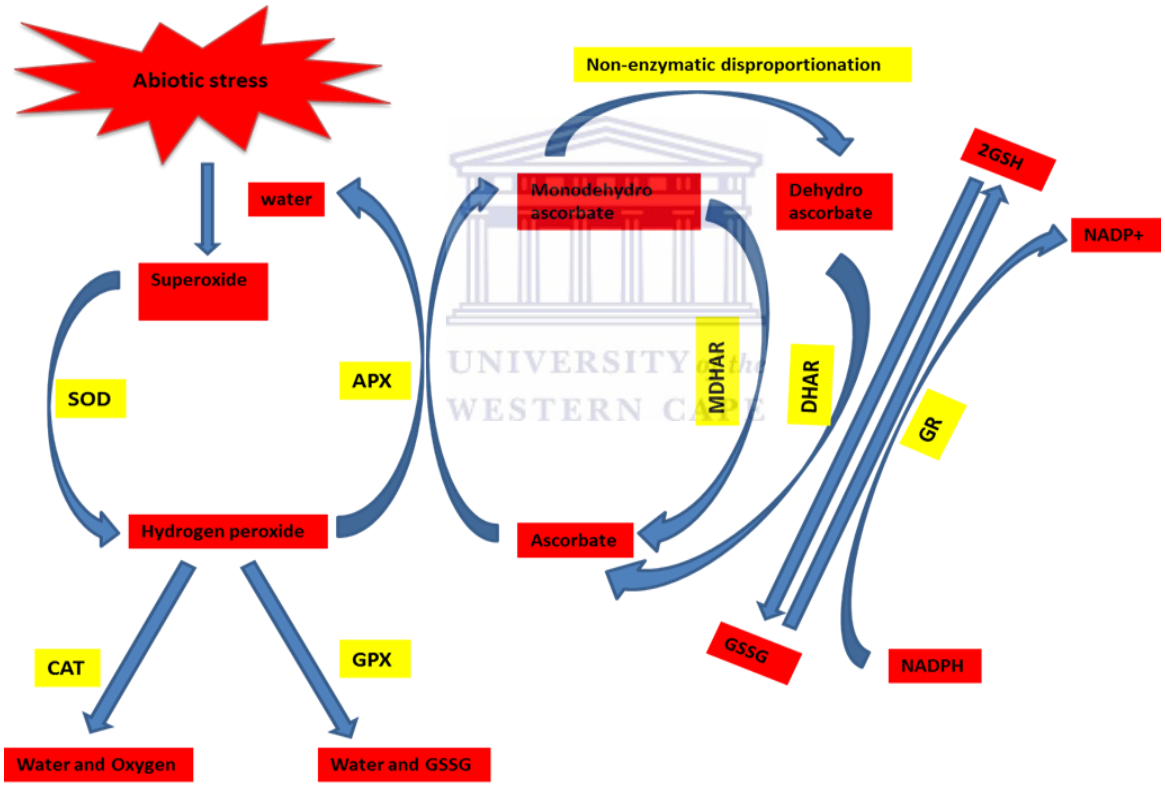

Figure 1.6: ROS and their scavenging by the antioxidant enzymes (adapted and modified from Gill and Tuteja, 2010).

1.6.3 Superoxide dismutase enzyme

Superoxide dismutase (SOD) activity was first discovered by McCord and Fridovich in 1969. SODs are multimeric metallo-enzymes that effectively scavenge the $O_2^{\bullet-}$ radicals (Scandalios, 1993). These enzymes catalyse the dismutation of $O_2^{\bullet-}$ produced as a result of oxidative stress (del Rio *et al.*, 1978) and therefore protect cells against oxidative damage caused by $O_2^{\bullet-}$ radicals. The scavenging of $O_2^{\bullet-}$ also reduces the formation of the $OH^{\bullet}$ radical (which is the most toxic reactive species) via the Haber-Weiss reaction (Arora *et al.*, 2002). SOD enzymes are found in almost all cellular compartments where $O_2^{\bullet-}$ is synthesised (Alscher *et al.*, 2002) and are found in all aerobic organisms as well as some aerotolerant,

anaerobic and obligate organisms (Fridovich, 1986). The metal cofactors in the SOD enzymes are active during $O_2^{\bullet-}$ radical catalysis.

SODs are classified into 3 isoforms in plants based on their metal cofactor at the active site which contains either iron, manganese or copper-zinc and they are designated as FeSOD, MnSOD and CuZnSOD. FeSOD and MnSOD are the most ancient SOD isoforms (Alscher *et al.,* 2002). FeSODs were previously discovered in prokaryotes and were later reported to exist in all plants (Scandalios, 1993). They are located in the chloroplast and a potential chloroplastic targeting sequence was found in a soybean FeSOD (Kliebenstein *et al.,* 1998). MnSODs occur in both eukaryotes and prokaryotes and they are localised in the mitochondria and peroxisomes. MnSODs have been detected in the mitochondria of several plant species including tobacco, watermelon and spinach (Alscher *et al.,* 2002). CuZnSODs occur in eukaryotes and certain prokaryotes, these are the most abundant SODs in plants (Odén *et al.,* 1992) located in the chloroplast, cytosol and the peroxisomes. CuZnSOD also occurs in the extracellular space (Alscher *et al.,* 2002). A cytosolic CuZnSOD has been previously isolated from pea (Pitcher *et al.,* 1992).

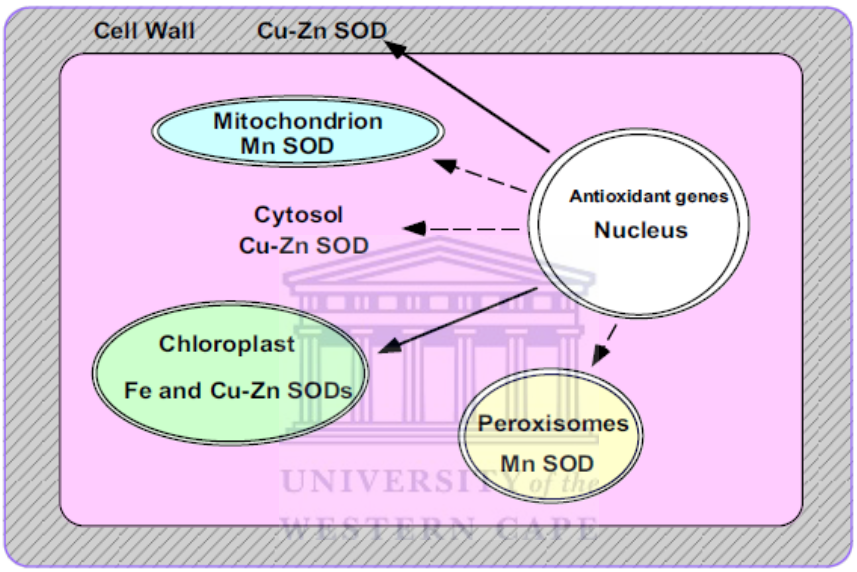

Figure 1.7: The localisation of the different SOD isoforms in different compartments of a cell (Alscher *et al.*, 2002).

In higher plants, different SOD isoforms have been isolated. In *Arabidopsis*, seven cDNAs and genes encoding SOD, including three CuZnSODs (CSD1, CSD2 and CSD3), three FeSODs (FSD1, FSD2 and FSD3) and one MnSOD (MSD1) have been identified (Kliebenstein *et al.*, 1998). A Mn-containing SOD was detected in pea leaves in the peroxisomes (del Rio *et al.*, 1983). A CuZnSOD cDNA clone was isolated from *Spinacia oleracea* L. leaves (Sakamoto *et al.*, 1993). Several CuZnSODs were identified in several species including spinach leaves, wheat germ and pea seeds (del Rio *et al.*, 1978). Kernodle and Scandalios (1996) identified ten SOD isozymes in maize including four cytosolic CuZnSODs, four mitochondrial associated MnSODs, as well as a chloroplastic associated CuZnSOD and FeSOD.

SODs can be classified into two phylogenetic families, with FeSOD and MnSOD being related based on their structural homologies and their degree of amino acid sequences whereas the CuZnSOD is not related to them (Scandalios, 1993) and they can also be

distinguished experimentally according to their sensitivity to the inhibitors H_2O_2 and cyanide (CN). MnSOD is resistant to both inhibitors, CuZnSOD is sensitive to both inhibitors and FeSOD is resistant to CN but sensitive to H_2O_2 (Bowler *et al.*, 1992).

A number of studies have shown the importance of SODs by either reducing oxidative damage or increasing stress tolerance. The SOD enzyme activity can increase during stress to enhance tolerance against oxidative stress, but in some cases the activity of this enzyme decreases in response to oxidative stress (Scandalios, 1993). The overexpression of SOD enzymes can also protect plants from oxidative damage. Drought strongly increased the induction of the cytosolic CuZnSOD whereas there was no effect on the chloroplastic CuZnSOD in tomato (Bowler *et al.*, 1992). The overexpression of MnSOD in maize chloroplast increased the antioxidant capacity in the leaves in response to chilling and oxidative stress (Van Breusegem *et al.*, 1999).

1.7 Aims and objectives of the study

The objective in this study was to impose osmotic stress in soybean using sorbitol treatment. Then exogenous NO was supplied with the aim of:

- Determining the role of the exogenous NO in osmotic stress responses.
- Determining the effects of exogenous NO on SOD activity during osmotic stress, and also
- Identifying the SOD isoforms that are expressed in response to osmotic stress and exogenous NO supply.

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Chapter 2

Nitric oxide improves soybean tolerance during osmotic stress

2.1 Abstract

Important crop plants such as soybean grow in external environments and they are susceptible to environmental stresses such as osmotic stress (secondary stress occurring due to drought). Osmotic stress occurs as a result of water loss to external environments and the inability of plants to take up water via roots. This limits plant growth and development and also reduces crop yield. In plants, nitric oxide (NO) is a signaling molecule that has been implicated in mediating plant physiological responses induced by various biotic and abiotic stresses and also enhancing stress tolerance. This study investigated the effects of exogenously applied NO on soybean during osmotic stress by measuring cell death, lipid peroxidation and the superoxide ($O_2^{\bullet-}$) radical content in the presence or absence of exogenous NO. The soybean plants were treated for 48 hours with 300 mM sorbitol (to induce osmotic stress) and in addition supplemented with 10 µM DETA/NO (NO donor). Osmotic stress extensively increased $O_2^{\bullet-}$ levels, which ultimately resulted in oxidative damage (increased lipid peroxidation) and cell death. Exogenous NO was able to alleviate osmotic stress-induced injuries by reducing the $O_2^{\bullet-}$ levels, lipid peroxidation and cell death. These results suggest that exogenously applied NO improves soybean tolerance to osmotic stress by breaking the oxidative chain reaction through a process of $O_2^{\bullet-}$ scavenging, thereby stopping the propagation of lipid peroxidation and preventing cell death.

2.2 Introduction

Soybean (*Glycine max* L. Merr.) is one of the world's leading oil seed crop with high protein and oil contents essential for human and animal feed (Manavalan *et al.,* 2009; Toorchi *et al.,* 2009). Consumption of soybean based food products is increasing worldwide because of the beneficial effects for human health (Friedman and Brandon, 2001). Important soybean products are ink, cosmetic products, soaps and it has also been recently implemented in biodiesel production (Pimentel and Patzek, 2008). Most importantly, soybean has the ability of fixing atmospheric nitrogen in a symbiotic relationship with nitrogen fixing bacteria (Toorchi *et al.,* 2009), improving soil fertility.

Soybean, like any other plants growing in external environments, is susceptible to abiotic stresses, which are environmental conditions affecting plant growth and development and ultimately reducing plant productivity (Cramer *et al.,* 2011). According to Bray *et al.,* (2000) abiotic stress reduced the yield of most crops by more than 50%. Major abiotic stresses such as drought, salinity, extreme temperatures and heavy metals have detrimental effects on plants. Amongst these stresses, drought is one of the major plant stresses. Drought may trigger a series of morphological, physiological, biochemical and molecular changes in plants (Shehab *et al.,* 2010). Drought was shown to reduce soybean productivity by 40% compared to its normal yield (Specht *et al.,* 1999). Drought, salinity and cold stress induce cellular responses such as: cell dehydration, osmotic imbalances, reduction of osmotic potential and inhibition of photosynthesis (Desikan *et al.,* 2003; Mahajan and Tuteja, 2005; Shehab *et al.,* 2010) and that can result in osmotic stress, illustrated in figure 2.2 (Beck *et al.,* 2007; Toorchi *et al.,* 2009).

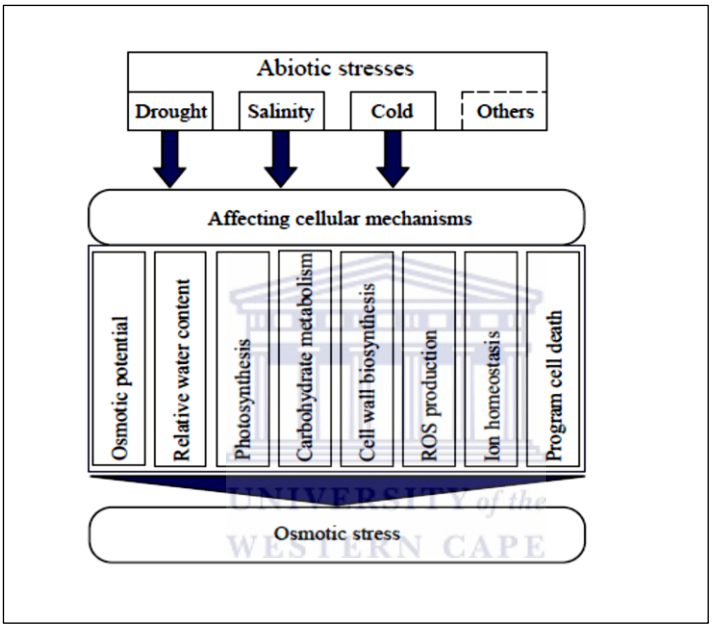

Figure 2.2: Cellular responses occurring due to abiotic stresses such as drought, salinity, cold, etc., inducing osmotic stress in plant cells (Nouri-Delavar, 2011).

During osmotic stress, water and ion homeostasis is disturbed and this increases the levels of reactive oxygen species (ROS), causing damage to cellular organelles (Moran *et al.*, 1994; Zhu, 2001). ROS are active oxygen molecules which are by-products of metabolic processes (Shehab *et al.*, 2010) and they can exist in four basic forms: the superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) and singlet oxygen ($^1O_2$) (Cruz de Carvalho, 2008). Under normal conditions, ROS are formed as by-products of metabolic processes such as photosynthesis and respiration in different cell compartments (Gill and Tuteja, 2010; Wang *et al.*, 2010) and they are scavenged by the antioxidant system (Foyer and Noctor, 2005; Cruz de Carvalho, 2008) of plants to avoid oxidative injury. During abiotic stress such as drought, ROS overwhelm the capacity of the antioxidant system and results in oxidative stress (Cruz de Carvalho, 2008; Filippou *et al.*, 2011). Enhanced ROS levels can cause oxidative damage by oxidising proteins, damaging nucleic acids, causing lipid peroxidation (Foyer and Noctor, 2005) and ultimately resulting in cell death (Gill and Tuteja,

2010; Sharma *et al.*, 2012). ROS such as H_2O_2 are important signal molecules mediating responses to abiotic stress but high levels of H_2O_2 can mediate programmed cell death (PCD) in plants (Bhattacharjee, 2005).

Nitric oxide (NO) is a free radical bioactive molecule (Neill *et al.*, 2003; Arasimowicz and Floryszak-Wieczorek, 2007; Gou *et al.*, 2009) which is important for signaling of plants and animals (Beligni *et al.*, 2002; Garcia-Mata and Lamattina, 2002). In plants, NO is involved in several physiological processes including the induction of seed germination (Beligni and Lamattina, 2000); regulation of plant maturation, photosynthesis and senescence (Leshem *et al.*, 1998); controlling stomatal movements (Garcia-Mata and Lamattina, 2001) and also inducing plant programmed cell death (Siddiqui and Al-Whaibi, 2011). NO also plays an important role in plant responses to abiotic stresses such as drought (Garcia-Mata and Lamattina, 2002), salt (Zhao *et al.*, 2004) and heat (Uchida *et al.*, 2002).

NO can be induced during stress to regulate plant responses (Hao *et al.*, 2008), as shown for an increase in NO production during dehydration of maize seedlings. Exogenous application of NO can increase stress tolerance, as evidenced from the use of NO donors such as sodium nitroprusside (SNP) in which the NO donor increased tolerance against drought (Garcia-Mata and Lamattina, 2001). NO can enhance abiotic stress tolerance through signaling mechanisms that regulate the expression of genes involved in stress tolerance (Qiao and fan, 2008). NO can modulate oxidative stress either by directly acting as an antioxidant regulating ROS toxicity and maintaining the cellular redox homeostasis (Qiao and Fan. 2008), or indirectly by inducing ROS-scavengers i.e. antioxidant defence system (Lamattina *et al.*, 2003). NO has antioxidant properties that can inhibit lipid peroxidation (Boveris *et al.*, 2000) and modulate the formation of $O_2^{\bullet-}$ (Caro and Pantarulo, 1998). ROS-mediated damages (including cell death, ion leakage and DNA fragmentation) caused by drought stress were reduced by

exogenous NO (Beligni and Lamattina, 1999). Therefore nitric oxide has the ability of alleviating damages caused by osmotic stress (Qiao and Fan. 2008).

In this study, the effects that osmotic stress has on soybean in terms of regulating cell viability, lipid peroxidation levels and the superoxide radical content were evaluated. The role of DETA/NO (NO donor) on cell death, lipid peroxidation and superoxide content in sorbitol- (osmotic stress inducer) treated plants was also determined in soybean leaves, nodules and roots treated for a period of 48 hours.

2.3 Materials and methods

2.3.1 Materials

Soybean seeds used for this work were kindly provided by Pannar Seeds (Greytown, South Africa). The Rhizobium inoculum *Bradyrhizobium japonicum*, which is a commercial peat-based HiStick2 soybean inoculant, was supplied by Becker Underwood Ltd (West Sussex, United Kingdom). The filtered Silica sand (98% SiO_2) was purchased from Rolfes® Silica (Pty) Ltd (Brits, North West, South Africa). All chemicals were purchased from Sigma-Aldrich and Bio-Rad, unless otherwise stated.

2.3.2 Plant growth

Soybean (*Glycine max* L. Merr. cv. PAN626) seeds were surface sterilized in 0.35% (v/v) sodium hypochlorite and 0.1% (v/v) Tween-20 for 10 minutes and washed five times with distilled water. The seeds were allowed to imbibe in distilled water for one hour at room temperature and then after inoculated with *Bradyrhizobium japonicum*. The seeds were sown

in one litre of filtered silica sand that had been pre-soaked with distilled water in 15 cm diameter plastic pots. The plants were grown in a greenhouse between early March and mid-April (average day temperature of 24°C and average night temperature of 15°C) under natural light conditions and they were watered with distilled water during germination until they reached VC stage (when unifoliolate leaves are fully expanded and the first node is visible). At VC stage, the plants were supplied with nitrogen-free nutrient solution [3 mM $CaCl_2$, 2 mM $MgSO_4$, 1 mM K_2SO_4, 1 mM K_2HPO_4 buffer (pH 7.2), 50 µM FeNaEDTA, 25 µM H_3BO_3, 2 µM $MnSO_4$, 2 µM Na_2MoO_4, 2 µM $CuSO_4$, 2 µM $ZnSO_4$, 0.1 µM $CoSO_4$ and 10 mM 4-(2-hydroxethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 7.3] at three day intervals until they reached the V3 stage (third trifoliolate leaf).

2.3.3 Plant treatment

Plants of the same phenological stage and similar height were selected for all experiments. The treatments were done for a period of 48 hours. The treatments consisted of the nitrogen-free nutrient solution supplemented with either sorbitol (osmotic stress inducer) at a final concentration of 300 mM, sorbitol at a final concentration of 300 mM combined with 2,2'-(hydroxynitrosohydrazono)bis-ethanimine (DETA/NO, NO donor) at a final concentration of 10 µM and sorbitol at a final concentration of 300 mM combined with diethylenetriamine (DETA, negative control for NO) at a final concentration of 10 µM. A control containing nitrogen-free nutrient solution only (untreated) was used and all the treatments were done at pH 7.2. After 48 hours of treatment, the plants were harvested. Freshly harvested plants (including leaves, nodules and roots) were used for measuring superoxide content and for cell viability analysis and the rest of the tissue (leaves, roots and nodules) was snap-frozen in liquid nitrogen, then stored at -80^0C and used for lipid peroxidation assays.

2.3.4 Cell viability analysis

The loss of cell viability (signifying cell death) was evaluated using the Evan's Blue staining method. For the assay, 100 mg of freshly harvested leaves, roots and nodule tissues from each treatment were stained with 0.25% (w/v) aqueous solution of Evans Blue at room temperature for approximately 45 minutes. The leaves, roots and nodules were then washed with distilled water several times and left overnight in distilled water. The leaves, roots and nodules were then incubated with 1% (w/v) SDS at 55^0C for one hour. The absorbance of extract was measured at 600 nm to determine the level of uptake of the Evans Blue by the cells.

2.3.5 Lipid peroxidation

Lipid peroxidation was measured as the amount of malondialdehyde (MDA) produced by the thiobarbituric acid (TBA) reaction. The leaves, roots and nodule tissues (100 mg for each organ) for each treatment were ground into a fine powder using liquid nitrogen and then homogenized in 400 µl cold 6 % (w/v) TCA. The homogenate was centrifuged at 12 000 x *g* for 15 minutes at room temperature. The supernatant was used to determine lipid peroxidation according to the modified method from Heath and Packer (1968), 100 µl of the supernatant was resuspended with 400 µl of 0.5% (w/v) TBA prepared in 20% (w/v) TCA. The samples were incubated at 95^0C for 30 minutes. The reaction was stopped by cooling the samples on ice for 5 minutes and then centrifuged for 10 minutes at 10 000 x *g* at room temperature. The absorbance of the resulting supernatant was measured at 532 nm and at 600 nm. The non-specific absorbance at 600 nm was subtracted from that recorded at 532 nm. The concentration of MDA was calculated using an extinction coefficient 155 $mM^{-1}cm^{-1}$ and expressed as $nmol.g^{-1}$ fresh weight.

2.3.6 Measurement of superoxide content

For the superoxide radical assay, fresh leaves, nodules and roots were used. Approximately 40 mg of the leaf, nodule and root tissues (from each treatment) were excised and homogenized in 400 µl of 0.12 mM XTT in 50 mM phosphate buffer (pH 8.2) using a pestle. In the control tubes, 100 Units of SOD enzyme (Sigma-Aldrich) were added to the homogenates (to inhibit superoxide generation in the plants). In experimental tubes, the homogenates were incubated along with the control tubes for 20 minutes in the dark at room temperature. After incubation, the assay solutions were centrifuged at 13 000 x *g* for 5 minutes. The absorbance of the resulting supernatant was measured at 450 nm and 470 nm for 30 minutes, reading every 5 minutes. Absorbance of the control tubes were subtracted from the absorbance of the experimental tubes. Superoxide content was expressed as micromoles per minute using the extinction coefficient for the XTT formazan product of 23, 600 M^{-1} cm^{-1}. The superoxide estimations were carried out in duplicates.

2.3.7 Statistical analysis

One-way analysis of variance (ANOVA) test was used for all data to evaluate statistical validity of the results and means were compared according to the Tukey-Kramer test at 5% level of significance, using GraphPad Prism 5.03 software. All results are the mean of at least three analysis replicates (n=3).

2.4 Results

2.4.1 The effects of NO on osmotic-stress induced cell death

Plants experiencing abiotic stress may suffer cell death due to increased levels of ROS. NO has protective effects when supplied to plants experiencing abiotic stress (Lamattina *et al.,* 2001) and that may prevent cell death. However, NO has also been reported to have cell death inducing effects (Beligni and Lamattina, 1999). Thus this study evaluated the effects of osmotic stress (by treatment with sorbitol) and the application of the NO donor (via DETA/NO treatment) on cell viability for soybean leaves, nodules and roots using the Evan's Blue uptake method.

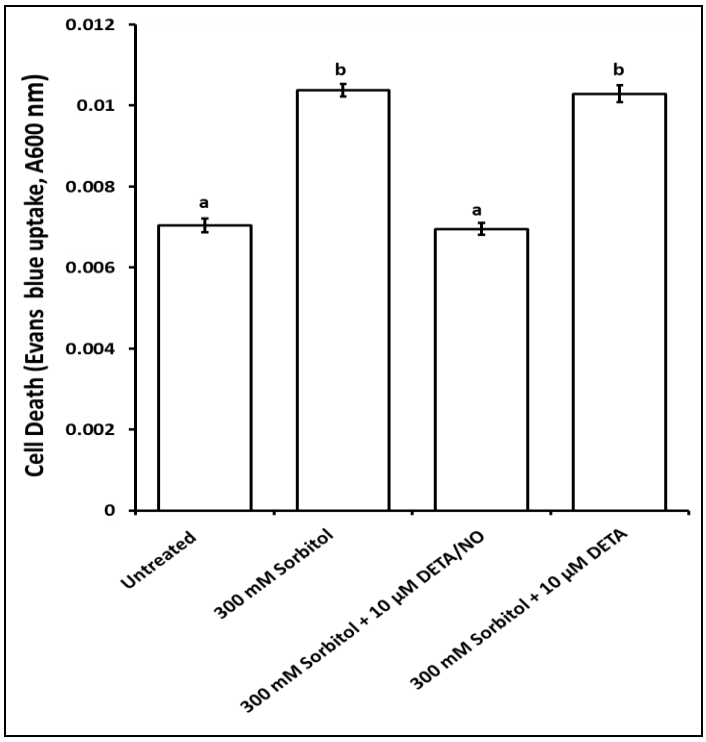

Figure 2.4.1.1: Changes in cell viability in soybean leaves in response to NO and osmotic stress. The assay was done on freshly harvested leaves after 48 hours treatment at V3 stage with either a nitrogen free nutrient solution only (untreated), 300 mM sorbitol, 300 mM sorbitol + 10 µM DETA/NO or 300 mM sorbitol + 10 µM DETA. Data shown are the means (±SE) of three independent experiments, different letters indicate mean values that are significantly different at $p<0.05$ using Tukey-Kramer test.

In the leaves, plants treated with 300 mM sorbitol experienced increased levels of cell death. This was indicated by an increase of ±47.5% in the Evan's Blue uptake compared to the untreated leaves (Figure 2.4.1.1). A combination of 300 mM sorbitol and 10 µM DETA/NO resulted in reduced levels of cell death, which was equivalent to the untreated leaves. Treatment with 300 mM sorbitol combined with 10 µM DETA did not reverse the effects of osmotic stress on cell viability triggered by sorbitol treatment, as DETA/NO did (Figure 2.4.1.1).

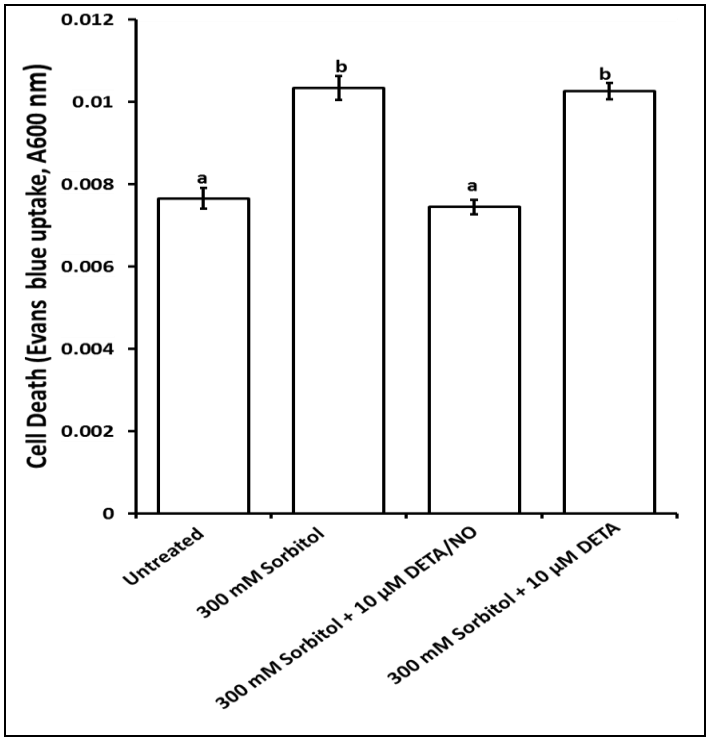

Figure 2.4.1.2: Changes in cell viability in soybean nodules in response to NO and osmotic stress. The assay was done on freshly harvested nodules after 48 hours treatment at V3 stage with either a nitrogen free nutrient solution only (untreated), 300 mM sorbitol, 300 mM sorbitol + 10 µM DETA/NO or 300 mM sorbitol + 10 µM DETA. Data shown are the means (±SE) of three independent experiments, different letters indicate mean values that are significantly different at $p<0.05$ using Tukey-Kramer test.

A similar pattern was observed with the nodules, cell death also increased by ±35% in sorbitol treated nodules compared to untreated nodules (Figure 2.4.1.2). Exogenous NO (i.e. 10 µM DETA/NO) in 300 mM sorbitol treated nodules, reduced cell death levels (to be equivalent with untreated nodules) and the NO control (10 µM DETA combined with 300 mM sorbitol) did not have any effects on cell viability as it was equivalent to sorbitol treated nodules (Figure 2.4.1.2).

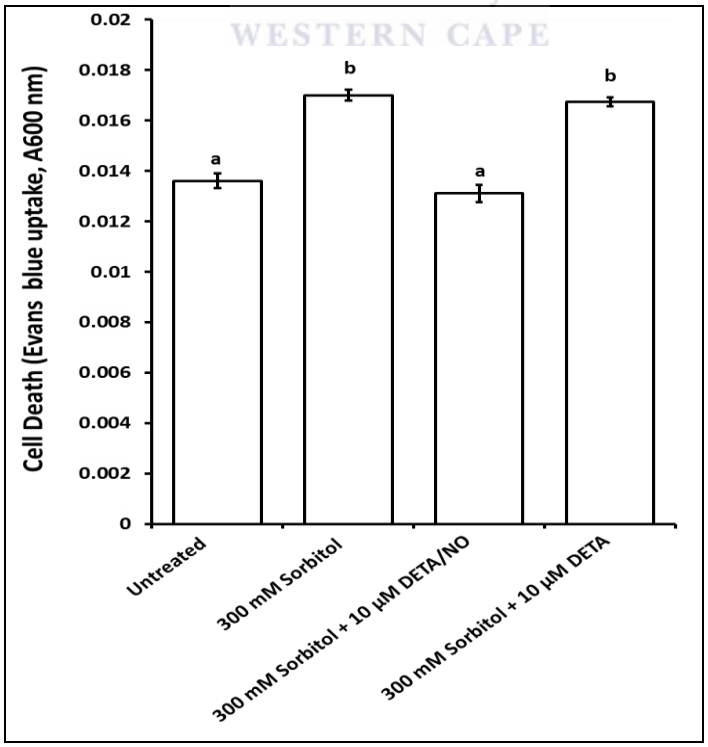

Figure 2.4.1.3: Changes in cell viability in soybean roots in response to NO and osmotic stress. The assay was done on freshly harvested roots after 48 hours treatment at V3 stage with either a nitrogen free nutrient solution only (untreated), 300 mM sorbitol, 300 mM sorbitol + 10 µM DETA/NO or 300 mM sorbitol + 10 µM DETA. Data shown are the means (±SE) of three independent experiments, different letters indicate mean values that are significantly different at $p<0.05$ using Tukey-Kramer test.

Cell death increased by ±25% in sorbitol treated roots compared to the untreated. Exogenous NO (i.e. DETA/NO) reduced cell death during osmotic stress to levels equivalent with untreated roots. Whereas, the NO control (i.e. DETA) did not augment the effects of cell

death caused by 300 mM sorbitol treatments (Figure 2.4.1.3). In the above results, the increase in cell death was occurring mostly in the leaves and least occurred in the roots during osmotic stress.

2.4.2 The effects of NO on the extent of lipid peroxidation levels during osmotic stress

Cell death occurs as a result of oxidative damage which can be characterised by cellular effects such as protein oxidation, lipid peroxidation and nucleic acid damage (Foyer and Noctor, 2005). In plants, NO enhances stress tolerance and it is expected to reverse the effects of oxidative damage. To determine the extent of oxidative damage induced by osmotic stress, the effects of various treatments on the MDA content (an indicator of lipid peroxidation and oxidative damage to membranes) was investigated in soybean leaves, nodules and roots.

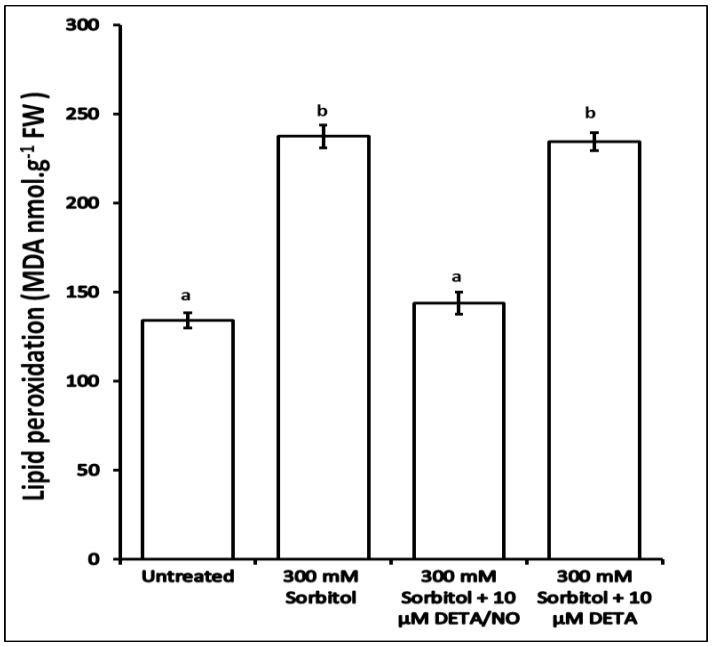

Figure 2.4.2.1: The effects of NO and osmotic stress response on lipid peroxidation levels in soybean leaves. The assay was done on leaves that were treated for 48 hours at V3 stage. Data shown are the means (±SE) of three independent experiments, different letters indicate mean values that are significantly different at $p<0.05$ using Tukey-Kramer test.

Lipid peroxidation increased in 300 mM sorbitol treated leaves. This was indicated by a ±77% increase in MDA levels when compared to untreated leaves. A similar trend (in terms of MDA levels) was observed in soybean leaves treated with 300 mM sorbitol and 10 µM DETA (Figure 2.4.2.1). Lipid peroxidation was reduced (to levels equivalent to untreated leaves) in plants treated with 300 mM sorbitol and 10 µM DETA/NO (Figure 2.4.2.1).

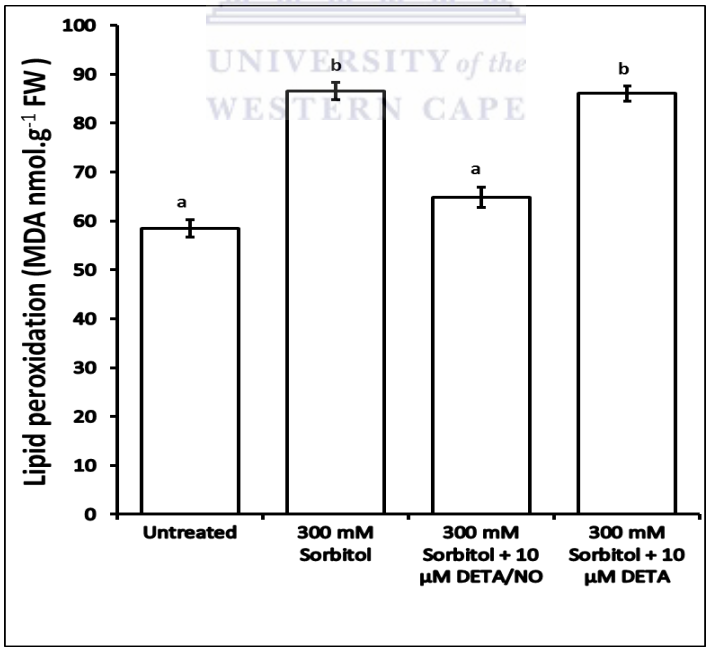

Figure 2.4.2.2: The effects of NO and osmotic stress response on lipid peroxidation levels in soybean nodules. The assay was done on nodules that were treated for 48 hours at V3 stage. Data shown are the means (±SE) of three independent experiments, different letters indicate mean values that are significantly different at $p<0.05$ using Tukey-Kramer test.

In the nodules, lipid peroxidation levels increased by ±51% in response to 300 mM sorbitol treatment or treatment with 300 mM sorbitol combined with 10 µM DETA when compared to the untreated nodules (Figure 2.4.2.2). Exogenous NO (i.e. 10 µM DETA/NO) in 300 mM sorbitol treated nodules, reduced the MDA content to a level equal to untreated nodules (Figure 2.4.2.2).

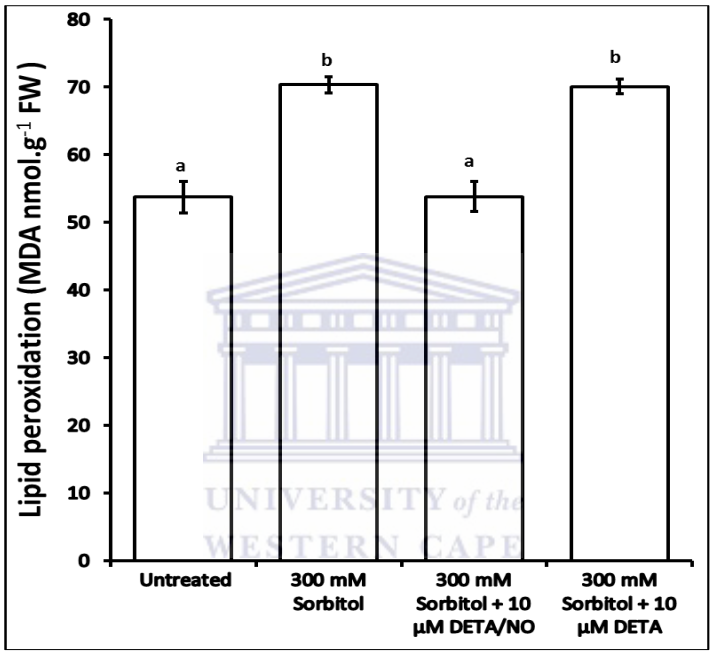

Figure 2.4.2.3: The effects of NO and osmotic stress response on lipid peroxidation levels in soybean roots. The assay was done on roots that were treated for 48 hours at V3 stage. Data shown are the means (±SE) of three independent experiments, different letters indicate mean values that are significantly different at $p<0.05$ using Tukey-Kramer test.

Roots treated with 300 mM sorbitol or a combination of 300 mM sorbitol and 10 µM DETA experienced lipid peroxidation at levels ±31% higher than the untreated (Figure 2.4.2.3). Whereas treatment with a combination of 300 mM sorbitol and 10 µM DETA/NO exhibited low levels of lipid peroxidation, which was equivalent to untreated controls (Figure 2.4.2.3). Oxidative damage was more marked in soybean leaves than the nodules and roots.

2.4.3 The $O_2^{\bullet-}$ content in response to osmotic stress and exogenous NO

In the view that oxidative damage occurs due to excessive accumulation of ROS, the data obtained from lipid peroxidation prompted further investigations to evaluate the effects of NO responses and osmotic stress on the $O_2^{\bullet-}$ levels in soybean leaves, nodules and roots.

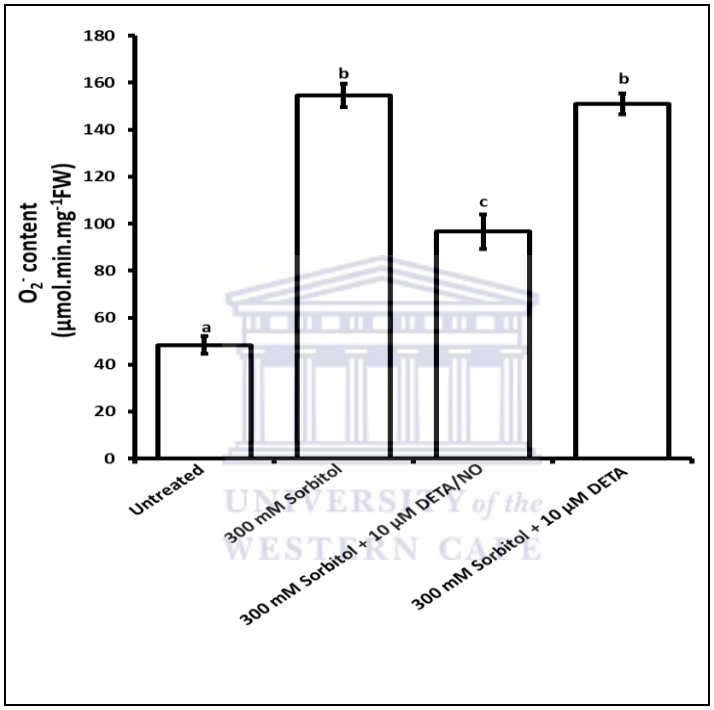

Figure 2.4.3.1: The effects of NO and osmotic stress on soybean leaf superoxide content. The assay was done on freshly harvested leaves after 48 hours treatment at V3 stage with either a nitrogen free nutrient solution only (untreated), 300 mM sorbitol, 300 mM sorbitol + 10 µM DETA/NO or 300 mM sorbitol + 10 µM DETA. Data shown are the means (±SE) of three independent experiments, different letters indicate mean values that are significantly different at $p<0.05$ using Tukey-Kramer test.

Compared to the untreated control, leaf $O_2^{\bullet-}$ content significantly increased by ±220% in response to treatment of plants with 300 mM sorbitol (Figure 2.4.3.1), whereas the increase of the $O_2^{\bullet-}$ content was only limited to ±100% in plants treated with 300 mM sorbitol combined with 10 µM DETA/NO. There was no significant difference in the level of superoxide contents between 300 mM sorbitol and 300 mM sorbitol plus 10 µM DETA treated leaves (Figure 2.4.3.1).

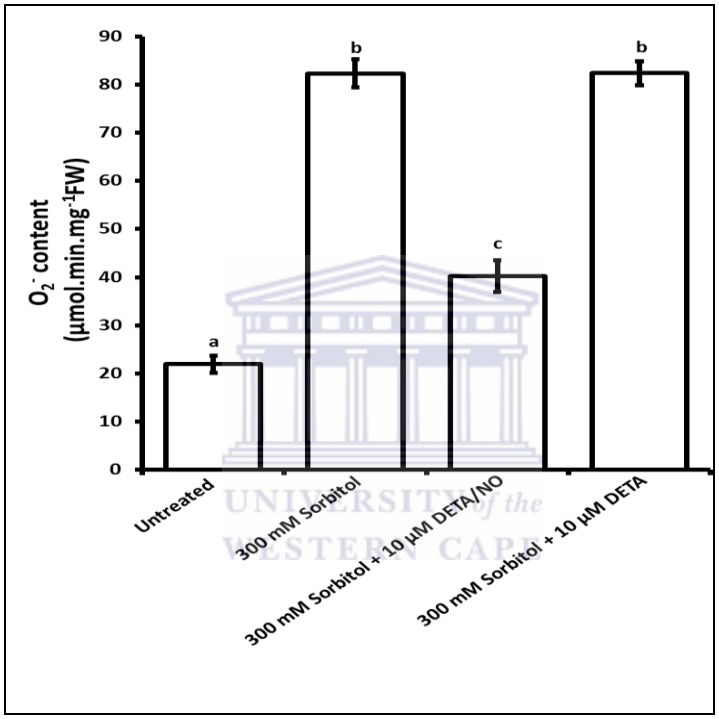

Figure 2.4.3.2: The effects of NO and osmotic stress on soybean nodule superoxide content. The assay was done on freshly harvested nodules after 48 hours treatment at V3 stage with either a nitrogen free nutrient solution only (untreated), 300 mM sorbitol, 300 mM sorbitol + 10 µM DETA/NO or 300 mM sorbitol + 10 µM DETA. Data shown are the means (±SE) of three independent experiments, different letters indicate mean values that are significantly different at $p<0.05$ using Tukey-Kramer test.

In the nodules, treatment with 300 mM sorbitol increased the superoxide content by ±274.8% compared to the untreated nodules (Figure 2.4.3.2). The increased $O_2^{\cdot-}$ levels were reduced by treatment of nodules with 300 mM sorbitol combined with 10 µM DETA/NO, by only a ±83.3% increase in superoxide levels compared to untreated nodules. Treatments with DETA did not have any effects when compared to the sorbitol treated nodules (Figure 2.4.3.2). A similar pattern was observed with the roots, the superoxide levels were increased by ±275% in sorbitol treated roots. But treatment of the roots with 300 mM sorbitol in combination with 10 µM DETA/NO increased the $O_2^{\cdot-}$ levels by ±88.4% compared to sorbitol treated roots (Figure 2.4.3.3). DETA treatments did not have any significant difference in the superoxide content when compared to sorbitol treated roots. Sorbitol-treated leaves had the highest

amount of the superoxide content and sorbitol-treated roots had the least amount of the superoxide content.

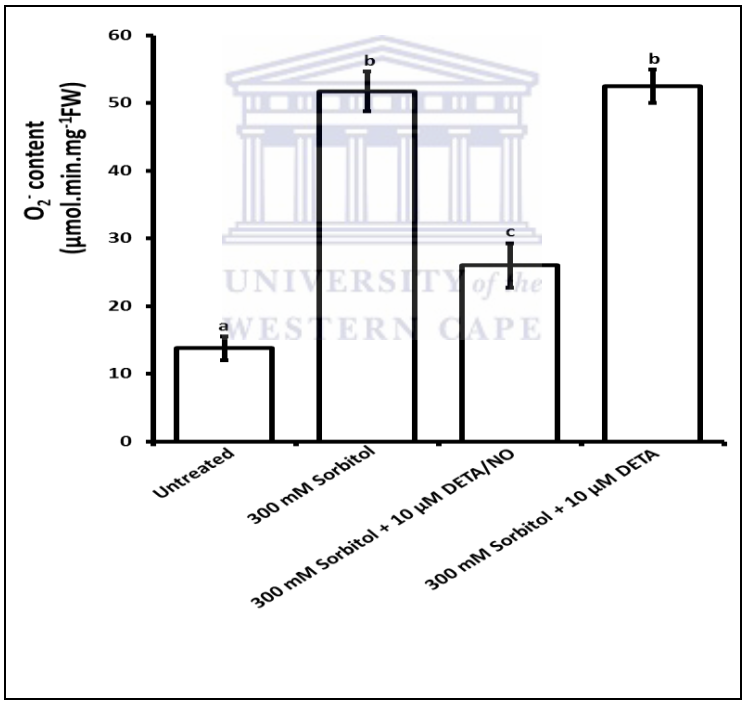

Figure 2.4.3.3: The effects of NO and osmotic stress on soybean root superoxide content. The assay was done on freshly harvested roots after 48 hours treatment at V3 stage with either a nitrogen free nutrient solution only (untreated), 300 mM sorbitol, 300 mM sorbitol + 10 µM DETA/NO or 300 mM sorbitol + 10 µM DETA. Data shown are the means (±SE) of three independent experiments, different letters indicate mean values that are significantly different at $p<0.05$ using Tukey-Kramer test.

2.5 Discussion

NO is an important plant signaling molecule that mediates plant responses to biotic and abiotic stresses. Several studies have reported the involvement of NO in responses to abiotic stresses such as drought (Uchida *et al.,* 2002) or heat (Leshem *et al.,* 1998); to physiological processes such as growth and development or apoptosis (Guo *et al.,* 2009). NO can prevent cell death in plants by acting as an antioxidant or antiapoptotic modulator (Chung *et al.,* 2001), however NO can also be toxic causing plant cell death. The toxic and protective

effects of NO during osmotic stress are concentration dependant (Wink and Mitchell, 1998). With these effects, it was necessary to evaluate the role of exogenous NO using 10 µM DETA/NO concentration as the NO donor in cell viability of soybean leaves, nodules and roots during osmotic stress. In this study sorbitol was used to induce osmotic stress and 300 mM sorbitol increased the levels of Evan's Blue uptake (which is an indication of cell death). However this shows that osmotic stress is detrimental for the survival of plants.

The results also suggest that DETA/NO releases NO and the NO released by 10 µM DETA/NO concentration is protective as it was able to reduce cell death levels induced by sorbitol treatment. Only high levels of NO have toxic effects in plants and that can impair leaf expansion, inhibit shoot and root growth and cause cell death (Leshem *et al.*, 1998); but that was not the case with the results obtained from this study. In a previous study, the use of 5 µM and 10 µM DETA/NO did not induce any detrimental effects on soybean nodule viability or functioning (Keyster *et al.*, 2011). Another study showed that high concentrations of 200 µM DETA/NO affected the nodule functionality by reducing plant growth parameters and nodule cell viability (Leach *et al.*, 2010). ROS-mediated cell death in plants can be triggered by high caspase-like cysteine protease activity (Solomon *et al.*, 1999) and NO may prevent cell death by scavenging ROS or regulating cysteine protease activity in response to osmotic stress.

Since osmotic stress resulted in increased levels of cell death, it was necessary to show that the plants had been exposed to oxidative stress and this could have been the events that lead to cell death. During abiotic stress, the increased accumulation of ROS molecules results in oxidation of polyunsaturated fatty acids (PUFA) producing secondary products such as malondialdehyde (MDA), which is the indicator of lipid peroxidation (Smirnoff, 1993). Lipid peroxidation is marker of membrane cellular damage occurring in the event of oxidative

stress (Sheokand *et al.,* 2010). In this study, lipid peroxidation was measured in terms of MDA content and the increased MDA levels in sorbitol treated leaves, nodules and roots clearly showed that the plants were exposed to osmotic stress and that membrane cellular damage occurred. Valentovič *et al.,* (2006) demonstrated increased lipid peroxidation levels in two maize cultivars in response to osmotic stress. Elevated lipid peroxidation levels were also reported by Niedzwiedz-Siegien *et al.,* (2004) during drought stress and by Esfandiari *et al.,* (2007) during salt stress in wheat seedlings.

However exogenous NO prevented sorbitol-induced lipid peroxidation. This once again shows that the NO concentration used in this study was effective in reducing oxidative damage other than having toxic effects. Similar protective effects of NO on relative membrane injury have been reported in several studies during osmotic stress (Tan *et al.,* 2008); drought stress (Garcia-Mata and Lamattina, 2001; Zhao *et al.,* 2008); and salt stress (Zhao *et al.,* 2004; Guo *et al.,* 2009). A possible reason for the protective effects of NO could be due to its highly reactive properties that enable it to scavenge accumulated ROS molecules during abiotic stress (Kopyra and Gwóźdź, 2003). Another possible reason could be that NO directly reacted with lipid radicals: lipid alcoxyl (LO) and lipid peroxyl ($LOO^{\cdot}$) and terminated lipid peroxidation mediated by these radicals (Beligni and Lamattina, 1999).

Under normal conditions, ROS effects in plants depend on maintaining the balance between ROS production and scavenging. During abiotic stress the increased levels of ROS induce membrane damage and even cell death. In this study, the superoxide radical in response to osmotic stress and exogenous NO was analysed in soybean leaves, nodules and roots. The superoxide radical ($O_2^{\cdot-}$) is the primary ROS molecule that is formed by reduction of electrons in oxygen molecules and this reaction can be catalysed by NADPH oxidase (Hancock *et al.,* 2001).

The results obtained from this study suggest that osmotic stress triggers excessive ROS production (evidenced by a significant increase in $O_2^{\bullet-}$ levels in sorbitol treatments). Increased $O_2^{\bullet-}$ content in response to osmotic stress was also reported by Tan *et al.*, (2008). The application of exogenous NO reduced the $O_2^{\bullet-}$ levels and this completely alleviated osmotic stress-induced oxidative damage as it was illustrated by reduced levels of lipid peroxidation and cell death. The protective role of NO in reducing the $O_2^{\bullet-}$ levels has been reported by Wang and Yang (2005) during Aluminium toxicity in roots of *Cassia tora* L. Possible reasons for the reduction of the superoxide content in response to NO could be that, NO directly scavenged $O_2^{\bullet-}$ to produce peroxynitrite ($ONOO^-$) which is less toxic in plant cells compared to animal cells (Delledonne *et al.*, 2001; Kopyra and Gwóźdź, 2004). This shows that NO has antioxidant properties that are important for regulating ROS toxicity and maintaining cellular redox homeostasis (Qiao and Fan, 2008). NO can also increase the scavenging capacity of antioxidant enzymes to scavenge ROS, and in this case it might have increased the SOD activity to scavenge $O_2^{\bullet-}$.

Compared to the untreated control, the $O_2^{\bullet-}$ content increased very rapidly in response to osmotic stress, whereas cell death in response to osmotic stress did not rapidly increase compared to the untreated. It is possible that in response to increased $O_2^{\bullet-}$ content, ROS scavenging capacity especially the SOD activity also increased and it was able to scavenge $O_2^{\bullet-}$, producing O_2 and H_2O_2. H_2O_2 has low toxicity compared to $O_2^{\bullet-}$ (Gadjev *et al.*, 2008) and low concentrations of H_2O_2 are important in signaling mediating plant responses during abiotic stresses. Therefore under these conditions it would be expected that sorbitol induced cell death levels will not be extremely high when compared to the untreated control. In response to NO treatments, cell death and lipid peroxidation were reduced to levels equivalent to the untreated plants, whereas that was not the case with the $O_2^{\bullet-}$ content. It is possible that the moderate increase in $O_2^{\bullet-}$ content observed in sorbitol treated plants in the

presence of DETA/NO was not sufficient to induce cell death and lipid peroxidation, or NO activated other ROS scavenging antioxidant enzymes to scavenge H_2O_2, preventing the formation of toxic ROS such as $OH^{\bullet}$.

In the untreated samples, the roots appear to have lower cell viability than the leaves and the nodules which have similar cell viability contents; this may be due to the differences in the responses of different tissue types to osmotic stress. The leaves seemed to be the most sensitive organs to sorbitol-induced osmotic stress (indicated by increased cell death and lipid peroxidation levels) compared to the nodules and roots, even though same treatment conditions were used. It is possible that the leaf organelles such the chloroplast or peroxisomes over-produced ROS and that led to high levels of lipid peroxidation and cell death. In a study by Guo *et al.*, (2009), leaves had high lipid peroxidation levels compared to roots during salt stress. The roots were the most sensitive organs than the leaves in terms of lipid peroxidation and ion leakage during osmotic stress (Valentovič *et al.*, 2006). This shows that plant responses to abiotic stress are dynamic and complex and they can depend on organ or tissue affected by stress (Dinney *et al.*, 2008).

The results showed no significant difference in plants treated with sorbitol and sorbitol combined with DETA on cell viability, lipid peroxidation and the $O_2^{\bullet -}$ content, whereas DETA/NO was very effective in enhancing osmotic stress tolerance. It can be concluded that DETA on its own has no influence other than releasing the exogenous NO which is the one that alleviated osmotic stress toxicity. For that reason DETA was used as the control for DETA/NO in this study, as it did not alter sorbitol-induced effects. DETA/NO was used as the suitable donor for NO because no toxic side effects have been reported unlike some donors such as SNP which has been shown to induce programmed cell death and suppress ROS scavenging capacity (Murgia *et al.*, 2004).

From the experimental evidence obtained, it can be concluded that osmotic stress induced by 300 mM sorbitol within 48 hours has an impact on increasing oxidative damage as that was observed by increased $O_2^{\bullet-}$ content, lipid peroxidation and also a loss of cell viability. Exogenous application of 10 µM DETA/NO was able to attenuate osmotic stress injuries by reducing the $O_2^{\bullet-}$ content, lipid peroxidation levels and cell death. These results suggest that exogenously applied NO improves soybean tolerance to osmotic stress by breaking the oxidative chain through a process of scavenging $O_2^{\bullet-}$, thereby stopping the propagation of lipid peroxidation and preventing cell death.

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Chapter 3

The role of nitric oxide in mediating superoxide dismutase activity during osmotic stress

3.1 Abstract

Superoxide dismutase (SOD) plays an important role in plants by scavenging superoxide radicals ($O_2^{\bullet-}$) produced during osmotic stress. The scavenging of $O_2^{\bullet-}$ by SOD enzyme activity is modulated by a signaling molecule, Nitric Oxide (NO). The mechanism of how NO mediates SOD activity during osmotic stress is not fully understood. Hence the soybean (*Glycine max* L. Merr.) leaves and roots exposed to osmotic stress (using 300 mM sorbitol) and exogenous NO (i.e. 10 µM DETA/NO) for one and seven days, were used to investigate the effects of exogenous NO on SOD isoform activities and on oxidative damage during osmotic stress. In order to analyse changes in SOD isoform activities, protein extracts were subjected to native PAGE and stained for SOD activity, the inhibitors: hydrogen peroxide (H_2O_2) and potassium cyanide (KCN) were used to identify the SOD isoforms. Oxidative damage was determined by measuring lipid peroxidation levels using the MDA assay. In leaves and roots examined, two SOD isoforms i.e. MnSOD and CuZnSOD were detected and FeSOD could not be identified. During short-term osmotic stress (one day treatment), three MnSOD and nine CuZnSOD isoforms were detected in the leaves, the roots induced one MnSOD and eight CuZnSOD isoforms. In response to long-term osmotic stress (seven days treatment), three MnSOD and nine CuZnSOD isoforms were expressed by leaves and the roots induced one MnSOD and nine CuZnSOD isoforms. Differential activity responses of SOD isoform activities occurred in response to osmotic stress and exogenous NO. The total

SOD activity decreased in response to osmotic stress, except for few isoform activities that increased. When NO was supplied, the total SOD activity was up-regulated even though the activity of few isoforms decreased. Osmotic stress enhanced oxidative damage by increasing lipid peroxidation levels and exogenous NO was able to alleviate the damage caused by osmotic stress by reducing lipid peroxidation. These results suggest that exogenous application of NO protects soybean from oxidative damage during osmotic stress, by increasing SOD enzymatic activity of specific isoforms to scavenge the $O_2^{\bullet-}$ radicals thus reducing lipid peroxidation.

3.2 Introduction

Superoxide dismutase (SOD, EC 1.15.1.1) is a significant enzyme in detoxification of ROS molecules during oxidative stress (Gupta *et al.,* 1993). SOD enzymes constitute the first line of defence by converting superoxide ($O_2^{\bullet-}$) forming oxygen and another type of reactive molecule, which is H_2O_2 (Tsang *et al.,* 1991; Gupta *et al.,* 1993; Scandalios, 1993; Alscher *et al.,* 2002). $O_2^{\bullet-}$ is a reactive oxygen molecule that can be formed during stress conditions due to reduction of molecular oxygen by electrons (Arora *et al.,* 2002). The $O_2^{\bullet-}$ radicals are formed in different cell compartments such as mitochondria, chloroplast, peroxisomes, cytosol, etc. (Alscher *et al.,* 2002) and it is important that the SOD activity is available in these compartments to scavenge $O_2^{\bullet-}$ (Takahashi and Asada, 1983).

Plant SODs exist in three basic isoforms which are classified based on their metal co-factor binding at the active site, which may contain either iron, manganese or copper/zinc and they are designated as FeSOD, MnSOD and CuZnSOD (Alscher *et al.,* 2002). These metal cofactors bind to the enzymes at their catalytic site and aid in the catalysis of $O_2^{\bullet-}$ (Attar *et al.,* 2006). SOD isoforms can be classified by their sensitivity to inhibitors H_2O_2 and cyanide

(Odén *et al.,* 1992). CuZnSOD is sensitive to both inhibitors, MnSOD is insensitive to both inhibitors and FeSOD is sensitive to H_2O_2 but insensitive to cyanide (Bowler *et al.,* 1992). FeSOD and MnSOD are evolutionary related as they share the same sequence similarity and structure whereas CuZnSOD is distinct from them (Kliebenstein *et al*., 1992; Scandalios, 1993).

SOD isoforms are located in different cell compartments and their localisation often determines the function of the isoform. FeSOD is located in the chloroplast (Alscher *et al.,* 2002) where it is essential for early chloroplast development in *Arabidopsis* (Myouga *et al.,* 2008); FeSOD can also protect the chloroplast from photooxidative damage during photosynthesis (Zhang *et al.,* 2011). MnSOD is localised in the mitochondria and peroxisomes (Alscher *et al.,* 2002; del Río *et al.,* 2003). The *Arabidopsis* MnSOD is required for maintaining mitochondrial redox homeostasis (Morgan *et al.,* 2008). CuZnSOD is found mainly in the chloroplast, cytosol and peroxisomes (Alscher *et al.,* 2002). Ogawa *et al.,* (1996, 1997) identified two CuZnSOD isoforms, one located in the apoplast, which is essential for lignification and the other one in the nucleus which protects against mutations caused by $O_2^{\bullet-}$ in plant cells.

During abiotic stress the production of $O_2^{\bullet-}$ is increased and hence plants rely on the SOD enzyme activity to detoxify this reactive molecule (Kliebenstein *et al.,* 1998). The role of SOD in reducing oxidative stress has been well documented in various studies. During oxidative stress, the levels of SOD may increase to enhance stress tolerance. Brou *et al.,* (2007) demonstrated an increased SOD activity in cowpea plants contributing to protection against oxidative stress. An increased SOD activity was reported in the leaves of *Brassica napus* L. during drought treatment (Abedi and Pakniyat, 2010). Other studies have shown that SOD activity can be reduced in response to abiotic stress. The SOD activity of sunflower

seedlings and grass plants (*Aegilops squarrosa*) was reduced during water stress (Badiani *et al.,* 1990; Quartacci and Navari-Izzo, 1992).

The protective role of the SOD activity in plants has been explored using transgenic studies by over-expression of different SOD transgenes to enhance oxidative stress tolerance (Bowler *et al.,* 1994; Alscher *et al.,* 1997; Scandalios, 1997). The overexpression of MnSOD increased tolerance to salt and oxidative stress in tomato (Wang *et al.,* 2007); the overproduction of FeSOD in the chloroplast of transgenic tobacco enhanced tolerance to oxidative stress induced by methyl viologen (Van Camp *et al.,* 1996).

The increased scavenging capacity of the antioxidant enzymes can increase oxidative stress tolerance. Nitric oxide (NO) is an important signaling molecule that acts as an antioxidant to scavenge reactive molecules and increase stress tolerance (Neill *et al.,* 2008). NO can also enhance the antioxidant capacity in plants by increasing the antioxidant enzymes such as SOD (to convert $O_2^{\bullet-}$ to H_2O_2), catalase and ascorbate peroxidase to both remove H_2O_2 (Neill *et al.,* 2008). Researchers have applied exogenous NO donors to plants in order to evaluate its role in plant growth and stress tolerance (Tan *et al.,* 2008). The results showed that, exogenous NO enhances tolerance to salt (Uchida *et al.,* 2002); chilling (Neill *et al.,* 2002b) and drought (Garcia-Mata and Lamattina, 2001). In a recent study, an NO donor 2,2'-(hydroxynitrosohydrazono)bis-ethanimine (DETA/NO) increased the enzymatic activity of ascorbate peroxidase in soybean nodules and reduced the H_2O_2 content (Keyster *et al.,* 2011). Other NO donors such as SNP (sodium nitroprusside) also increased the antioxidant capacity of SOD, catalase and peroxidase enzymes in maize seedlings during waterlogging (Wang *et al.,* 2011). These results suggest that the activation of antioxidant enzymes is the mechanisms used by NO to enhance protection against oxidative stress (Hayat *et al.,* 2010).

However not much is being reported about the role of NO on SOD activity during osmotic stress. Hence the aim of this study was to determine the effects of exogenous NO on soybean SOD enzyme activity using in-gel assay, during short-term (one day) and long-term (seven days) osmotic stress. The study also aimed at identifying SOD isoforms induced by soybean leaves and roots. This study further analysed the role of SOD activity on oxidative stress damage, in response to exogenous NO and osmotic stress by measuring lipid peroxidation.

3.3 Materials and methods

3.3.1 Materials

Soybean seeds used for this work were kindly provided by Pannar Seeds (Greytown, South Africa). The Rhizobium inoculum *Bradyrhizobium japonicum* which is a commercial peat-based HiStick2 soybean inoculant was supplied by Becker Underwood Ltd (West Sussex, United Kingdom). The filtered silica sand (98% SiO_2) was purchased from Rolfes® Silica (Pty) Ltd. (Brits, North West, South Africa). All chemicals were purchased from Sigma-Aldrich or Bio-Rad, unless otherwise stated.

3.3.2 Plant growth

Soybean (*Glycine max* L. Merr. cv. PAN626) seeds were surface sterilized in 0.35% (v/v) sodium hypochlorite and 0.1% (v/v) Tween-20 for 10 minutes and washed five times with distilled water. The seeds were allowed to imbibe in distilled water for one hour at room temperature and then after inoculated with *Bradyrhizobium japonicum*. The seeds were sown in one litre of filtered Silica sand that has been pre-soaked with distilled water in 15 cm diameter plastic pots. The plants were grown in a greenhouse between early March and mid-

April (average day temperature of 24°C and average night temperature of 15°C) under natural light conditions and they were watered with distilled water during germination until they reached VC stage (when unifoliolate leaves are fully expanded and the first node is visible). At the VC stage, the plants were supplied with nitrogen-free nutrient solution [3 mM $CaCl_2$, 2 mM $MgSO_4$, 1 mM K_2SO_4, 1 mM K_2HPO_4 buffer (pH 7.2), 50 µM FeNaEDTA, 25 µM H_3BO_3, 2 µM $MnSO_4$, 2 µM Na_2MoO_4, 2 µM $CuSO_4$, 2 µM $ZnSO_4$, 0.1 µM $CoSO_4$ and 10 mM 4-(2-hydroxethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 7.3] at three day intervals until they reached the V3 stage (third trifoliolate leaf).

3.3.3 Plant treatment

Plants of the same phenological stages and similar heights were selected for all experiments. The treatments were done for one day (short-term) and seven days (long-term). For long-term periods, treatments were performed once the plants were on V3 stage and they consisted of the nitrogen-free nutrient solution supplemented with either sorbitol (osmotic stress inducer) at a final concentration of 300 mM, sorbitol at a final concentration of 300 mM combined with 2,2'-(hydroxynitrosohydrazono)bis-ethanimine (DETA/NO, NO donor) at a final concentration of 10 µM and sorbitol at a final concentration of 300 mM combined with diethylenetriamine (DETA, negative control for NO) at a final concentration of 10 µM.. A control containing nitrogen-free nutrient solution only (untreated) was used and all the treatments were done at pH 7.2. The plants were treated at three day intervals for seven days and harvested 24 hours after the last treatment. For short-term (one day), treatments started at V3 stage also (treated together with the last treatment of long-term treated plants). The same treatment conditions used for long-term plants were also used for short-term treatments, which consisted of: untreated, 300 mM sorbitol, 300 mM sorbitol combined with 10 µM

DETA/NO and 300 mM sorbitol combined with 10 µM DETA, all done at pH 7.2. The short-term treated plants were harvested on the same day as the long-term treated plants. After harvesting the plant tissues (leaves and roots) were snap-frozen in liquid nitrogen and the material was stored at -80^{0}C until use for SOD in-gel activity assays and lipid peroxidation assays.

3.3.4 Protein extraction

For protein extraction, leaves and roots were ground into fine powder using liquid nitrogen and then 100 mg of the tissue samples were homogenised with 400 µl of the cold homogenising buffer [40 mM K_2HPO_4, pH 7.4, 1 mM ethylene di-amine tetra-acetic acid (EDTA), 5% (w/v) polyvinylpyrrolidone (PVP) molecular weight = 40,000]. The homogenates were centrifuged at 12 000 x *g* for 15 minutes at 4^{0}C and the resulting supernatant was used as the extract for SOD in-gel activity assays.

3.3.5 Determination of protein concentration

The protein concentrations were determined according to Bradford (1976), using the RC DC Protein Assay Kit 11 (Bio-Rad Laboratories, Inc., Hercules, CA) as instructed by manufacture. Bovine serum albumin (BSA) was used as the standard.

3.3.6 Determination of SOD in-gel activity

Plant extracts containing equal amounts of proteins were subjected to native polyacrylamide gel electrophoresis (PAGE) under non-reducing and non-denaturing conditions as described

by Laemmli (1970), except that SDS was omitted from all the buffers used. The SOD native PAGE was performed on a 10 % resolving gel and 5 % stacking gel at 80 V and 4^0 C. The amount of protein used for the leaves was 50 µg and for the roots 70 µg. After electrophoresis, the SOD activity was detected by photochemical staining with riboflavin, N,N,N,N-tetramethylethylenediamine (TEMED) and Nitrotetrazolium blue chloride (NBT) as described by Beauchamp and Fridovich (1971). The gels were stained with 50 mM potassium phosphate buffer (pH 7.8) containing 2.5 mM NBT for 20 minutes in the dark at room temperature. This was followed by incubation with 50 mM potassium phosphate buffer (pH 7.8) containing 28 µM Riboflavin and 28 mM TEMED in the dark for another 20 minutes at room temperature. After staining, the gels were exposed to light until the SOD activity bands became visible. The gel images were captured and analysed for band intensities by densitometry using Alpha Ease FC software (Alpha Innotech Corporation).

3.3.7 Determination of metallic cofactors of the SOD isoforms

Identification and characterization of SOD isoenzymes was accomplished by selective inhibition with KCN and H_2O_2. The gels were incubated with 50 mM potassium phosphate buffer (pH 7.8) containing 6 mM KCN as an inhibitor of CuZnSOD activity or 5 mM H_2O_2 as an inhibitor of CuZnSOD and FeSOD activities for 20 min before staining for SOD activity. MnSOD isoform activity is insensitive to both the inhibitors.

3.3.8 Lipid peroxidation

Lipid peroxidation was measured as the amount of malondialdehyde (MDA) produced by the thiobarbituric acid (TBA) reaction. Leaf and root tissues (100 mg) for each treatment were

ground into fine powder using liquid nitrogen and then homogenized in 400 µl cold 6 % (w/v) TCA. The homogenate was centrifuged at 12 000 x *g* for 15 minutes at room temperature. The supernatant was used to determine lipid peroxidation according to the modified method from Heath and Packer (1968), 100 µl of the supernatant was resuspended with 400 µl of 0.5% (w/v) TBA prepared in 20% (w/v) TCA. The samples were incubated at 95^0C for 30 minutes. The reaction was stopped by cooling the samples on ice for 5 minutes and then centrifuged for 10 minutes at 10 000 x *g* at room temperature. The absorbance of the resulting supernatant was measured at 532 nm and at 600 nm. The non-specific absorbance at 600 nm was subtracted from that recorded at 532 nm. The concentration of MDA was calculated using an extinction coefficient 155 $mM^{-1}cm^{-1}$ and expressed as $nmol.g^{-1}$ fresh weight.

3.4 Results

3.4.1 Determining the effects of osmotic stress and exogenous NO on SOD activity

SOD isoforms and their activities in response to exogenous NO and also short-term and long-term osmotic stress were determined using 10 % native PAGE and stained for SOD activity (Beauchamp and Fridovich, 1971). SOD isoforms were identified using the inhibition assay as described in the method section 3.3.7, and the results are shown in Figure 3.4.1. The isoforms were named according to their relative position from the top of the gel. Based on the inhibition assay only CuZnSOD and MnSOD were induced in soybean leaves and roots, FeSOD could not be detected and CuZnSOD was the most abundant SOD. To better analyse the activity of the individual SOD isoforms in response to various treatments, the pixel intensity of the SOD bands was applied using the Alpha Ease FC Software.

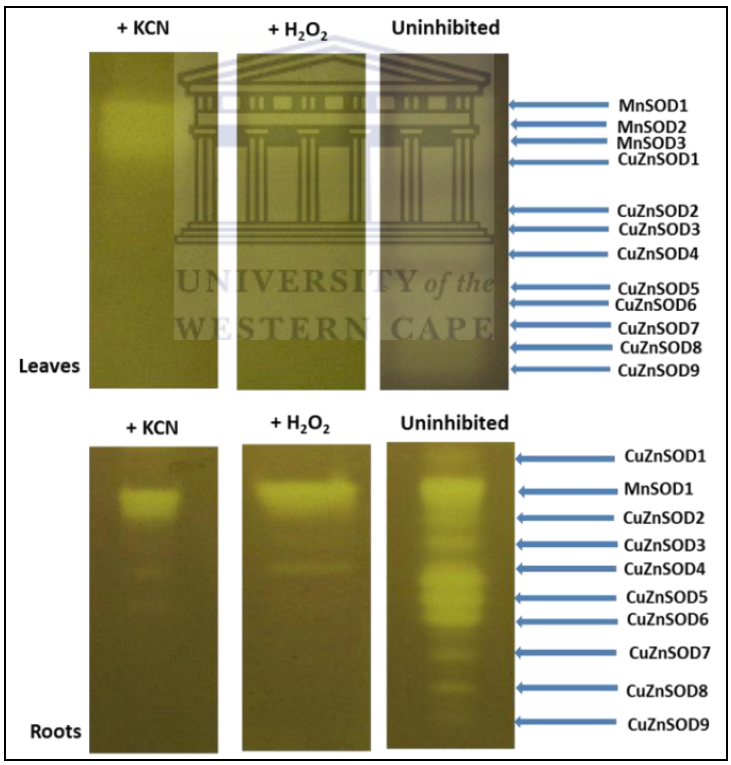


Figure 3.4.1: Inhibition assay for SOD isoform identification from sorbitol treated leaf (50 µg) and root (70 µg) protein extracts. The SOD isoforms were identified by pre-incubation of the 10 % native PAGE gels with the inhibitors: 6 mM KCN to inhibit CuZnSOD or 5 mM H_2O_2 to inhibit both CuZnSOD and FeSOD. An uninhibited gel was kept as a control for comparison and identification. The arrows indicate different isoforms identified in soybean leaves and roots.

3.4.2 Leaf SOD activity in response to short-term osmotic stress and exogenous NO

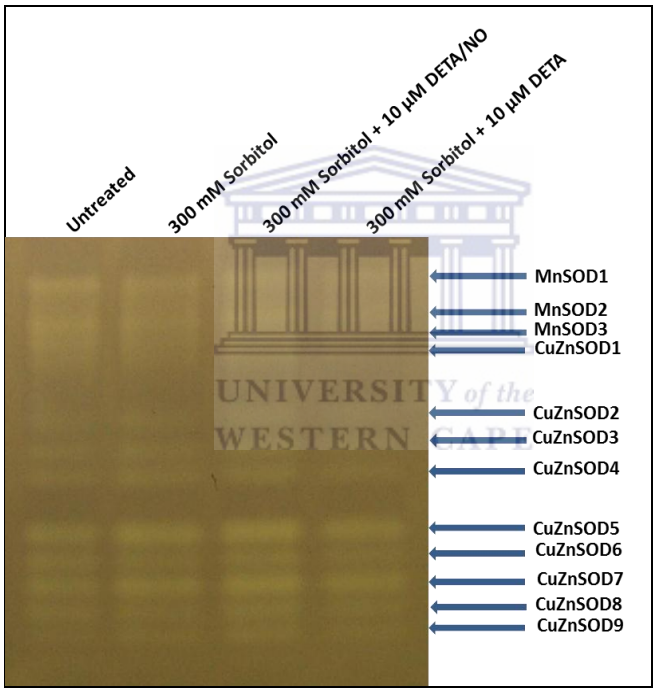
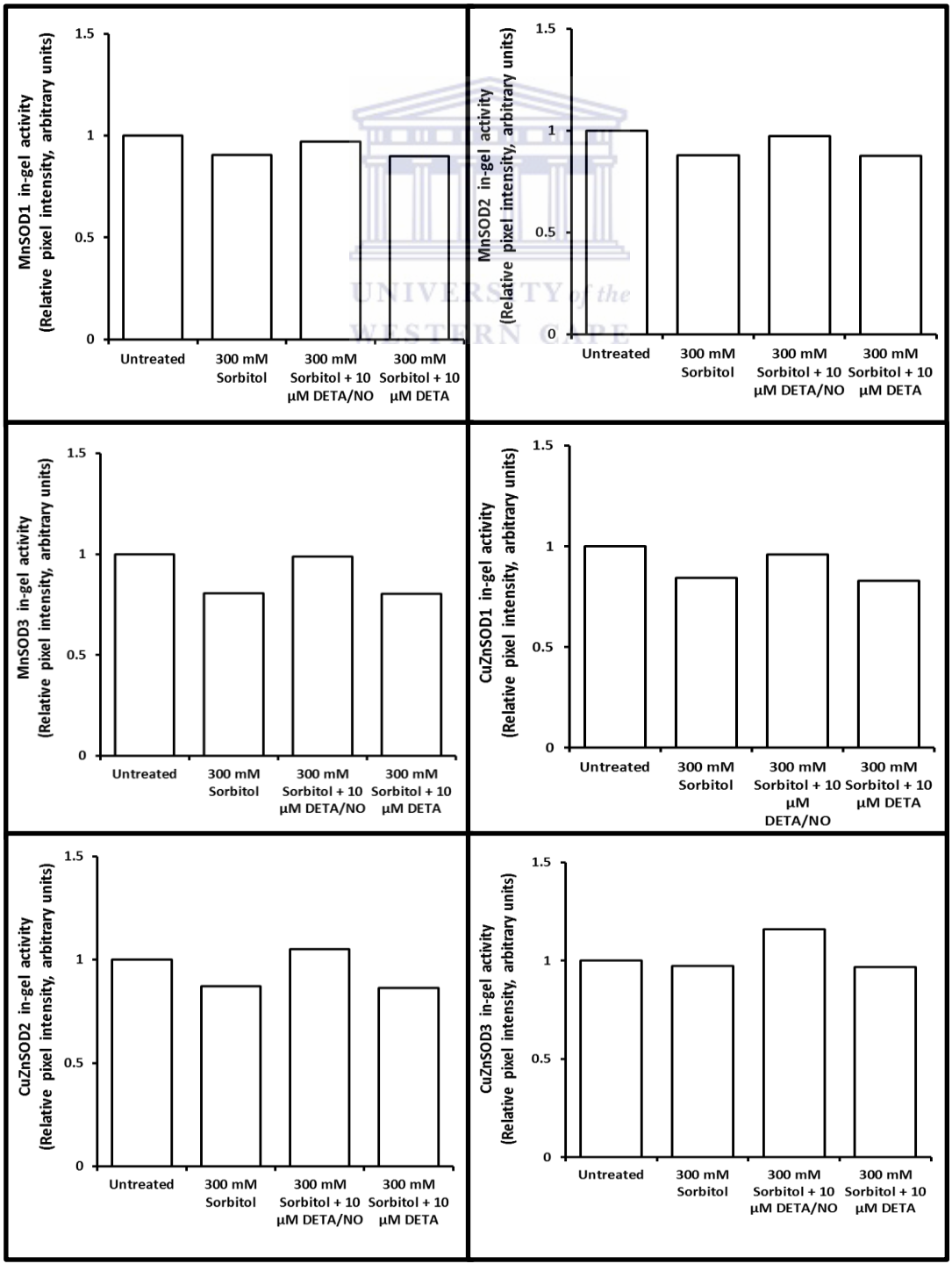


Figure 3.4.2.1: The effects of short-term osmotic stress and exogenous NO on SOD activity of soybean leaves. Equal amounts of the leaf protein extracts (50 µg) were separated by 10 % native PAGE. From the left, lane 1: untreated control; lane 2: 300 mM sorbitol; lane 3: 300 mM sorbitol + 10 µM DETA/NO and lane 4: 300 mM sorbitol + 10 µM DETA. The gel was stained for SOD activity as described by Beauchamp and Fridovich (1971).

The isozyme banding pattern for the short-term osmotic stressed leaves detected the presence of 12 SOD isoforms. Out of the 12 SOD isoforms detected, 3 of them were identified as MnSOD (i.e. MnSOD1, MnSOD2 and MnSOD3) due to their insensitivity to both KCN and H_2O_2 inhibitors. The rest of the isoforms were identified as CuZnSOD (i.e. CuZnSOD1-CuZnSOD9) as they were sensitive to both inhibitors (Figure 3.4.2.1). Differential changes in SOD activity in response to various treatments were observed and to better analyse the activity of SOD isoforms in response to various treatments, relative pixel intensity was employed.



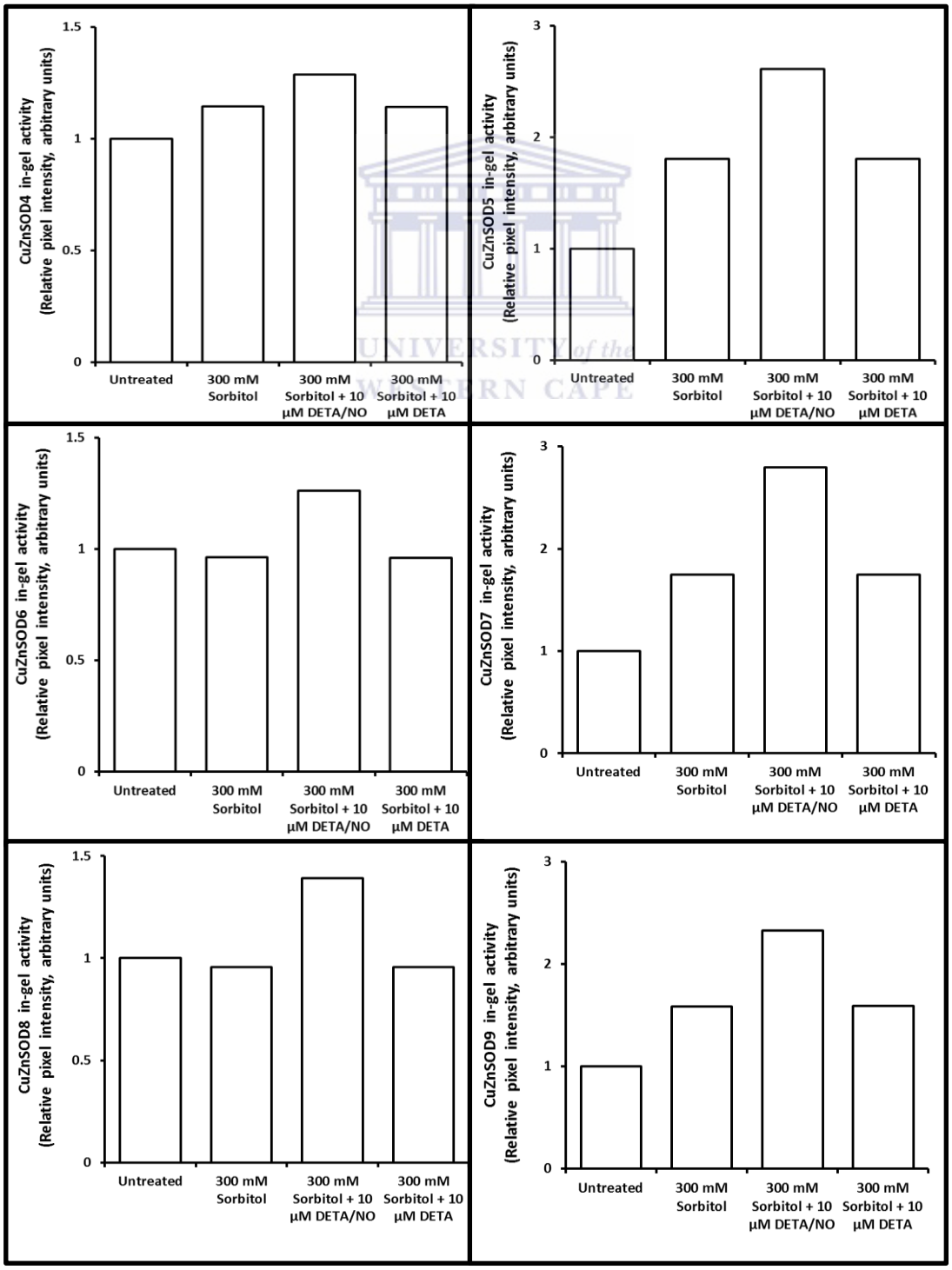


Figure 3.4.2.2: Leaf SOD isoform activities in response to short-term osmotic stress and exogenous NO, determined by Integrated Density Values with the intensity ratio of a SOD isoform relative to its specific untreated SOD isoform. The relative pixel intensity values are determined using the Alpha Ease FC software and the SOD activities are expressed as arbitrary units.

Based on the relative pixel intensity results (Figure 3.4.2.2), all the MnSOD isoenzyme activities decreased in response to sorbitol and sorbitol + DETA treatments when comparing to untreated leaves and exogenous NO increased MnSOD activity during osmotic stress to be almost equal to untreated leaves. A similar activity was observed for CuZnSOD1. Compared to untreated leaves; CuZnSOD2, CuZnSOD3, CuZnSOD6 and CuZnSOD8 activities were all reduced by osmotic stress, but exogenous NO increased the activities of these isoforms to levels higher than the untreated. The activities of CuZnSOD4, CuZnSOD5, CuZnSOD7 and CuZnSOD9 increased in response to sorbitol treatments when compared to untreated and application of exogenous NO further increased the activity of these isoforms.

3.4.3 Root SOD activity in response to short-term osmotic stress and exogenous NO

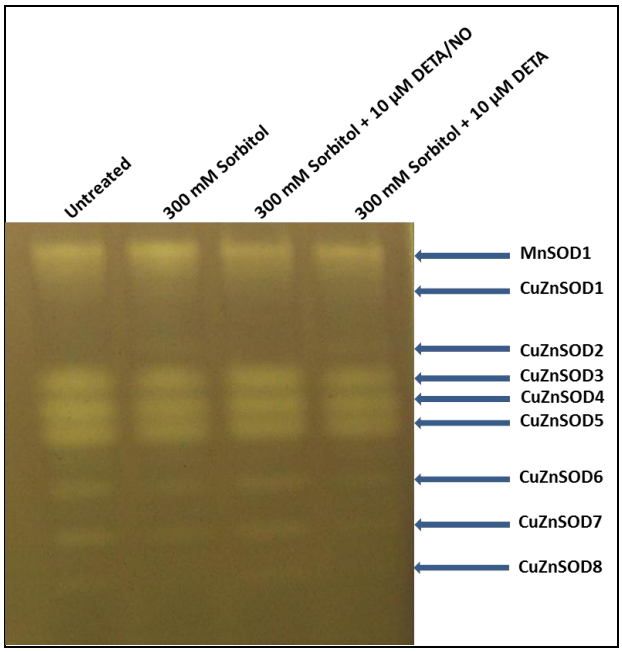
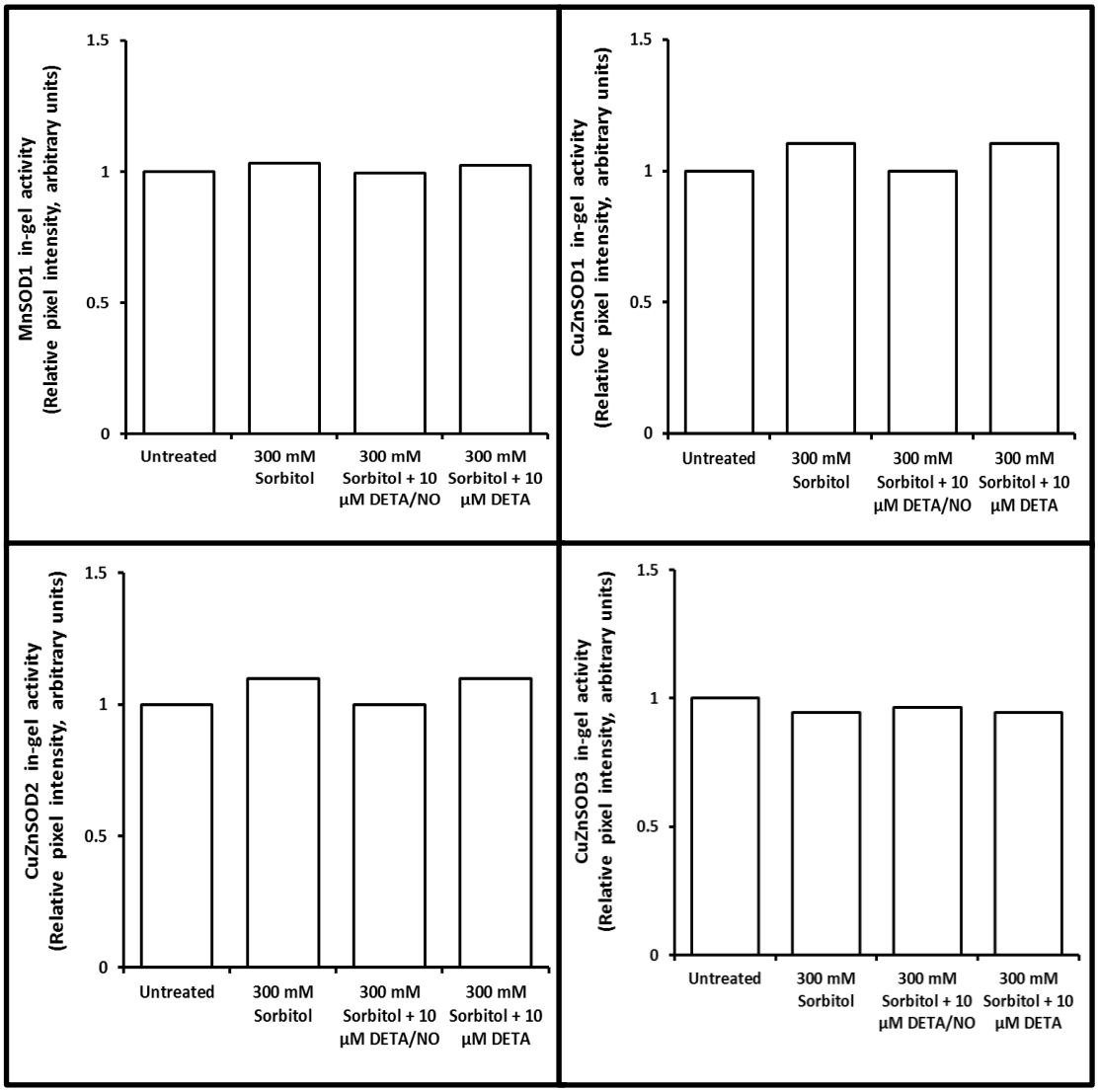


Figure 3.4.3.1: The effects of short-term osmotic stress and exogenous NO on SOD activity of soybean roots. Equal amounts of the root protein extracts (70 µg) were separated by 10 % native PAGE. From the left, lane 1: untreated control; lane 2: 300 mM sorbitol; lane 3: 300 mM sorbitol + 10 µM DETA/NO and lane 4: 300 mM sorbitol + 10 µM DETA. The gel was stained for SOD activity as described by Beauchamp and Fridovich (1971).

For the short-term osmotic stressed roots, nine SOD isoforms were detected. Only one from these isoforms was identified as MnSOD (i.e. MnSOD1) based on the fact that it was resistant to both KCN and H_2O_2. All the other isozymes that were observed on the gel were sensitive to both inhibitors and that led to identify them as CuZnSOD (i.e. CuZnSOD1-CuZnSOD8) (Figure 3.4.3.1). The roots had fewer isoforms compared to the leaves. Different activity responses occurred in various treatments and they were analysed using the relative pixel intensities.



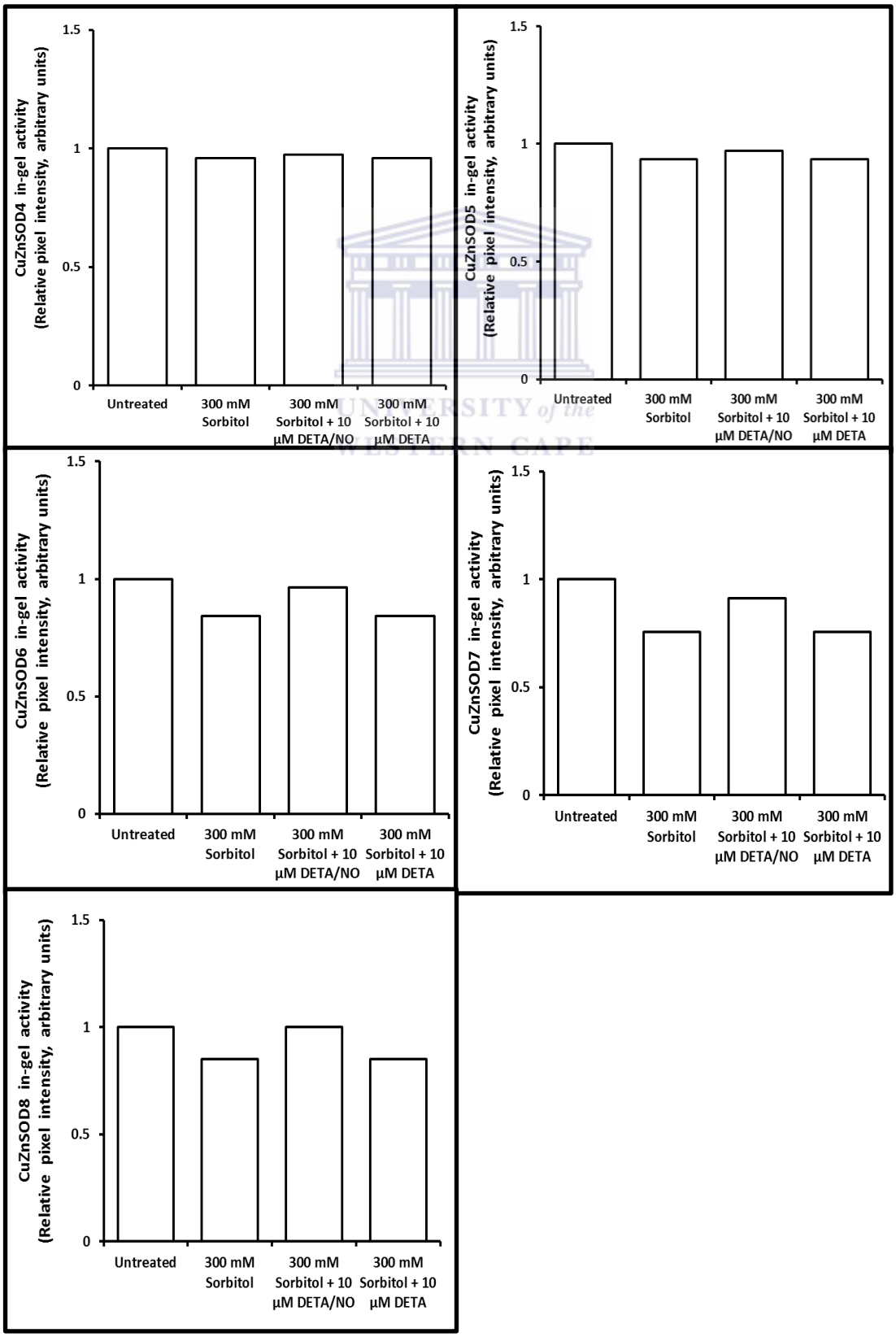


Figure 3.4.3.2: Root SOD isoform activities in response to short-term osmotic stress and exogenous NO, determined by Integrated Density Values with the intensity ratio of a SOD isoform relative to its specific untreated SOD isoform. The relative pixel intensity values are determined using the Alpha Ease FC software and the SOD activities are expressed as arbitrary units.

According to the relative pixel intensities (Figure 3.4.3.2), MnSOD1 showed an increased activity under osmotic stress and NO decreased the activity of this isoform to levels lower than the untreated roots. An increased activity during osmotic stress was observed for CuZnSOD1 and CuZnSOD2, whereas exogenous NO decreased the activities of these isoforms to intensities equal with untreated controls. The activities of CuZnSOD3-CuZnSOD8 isoforms were all reduced by sorbitol and sorbitol + DETA treatments but when NO was added the activity of these isoforms increased.

3.4.4 Leaf SOD activity in response to long-term osmotic stress and exogenous NO

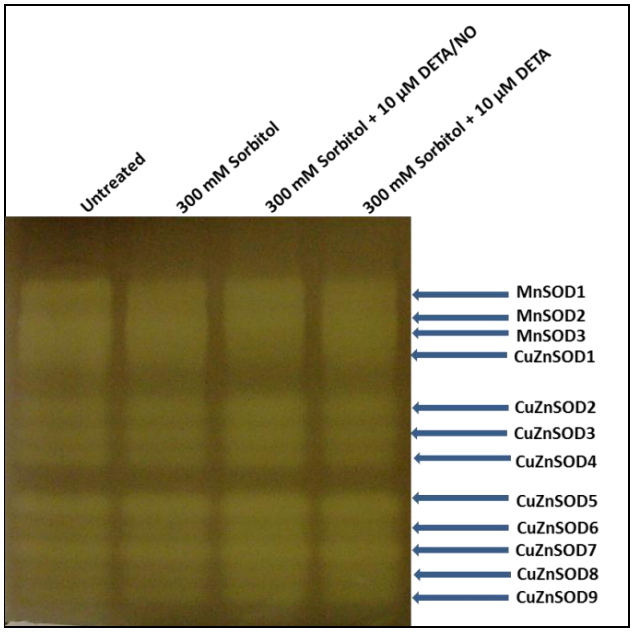
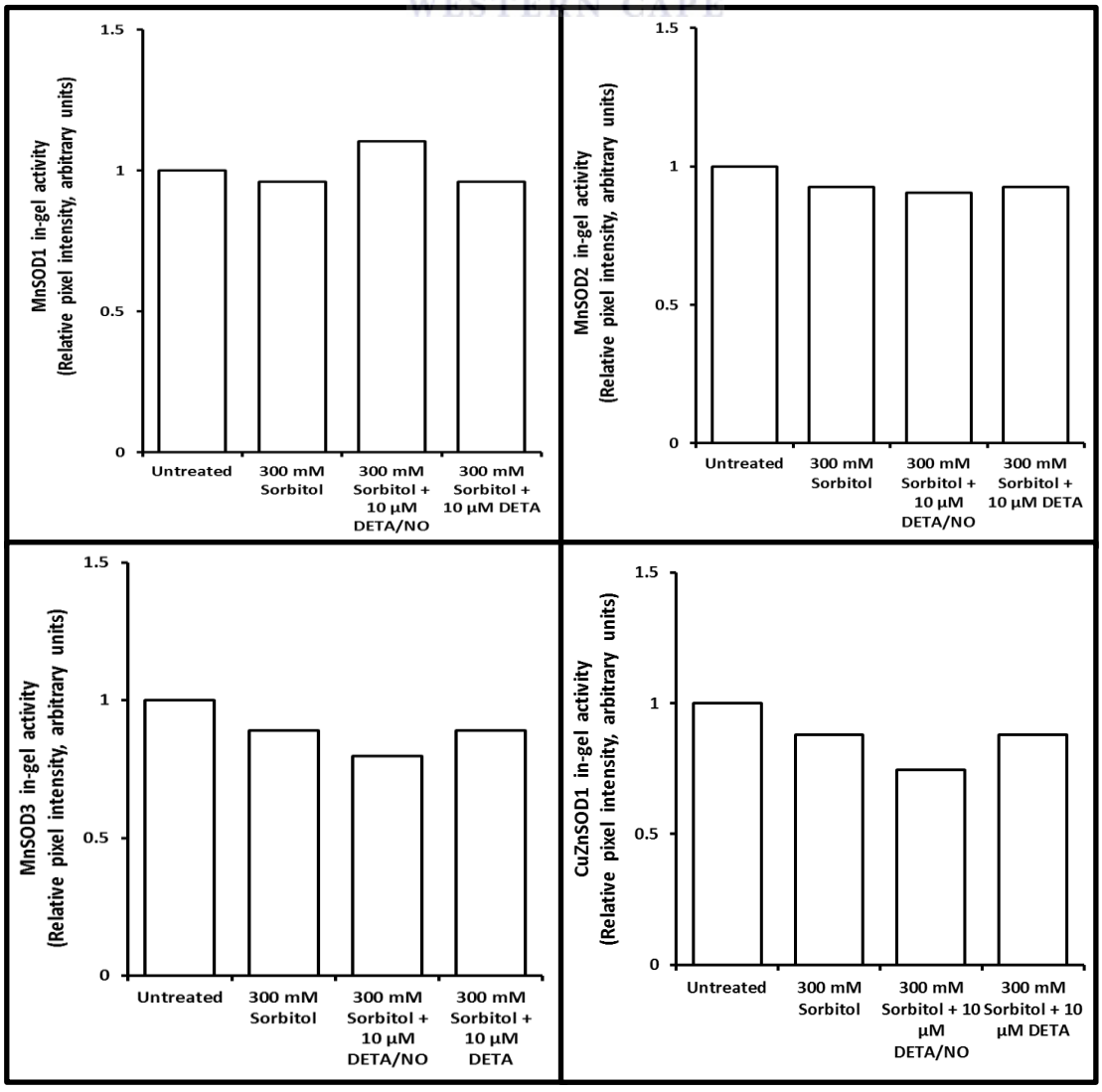
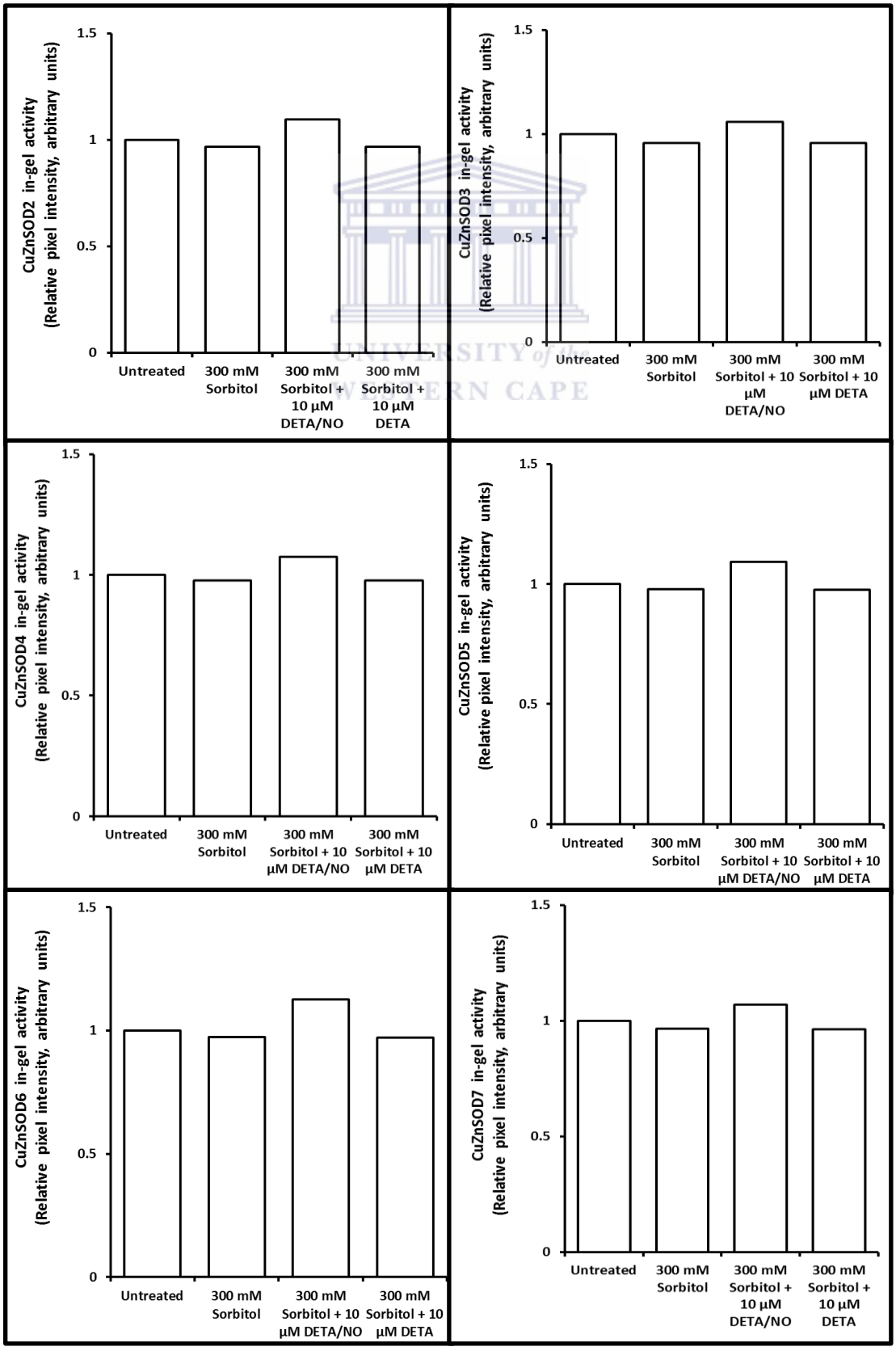


Figure 3.4.4.1: The effects of long-term osmotic stress and exogenous NO on SOD activity of soybean leaves. Equal amounts of the leaf protein extracts (50 µg) were separated by 10 % native PAGE. From the left, lane 1: untreated control; lane 2: 300 mM sorbitol; lane 3: 300 mM sorbitol + 10 µM DETA/NO and lane 4: 300 mM sorbitol + 10 µM DETA. The gel was stained for SOD activity as described by Beauchamp and Fridovich (1971).

The SOD in-gel activity for the long-term osmotic stressed leaves revealed the presence of 12 SOD isoforms. Based on the inhibition assay, these leaves expressed 3 MnSOD isoforms (i.e. MnSOD1, MnSOD2 and MnSOD3) as they were resistant to both KCN and H_2O_2. The other isoforms detected in these leaves were sensitive to both the inhibitors and for that they were identified as CuZnSOD (i.e. CuZnSOD1-CuZnSOD9) (Figure 3.4.4.1).





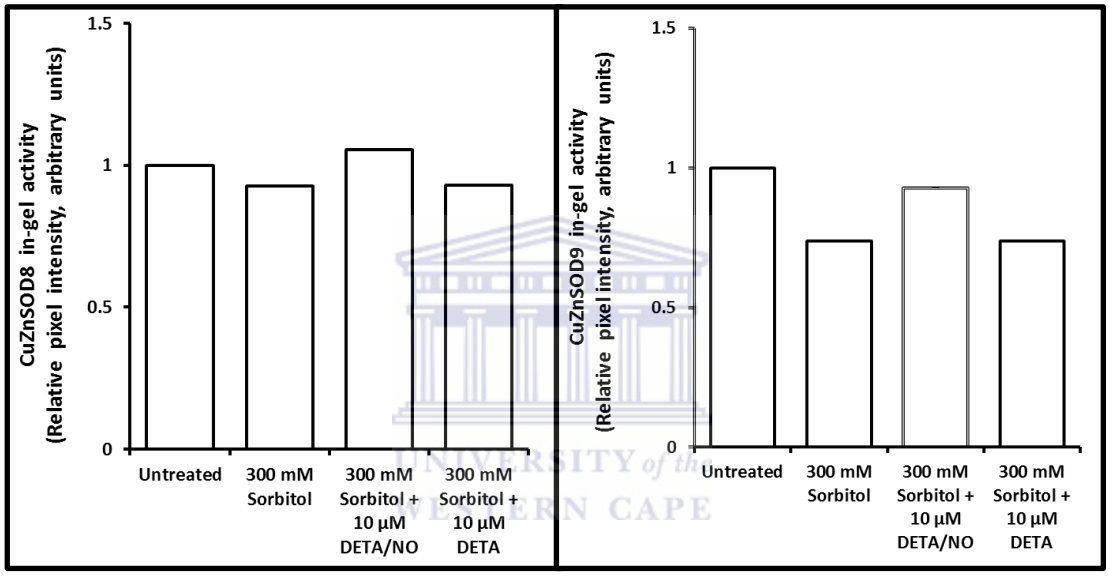


Figure 3.4.4.2: Leaf SOD isoform activities in response to long-term osmotic stress and exogenous NO, determined by Integrated Density Values with the intensity ratio of a SOD isoform relative to its specific untreated SOD isoform. The relative pixel intensity values are determined using the Alpha Ease FC software and the SOD activities are expressed as arbitrary units.

The relative pixel intensities for the long-term osmotic stressed leaves shows that, sorbitol treatments lowered the activities of all the SOD isoforms when compared with the untreated leaves. Similar effects were observed in sorbitol + DETA treatments. However in response to treatments with exogenous NO combined with sorbitol; the activities of MnSOD2, MnSOD3 and CuZnSOD1 were all decreased. Whereas the activity of all the other isoforms (i.e. MnSOD1 and CuZnSOD2-CuZnSOD9) was highly induced by NO treatments to levels higher than the untreated control, except for CuZnSOD9 (Figure 3.4.4.2).

3.4.5 Root SOD activity in response to long-term osmotic stress and exogenous NO

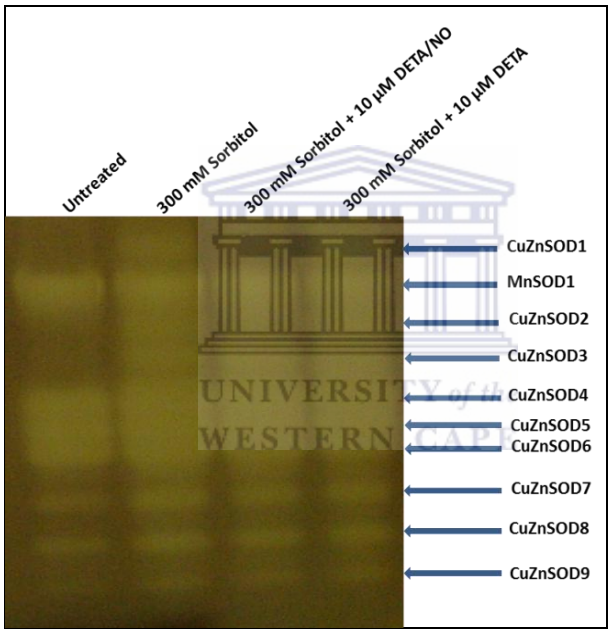
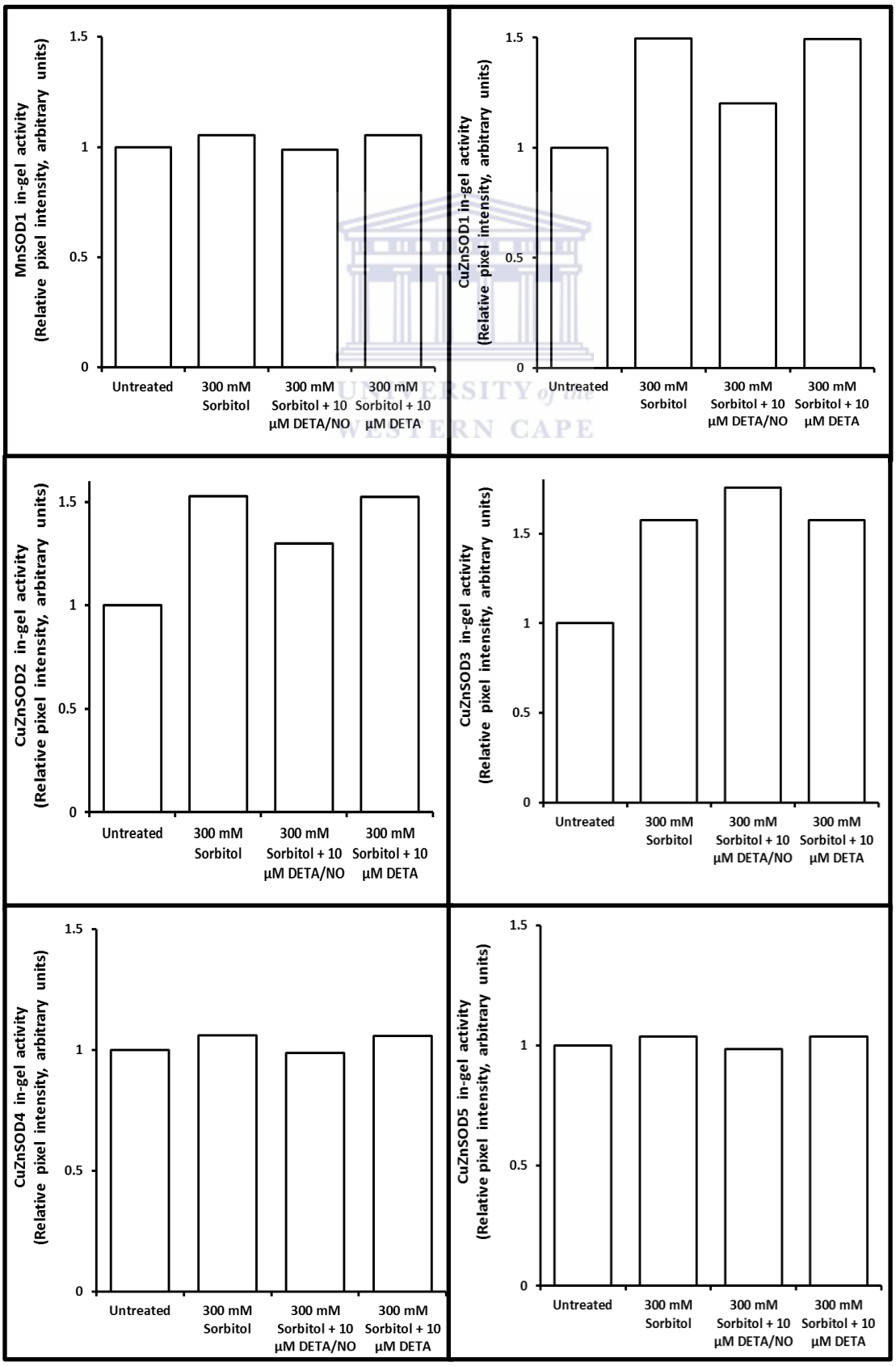


Figure 3.4.5.1: The effects of long-term osmotic stress and exogenous NO on SOD activity of soybean roots. Equal amounts of the root protein extracts (70 µg) were separated by 10 % native PAGE. From the left, lane 1: untreated control; lane 2: 300 mM sorbitol; lane 3: 300 mM sorbitol + 10 µM DETA/NO and lane 4: 300 mM sorbitol + 10 µM DETA. The gel was stained for SOD activity as described by Beauchamp and Fridovich (1971).

For the long-term osmotic stressed roots, the SOD in-gel activity showed that the roots expressed ten SOD isoforms. One belonged to the MnSOD family (i.e. MnSOD1) because of its sensitivity to both KCN and H_2O_2 inhibitors. The rest of the bands detected were sensitive to both inhibitors and they were identified as CuZnSOD isoforms (i.e. CuZnSOD1-CuZnSOD9) (Figure 3.4.5.1). An additional isoform (i.e. CuZnSOD1) was detected in the roots that were exposed to long-term osmotic stress and it was absent in short-term osmotic stressed roots. The SOD in-gel results also revealed that the long-term osmotic stressed plants had increased SOD expression levels compared to the short-term treated plants.



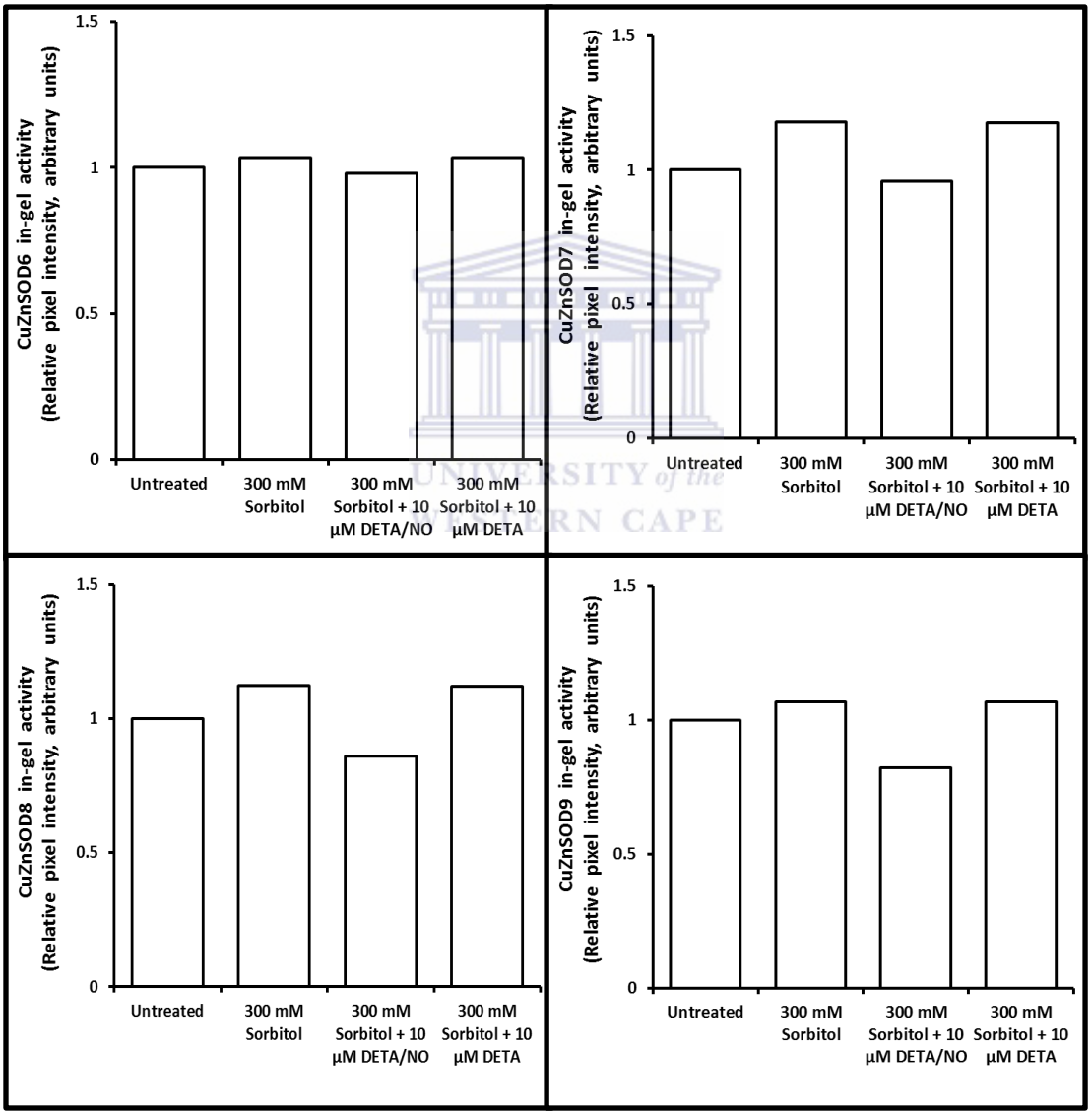


Figure 3.4.5.2: Root SOD isoform activities in response to long-term osmotic stress and exogenous NO, determined by Integrated Density Values with the intensity ratio of a SOD isoform relative to its specific untreated SOD isoform. The relative pixel intensity values are determined using the Alpha Ease FC software and the SOD activities are expressed as arbitrary units.

Based on the densitometry analysis (Figure 3.4.5.2), osmotic stress increased the relative intensity of all the SOD isoforms when compared to untreated roots, but in response to NO treatments the activity of these isoforms was reduced to levels lower than the untreated except for the CuZnSOD1 and CuZnSOD2 isoforms (their activities were reduced when compared to sorbitol treatments but not lower than the untreated control plants). CuZnSOD3

is the only isoform that had its activity increased in response to exogenous NO when compared to sorbitol treatments.

3.4.6 Measurement of lipid peroxidation levels

The SOD enzymatic activity is known to scavenge the superoxide radical content, which is the source of oxidative damage in plants. Lipid peroxidation levels in response to osmotic stress (sorbitol treatment) and exogenous NO (i.e. through DETA/NO) were analysed in soybean leaves and roots that were exposed to short-term and long-term osmotic stress to determine if the increased SOD activity of specific isoforms in response to NO can reduce oxidative damage and to also determine the relationship between SOD activity and lipid peroxidation.

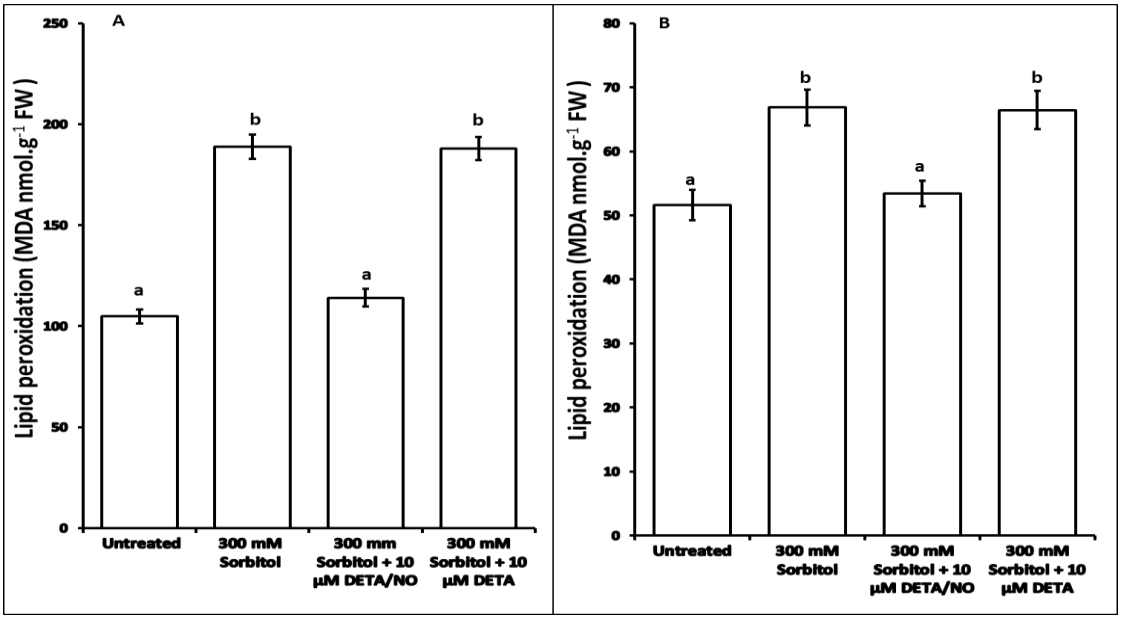


Figure 3.4.6.1: The effects of NO and short-term osmotic stress response on lipid peroxidation levels in soybean leaves (A) and roots (B). The assay was performed on soybean leaves and roots that were treated at V3 stage for a period of one day. Data shown are the means (±SE) of three independent experiments, different letters indicate mean values that are significantly different at $p<0.05$ using Tukey-Kramer test.

In the leaves (Figure 3.4.6.1, A), the levels of lipid peroxidation increased by approximately 80.2% in response to treatments with 300 mM sorbitol and 300 mM sorbitol combined with 10 µM DETA, when comparing to untreated leaves. The effects were reversed by additional DETA/NO in sorbitol treated leaves; the MDA levels were reduced to be equivalent to untreated leaves. The roots treated with 300 mM sorbitol experienced lipid peroxidation at levels ± 29.5% higher than the untreated roots (Figure 3.4.6.1, B). Exogenous NO (i.e. 10 µM DETA/NO combined with 300 mM sorbitol) decreased lipid peroxidation to levels equal to untreated roots. The NO control (i.e. 10 µM DETA combined with 300 mM sorbitol), did not augment the effects caused by 300 mM sorbitol on lipid peroxidation in the roots.

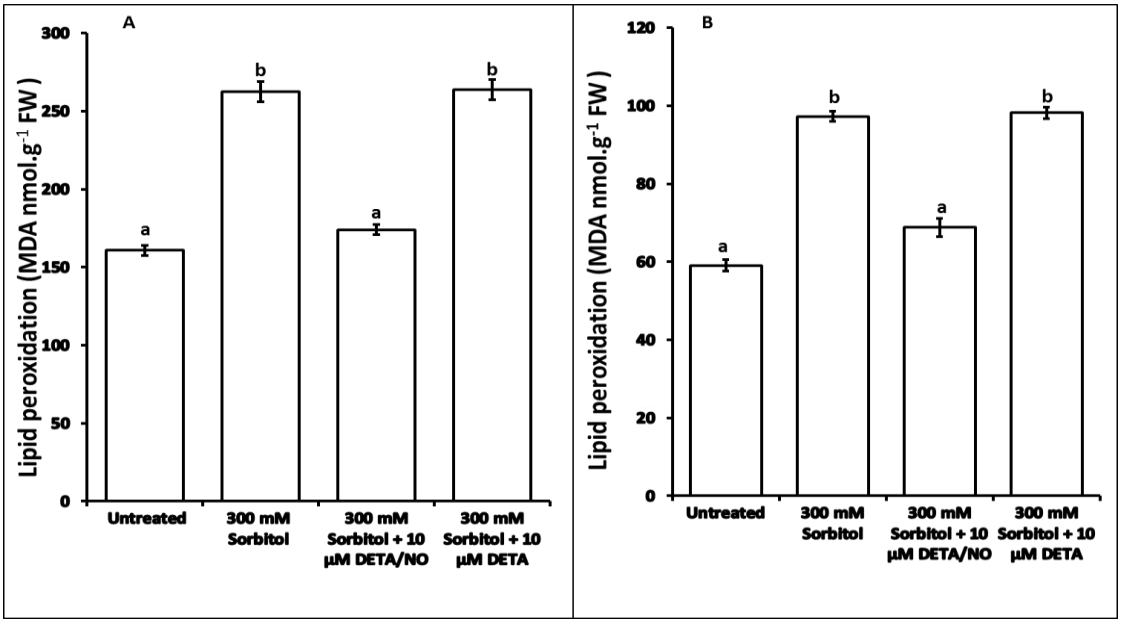


Figure 3.4.6.2: The effects of NO and long-term osmotic stress response on lipid peroxidation levels in soybean leaves (A) and roots (B). The assay was performed on soybean leaves and roots that were treated at V3 stage for a period of seven days. Data shown are the means (±SE) of three independent experiments, different letters indicate mean values that are significantly different at $p<0.05$ using Tukey-Kramer test.

In Figure 3.4.6.2 (A), lipid peroxidation increased by approximately 63.2% in leaves treated with 300 mM sorbitol when compared to untreated leaves. A similar trend was observed in leaves treated with 300 mM sorbitol combined with 10 µM DETA. Application of 10 µM DETA/NO in 300 mM sorbitol treated leaves reduced the levels of lipid peroxidation to an extent that it was equal to untreated leaves. In the roots treated with 300 mM sorbitol and also 300 mM sorbitol combined with 10 µM DETA, lipid peroxidation increased by ± 65% compared to untreated roots. Exogenous NO (i.e. 10 µM DETA/NO combined with 300 mM sorbitol) reduced the MDA content to be equivalent to untreated roots (Figure 3.4.6.2, B). The results also suggest that oxidative damage was more pronounced in long-term osmotic stressed plants compared to short-term treated plants as they had high MDA levels and the leaves had high lipid peroxidation levels than the roots.

3.5 Discussion

Plants subjected to stress conditions develop high antioxidant enzymes capacity like superoxide dismutase (SOD) to scavenge ROS and confer stress tolerance (Wang *et al.,* 2008). SOD is the significant enzyme in the detoxification system and scavenges superoxide ($O_2^{\bullet-}$) radicals (Lee and Lee, 2000), producing less harmful H_2O_2 (Tewari *et al.,* 2006). $O_2^{\bullet-}$ is a precursor of toxic and highly reactive oxygen by-products such as hydroxyl radicals and peroxynitrites (Halliwell and Gutteridge, 1999) and thus it's important that plants have sufficient SOD activity to control $O_2^{\bullet-}$ levels. Increased SOD activity enhances oxidative stress tolerance (Asada, 1999) and signaling molecules like nitric oxide (NO) are vital in enhancing SOD activity, this has been reported in several studies whereby NO donors increased SOD activity during osmotic stress (Tan *et al.,* 2008) or waterlogging (Wang et al., 2011).

Previous studies have shown that changes in SOD activity can depend on severity of stress, duration of stress or type of species (Salekjalali *et al.,* 2012). Hence the data presented in this chapter is obtained from investigating the effects of exogenous NO on SOD enzymatic activity in soybean leaves and roots during short-term and long-term osmotic stress. The in-gel activity assay was used to detect soybean specific SOD isoforms and their relative activity. This is an excellent assay for analysing relative changes in SOD activity (Janknegt *et al.,* 2007). Studying the regulation of individual SOD isoforms gives insight knowledge of how specific SOD isoforms contribute to total SOD activity during stress (Jithesh *et al.,* 2006).

The in-gel assays showed that only MnSOD and CuZnSOD activities were detectable and FeSOD activity could not be detected in soybean. Similar outcomes were observed in two soybean leaf cultivars subjected to ozone stress (Chernikova *et al.,* 2000) and in creeping bentgrass roots exposed to waterlogging stress (Wang and Jiang, 2007). MnSOD was the least mobile SOD isoform with low electrophoretic mobility while CuZnSOD had the highest mobility and was abundant, comprising the majority of the total SOD activity. These findings are in agreement with those obtained by Eyidoğan *et al.,* (2003).

The leaves induced more SOD isoforms than roots, meaning that oxidative stress occurred more in the leaves hence additional protection was required by the leaves. Long-term osmotic stressed plants had increased SOD expression levels compared to short-term treated plants. This is because sufficient SOD activity is required to scavenge highly accumulated $O_2^{\bullet-}$ levels during long-term stress. Whereas during short-term stress, a balance was maintained between ROS production and ROS scavenging.

3.5.1 The effects of exogenous NO on SOD activity during short-term osmotic stress

The leaves induced three MnSOD and nine CuZnSOD isoforms, (Figure 3.4.2.1). No isoform was newly expressed or inhibited in response to exogenous NO and short-term osmotic stress. Densitometry analysis indicates that the activity of each isoform responded differently to various treatments. The total activity of leaf SOD isoforms (i.e. MnSOD1 - MnSOD3 and CuZnSOD1, 2, 3, 6 and 8) was sensitive to osmotic stress, even though some isoforms (i.e. CuZnSOD4, 5, 7 and 9) were highly induced by osmotic stress (Figure 3.4.2.2). Differential responses of SOD isoforms have been observed in various plant species during different stress conditions (Jamal *et al.*, 2006).

One MnSOD and eight CuZnSOD isoforms were expressed by roots. Two isoforms (i.e. CuZnSOD1 and CuZnSOD2) were only induced during osmotic stress and NO inhibited the activity of these isoenzymes (Figure 3.4.3.1). In a recent study, a SOD isoform (i.e. SOD3) was only detected during drought and absent in normal conditions in barley leaves (Salekjalali *et al.*, 2012). Relative to untreated roots, total activity of the SOD isoforms (i.e. CuZnSOD3-CuZnSOD8) decreased during osmotic stress, although few isoforms were relatively increased (i.e. MnSOD1 and CuZnSOD1-CuZnSOD2) (Figure 3.4.3.2).

Treatment with 10 µM DETA/NO during osmotic stress, up-regulates SOD activity (except few isoforms that were inhibited). The increased SOD activity will reduce the $O_2^{\bullet-}$ content, improving osmotic stress tolerance. Short-term osmotic stress leads to differential regulation in SOD isoform activity, similar effects were observed by Wang *et al.*, (2004) during salt and osmotic stress. It is not known why SOD isoforms were differentially regulated but it can be assumed that during osmotic stress, the down-regulation of specific SOD isoforms is compensated by the up-regulation of other specific SOD isoforms (Zhang *et al.*, 2005).

3.5.2 The effects of exogenous NO on SOD activity during long-term osmotic stress

The leaves expressed three MnSOD and nine CuZnSOD isoforms. No records of novel isoforms were induced during various treatments (Figure 3.4.4.1). Compared with untreated leaves, osmotic stress decreased the activity of all the SOD isoforms. It is possible in this case that the $O_2^{\bullet-}$ content might have exceeded SOD scavenging capacity thus allowing the leaves to be sensitive to long-term osmotic stress. A decrease in SOD activity was reported by Panda and Khan (2004) during water stress in *Hydrilla verticillata* L. Nevertheless, differential regulation in SOD activity occurred in response to exogenous NO. Although the majority of SOD isoforms (i.e. MnSOD1 and CuZnSOD2-CuZnSOD9) had increased activity, the activity of some isoforms (i.e. MnSOD2-MnSOD3 and CuZnSOD1) was reduced by NO treatments (Figure 3.4.4.2).

The roots expressed one MnSOD and nine CuZnSOD isoforms. Three CuZnSOD isoforms (i.e. CuZnSOD1-CuZnSOD3), were highly induced during osmotic stress and down-regulated in the untreated roots. CuZnSOD1 is only specific to long-term osmotic stressed roots as it was absent in short-term treated roots (Figure 3.4.5.1). Based on the pixel graphs (Figure 3.4.5.2), all the SOD isoforms were increased by osmotic stress. This shows that plants can improve stress tolerance by increasing their antioxidant capacity. Increased activity of specific SOD isoforms has been reported by Lee and Lee (2000) during chilling stress, Abedi and Pakniyat (2010) during drought stress and Parida *et al.,* (2004) during salinity. All the SOD isoforms showed decreased activity during NO treatments except for CuZnSOD3 which displayed even greater activity in response to NO, suggesting its crucial role in osmotic stress tolerance.

Differential regulation in SOD activity occurred in response to exogenous NO. Some of the isoforms had increased activity under NO treatments, NO as a signaling molecule induces the

activation of antioxidant enzymes like SOD and promote the scavenging of $O_2^{\bullet-}$ to H_2O_2 (Hasanuzzman *et al.*, 2010). The role that exogenous NO has in increasing SOD activity has been described by Shi *et al.*, (2007) during salt stress and by Esim *et al.*, (2012) during chilling stress. However, most of the SOD isoform activities were reduced in response to NO, this could have occurred because they were not responsive to NO.

3.5.3 Measurement of lipid peroxidation levels

Lipid peroxidation was measured to determine how osmotic stress and exogenous NO affect membrane oxidative damage and also investigate the relationship between SOD activity and oxidative stress. The results obtained show that treatment with 300 mM sorbitol damaged cellular membranes as this is reflected by increased lipid peroxidation levels (Figure 3.4.6.1 and Figure 3.4.6.2). High MDA levels occur due to increased ROS production such as $O_2^{\bullet-}$ during osmotic stress (Tan *et al.*, 2008). High lipid peroxidation levels during osmotic stress could suggest that the $O_2^{\bullet-}$ production exceeded SOD scavenging capacity, even though some of the SOD isoforms were up-regulated during osmotic stress.

Detoxification of excess ROS produced during osmotic stress is important to reduce ROS-induced membrane lipid peroxidation (Mittler, 2002) and the induction of SOD is vital for $O_2^{\bullet-}$ detoxification. A 10 µM DETA/NO treatment during osmotic stress was able to protect soybean from oxidative damage, indicated by reduced MDA levels (Figure 3.4.6.1 and Figure 3.4.6.2). Increased SOD activity is often accompanied with an increase of H_2O_2 scavenging enzymes like APX, CAT or GPX (Koca *et al.*, 2006) and this mechanism can enhance oxidative stress tolerance. The role of NO in preventing oxidative damage by lowering MDA contents has been reported by Zhao *et al.*, (2008) during drought and by Xu *et al.*, (2010) during high light stress.

Oxidative stress is dependent on stress duration and the type of tissue. The roots and short-term treated plants had low MDA contents compared to the leaves and long-term treated plants. Over-production of ROS in the chloroplast and peroxisomes is suggested to be the major contributor to lipid peroxidation in the leaves during osmotic and salt stress (Foyer and Noctor, 2003). The low MDA levels suggest that the roots and short-term treated plants have better protection against oxidative damage and also signify osmotic stress tolerance.

In summary, differential regulation in SOD activity was induced during osmotic stress and exogenous NO. Some of the SOD isoforms were up-regulated and others were down-regulated in response to the treatments. Majority of the total SOD activity decreased during osmotic stress, suggesting its insignificant role in osmotic stress tolerance. Even the increased SOD activity during osmotic stress was not completely sufficient to prevent oxidative damage, which is indicated by increased MDA contents. In response to exogenous NO, the overall SOD activity increased although few SOD isoforms were decreased and this led to reduced oxidative damage. This study suggests that NO improves osmotic stress tolerance by increasing SOD activity of specific isoforms which is crucial for $O_2^{\bullet-}$ scavenging, thus preventing oxidative damage (by reducing MDA levels) in soybean leaves and roots. SOD converts $O_2^{\bullet-}$ to H_2O_2, which is another form of ROS and it can be toxic at high levels, therefore rapid scavenging of this oxidant is essential. NO can also increase H_2O_2 scavenging enzymes for osmotic stress tolerance; however this has not been established in this study and still needs to be investigated. Even though the pixel intensities had shown statistically significant differences, these differences may not be sufficient to contribute to a significant physiological effect and so there is a need for a more in-depth investigation on the physiological role of NO on osmotic stress which may be due to other molecular mechanisms distinct from SOD-mediated changes.

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Chapter 4

General conclusion and future work

4.1 General conclusion

Soybean is one of the most important crops with high protein and vegetable oil quantities essential for human feed. The consumption of soybean based food has been reported to increase worldwide (Friedman and Brandon, 2001) because of health beneficial effects. The productivity of this crop is affected during adverse environmental conditions such as abiotic stress. Major abiotic stress (drought, salinity and cold) cause osmotic stress and oxidative stress due to increased ROS production, which subsequently results in oxidative damage and plant cell death (Cruz de Carvalho, 2008). Abiotic stresses pose a threat to crop production and food security. According to Bray *et al.,* (2000), abiotic stresses cause more than 50% reduction in crop yield. The genetic engineering approach has been widely used to enhance crop tolerance to abiotic stresses. This makes a significant contribution towards improving crop yield and helps to attain sustainable food security (Athar and Ashraf, 2009).

In plants, abiotic stress tolerance can be enhanced by regulating the biosynthesis of signaling molecules such nitric oxide (NO). NO protects plants from the cytotoxic effects of abiotic stress by scavenging ROS or blocking oxidative damage mediated by ROS (Bavita *et al.*, 2012). NO can also protect plants from abiotic stress by enhancing plant antioxidant system to detoxify ROS induced by abiotic stress (Neill *et al.,* 2008). Plants possess enzymatic antioxidant mechanisms that regulate and detoxify ROS toxicity (Ashraf, 2009). The role of antioxidant enzymes like superoxide dismutase (SOD) has been elucidated towards increasing stress tolerance in plants during abiotic stress (Sharma *et al.,* 2012).

This study shows that osmotic stress induced cell death in soybean following the corresponding increased superoxide ($O_2^{\bullet-}$) content and lipid peroxidation. High $O_2^{\bullet-}$ levels and lipid peroxides indicate that osmotic stress triggers oxidative stress. This study further implicates the role exogenous NO in increasing soybean cell viability by reducing the $O_2^{\bullet-}$ content and lipid peroxidation. No toxic effects can be associated with the concentration of the NO donor used in this study i.e. 10 µM DETA/NO, as it was able to ameliorate the toxicity induced by osmotic stress. The increased cell viability would thus mean improved growth and productivity for soybean and therefore it can be concluded that NO increases soybean tolerance to osmotic stress.

In this study, SOD isoforms induced in soybean were characterized in terms of their responses to the various treatments. MnSOD and CuZnSOD seem to be the only isoforms playing a role against $O_2^{\bullet-}$ defence as FeSOD could not be detected. CuZnSOD is the most abundant isoform, suggesting that it may have essential roles for $O_2^{\bullet-}$ scavenging in soybean compartments such as the chloroplast, cytosol and peroxisomes (Alscher *et al.,* 2002). The presence of MnSOD suggests a role in protecting the mitochondria and peroxisomes against $O_2^{\bullet-}$ induced damage (Alscher *et al.,* 2002). It has also been established in this study that there are specific SOD isoforms only responsive to osmotic stress, for that they are assumed to be biomarkers for osmotic stress. A novel CuZnSOD1 isoform was only detected in long-term osmotic-stressed roots and it was inhibited by exogenous NO; its presence could suggest that the duration of stress exposure plays an important role for antioxidant enzyme responses.

This study also demonstrated that long-term stress exposure causes more sensitivity to osmotic stress in soybean (due to higher lipid peroxidation levels) than short-term stress. The most important aspect established in this study is the role played by exogenous NO in regulating SOD activity in response to osmotic stress. NO increased SOD activity during

osmotic stress, however a few SOD isoform activities decreased in response to exogenous NO. The increased SOD activity in response to NO can be correlated with reduced oxidative damage (shown by low lipid peroxidation levels during NO treatments).

From the outcomes obtained in this study, it can be suggested that exogenous NO improves osmotic stress tolerance in soybean by regulating and increasing the activity of specific SOD isoforms. The increased SOD activity maintains redox homeostasis by detoxifying $O_2^{\bullet-}$ to H_2O_2; thereby reducing oxidative damage and plant cell death (Figure 4.1). However, H_2O_2 scavenging enzymes that can be regulated downstream by NO cannot be ruled out in playing a role towards improving osmotic stress tolerance in soybean since that was not analysed in this study.

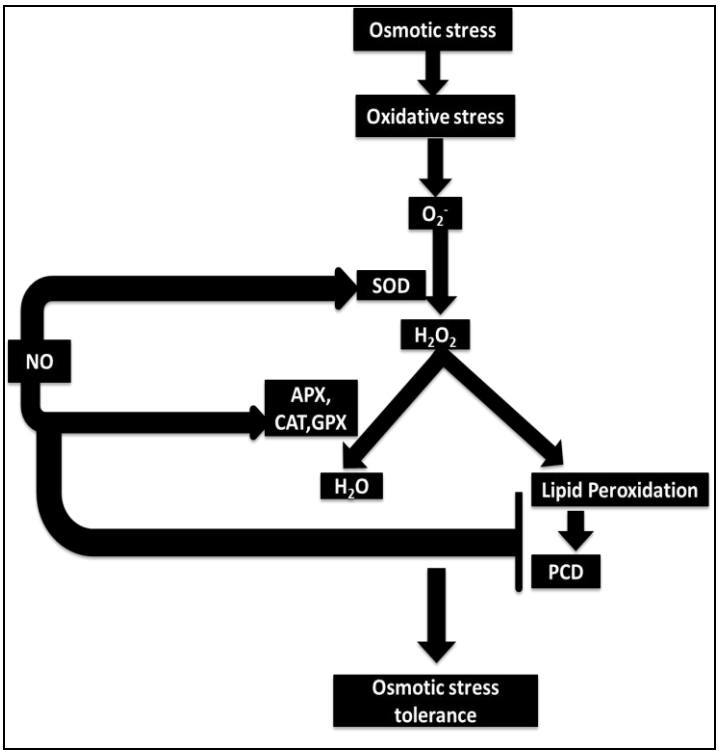

Figure 4.1: Illustration of NO mechanism in mediating SOD and other antioxidant enzymes to enhance osmotic stress tolerance by reducing ROS-induced oxidative stress and cell death.

4.2 Future work

This study characterised soybean SOD isoforms and therefore it would be useful to localise these isoforms in their subcellular compartments, as this would help to generate an understanding of how osmotic stress affects the different subcellular compartments. In this study it cannot be concluded that the protective outcomes of exogenous NO are solely due to the up-regulation of the specific SOD isoform activities, therefore other potential mechanisms by which exogenous NO exerts its protective effects still need to be further investigated and that can include studying other antioxidative enzymatic mechanisms in response to osmotic stress and exogenous NO in soybean using the same experimental approaches used in this study. Since NO mediates changes in SOD in-gel activity during osmotic stress, it would be necessary to identify the genes that code for these SOD isoforms and also analyse the changes in expression of these SOD-encoding genes in response to NO. This will help to establish if the changes in SOD in-gel activity occur at transcript level or not.

However we cannot rule out the possibility that these changes might be post-translational, therefore it would be required to confirm if any of the SOD isoforms are, for example, S-nitrosylated. This is mainly because recent evidence has shown that during stress conditions in plants, most proteins are S-nitrosylated by NO signaling (Lindemayr and Durner 2009). This will aid in understanding whether the changes that occurred in SOD in-gel activity in response to exogenous NO and osmotic stress were due to post-translational modifications or not. Transgenic studies expressing NO-inducible SOD genes can be an approach used to enhance soybean tolerance to osmotic stress. The information to be obtained from this future work can help in improving the genetic engineering of crop plants, and thus increase crop yield and enhance food security.

4.3 References

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