The inhibition of nitric oxide biosynthesis alters anti-oxidant capacity in soybean

Julian André Le Keur

Student Number: 3689777

Email: 3689777@myuwc.ac.za

A thesis submitted in partial fulfilment of the requirements for the degree of *Magister Scientiae* in the Department of Biotechnology, University of the Western Cape.

Supervisor: Dr. Marshall Keyster

Co-supervisor: Prof. Ndiko Ludidi

University of the Western Cape

Private Bag X17, Bellville 7535, South Africa

Telephone: ++27-21- 959 2255/959 2762 *Fax*: ++27-21- 959 1268/2266

Email: jvanbeverdonker@uwc.ac.za

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ACKNOWLEDGEMENTS

It is with immense gratitude that I acknowledge the support of my supervisor **Dr. Marshall Keyster** of the Department of Biotechnology at the University of the Western Cape, whose door stood open whenever I ran into a technical hitch or needed advice about my research or writing. Thank you for your guidance, know-how and constant encouragement.

I am thankful to my co-supervisor **Prof. Ndiko Ludidi** of the Department of Biotechnology at the University of the Western Cape for his expertise, guidance and input as well as the major role he played throughout my research project.

I cannot find words to express my appreciation towards **Arun** and **Anushka Gokul** for their support, guidance and encouragement throughout my M.Sc.

To the **Environmental (EBL) and Plant Biotechnology Laboratory, my academic peers as well as the National Research Foundation (Bursary funding)**; thank you all for the specific roles that you have played throughout my M.Sc. and for the opportunity to successfully complete my Masters dissertation.

I would like to give a special word of thanks to my best friend and confidant, **Clint Mercuur**, for all his love and support during my M.Sc. and always believing in me. Thank you for always looking out for me and for encouraging me every day.

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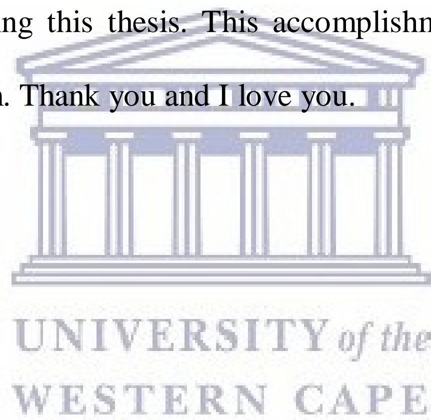
Thank you to my **brother, Nathan Le Keur** for the important role you have played throughout my life, for always having my back and being there for me when I needed it the most.

Finally I dedicate this master's dissertation to **my parents, Kelcy and Keith Le Keur,** who have given me the opportunity of an education from the best schools and academic institutions (The University of Stellenbosch and the University of the Western Cape) as well as their support throughout my life. I must express my very profound appreciation for their unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Thank you and I love you.



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LIST OF ABBREVIATIONS

APX	Ascorbate peroxidase
CAT	Catalase
cPTIO	2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
Cu/ZnSOD	Copper/Zinc SOD
ETC	Electron transport chain
FeSOD	Iron SOD
H_2O_2	Hydrogen peroxide
HR	Hypersensitive response
L-NAME	Nω-Nitro-L-arginine methyl ester hydrochloride
MDA	Malondialdehyde
MnSOD	Manganese SOD
NBT	Nitrotetrazolium Blue chloride
NO	Nitric oxide
NOS	Nitric oxide synthase
$OH^{\bullet}$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
$^1O_2$	Singlet oxygen
O_2^-	Superoxide radical
PAGE	Polyacrylamide gel electrophoresis
PCD	Programmed cell death
ROS	Reactive oxygen species

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RNS	Reactive nitrogen species
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine

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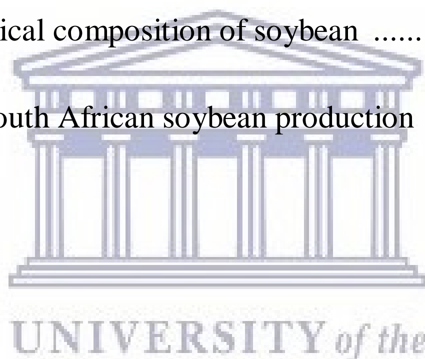
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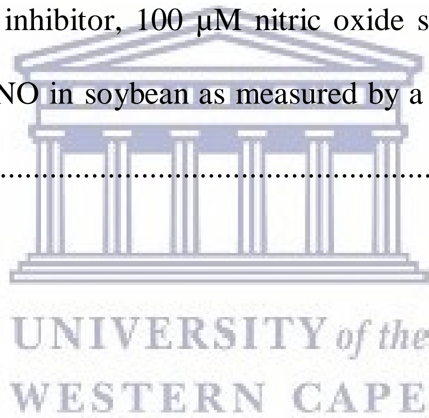
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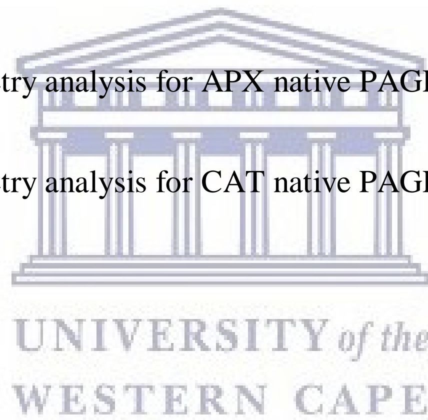

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The inhibition of nitric oxide biosynthesis alters anti-oxidant capacity in soybean

Julian André Le Keur

KEYWORDS

Soybean

Nitrate reductase

Nitric oxide donor

Nitric oxide scavenger

Nitric oxide synthase

Oxidative stress

Reactive oxygen species

Hydrogen peroxide

Lipid peroxidation

Antioxidant enzymes

Ascorbate peroxidase

Superoxide dismutase

Catalase

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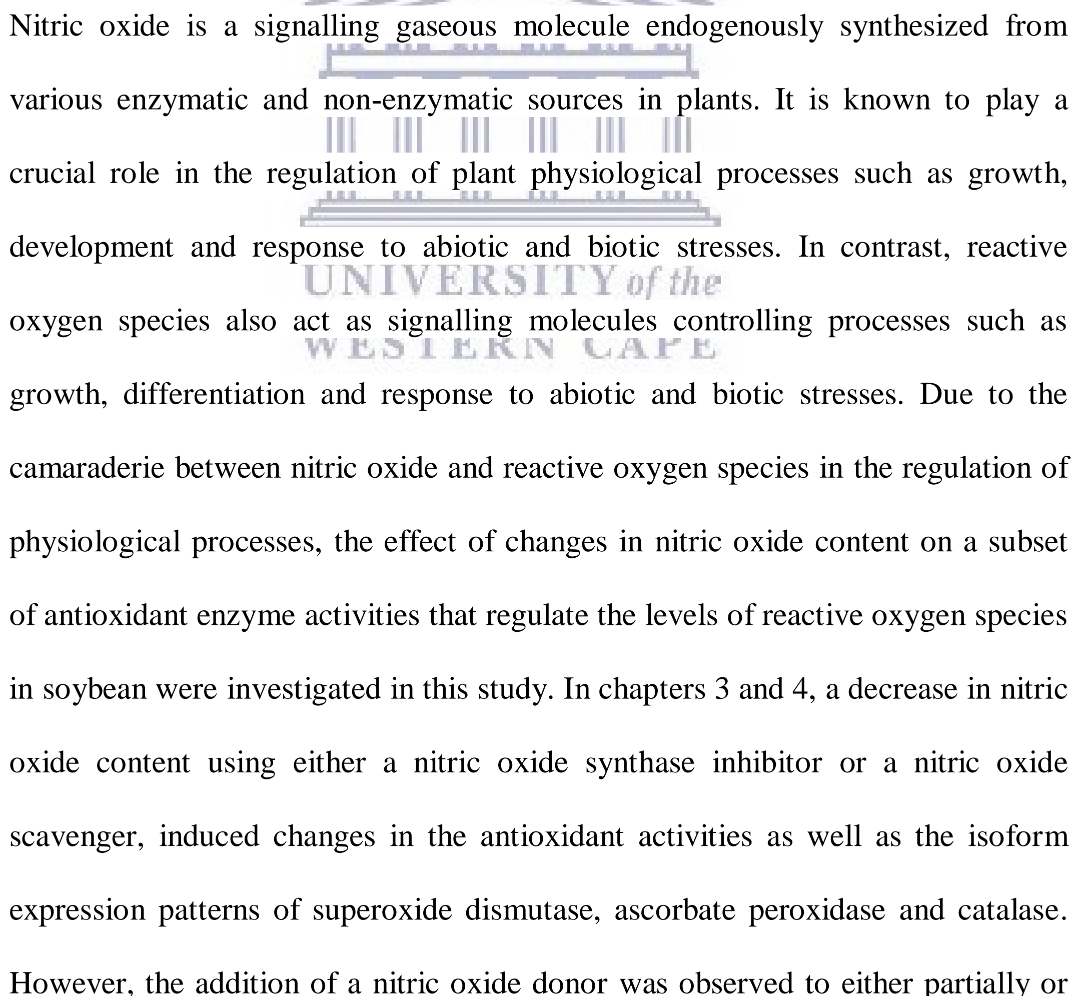
The inhibition of nitric oxide biosynthesis alters anti-oxidant capacity in soybean

Julian André Le Keur

M.Sc. Dissertation, Department of Biotechnology, University of the Western Cape

Abstract


Nitric oxide is a signalling gaseous molecule endogenously synthesized from various enzymatic and non-enzymatic sources in plants. It is known to play a crucial role in the regulation of plant physiological processes such as growth, development and response to abiotic and biotic stresses. In contrast, reactive oxygen species also act as signalling molecules controlling processes such as growth, differentiation and response to abiotic and biotic stresses. Due to the camaraderie between nitric oxide and reactive oxygen species in the regulation of physiological processes, the effect of changes in nitric oxide content on a subset of antioxidant enzyme activities that regulate the levels of reactive oxygen species in soybean were investigated in this study. In chapters 3 and 4, a decrease in nitric oxide content using either a nitric oxide synthase inhibitor or a nitric oxide scavenger, induced changes in the antioxidant activities as well as the isoform expression patterns of superoxide dismutase, ascorbate peroxidase and catalase. However, the addition of a nitric oxide donor was observed to either partially or

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fully reverse the changes in the above mentioned antioxidant activities. Thus, we concluded that endogenously synthesized nitric oxide is a crucial component of plant signalling to induce ROS scavenging pathways. It was thus concluded that NO is a crucial component for redox homeostasis as it plays an essential role in regulating the physiological and biochemical processes in plants by activation of antioxidant enzymes.

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Chapter 1

General Introduction and Literature review

1.1. Introduction

Abiotic stress refers to the impact of non-living factors on an environment, either being beneficial or detrimental depending on the location of the environment (Anjum *et al.*, 2011; Gong, Rao and Yu, 2013). Abiotic stressors include both the chemical and physical components of the environment (Diffen, 2016). These stressors have an effect on the growth, maintenance and reproduction of organisms and examples include water, light, temperature, humidity, salinity and soil pH (Rodziewicz *et al.*, 2013). Lesser-known stressors typically occur somewhat on a smaller scale. These include poor edaphic conditions such as rock content and radiation (Satyanarayana, Johri and Prakash, 2012).

The impact of abiotic factors on the environment differs significantly. For example, the absence of sunlight prevents plants from photosynthesising, which ultimately results in death (Bita and Gerats, 2013). Plants adapt very differently from one another, even from plants that occupy the same area. This was shown in a study by Power (1992), who concluded that a plant species responds to environmental stress signals in their own unique way, even if a different plant species have become accustomed to exactly the same environmental conditions. The first line of defence against abiotic stress is in the plant root system (Brussaard *et al.*, 2007). Any damage to these structures as well as any other part of the plant will result in oxidative stress, which is the accumulation of highly reactive compounds known as reactive oxygen species (ROS). These highly

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reactive compounds are toxic and are known to be harmful to the cellular integrity of the plant if not properly regulated.

1.2. Reactive Oxygen Species

When exposed to an abiotic stress, plants experience oxidative stress. This is in part due to the accumulation of ROS within the plant. Initially, ROS were thought to be the by-products of aerobic metabolism, which is subsequently removed by the plants antioxidant system (Bailey-Serres and Mittler, 2006). Reactive oxygen species play an essential role in the regulation of biological processes such as growth, development, response to environmental stimuli as well as programmed cell death (PCD); (Bailey-Serres and Mittler, 2006). The accumulation of ROS can be detrimental to plants and as a result elicits the induction of various cellular mechanisms. The latter includes lipid peroxidation, protein oxidation and nucleic acid damage which causes the deregulation, over-flow or disruption of electron transport chains (ETCs) within certain organelles and ultimately PCD if not properly regulated (Jack, 2012; Groß, Durner and Gaupels, 2013).

Examples of ROS that are responsible for oxidative damage include the superoxide radical ($O_2^{-\bullet}$), hydroxyl radical (OH^-), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) (Apel and Hirt, 2004). The over production of these ROS leads to the formation of lipid peroxidation by-products such as malondialdehyde (MDA) which is a strong oxidizing compound that can be detrimental to a plant's cellular integrity (figure 1.1); (Groß *et al.*, 2013). Thus, the evolution of plants has allowed for the development of sophisticated means

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for overcoming ROS toxicity (Bailey-Serres and Mittler, 2006; Groß *et al.*, 2013). One mechanism which plants possess to overcome ROS toxicity is the antioxidant system also known as the Foyer-Halliwell-Asada cycle (Groß *et al.*, 2013). However, reactive compounds are not always toxic to plants. When regulated, ROS play a fundamental role in plant intracellular redox signalling (Siddiqui, Al-Whaibi and Basalah, 2011; Jack, 2012). Therefore, a plants' survival is ensured when ROS concentrations remain within that of its "oxidative window" so as not to cause oxidative damage.

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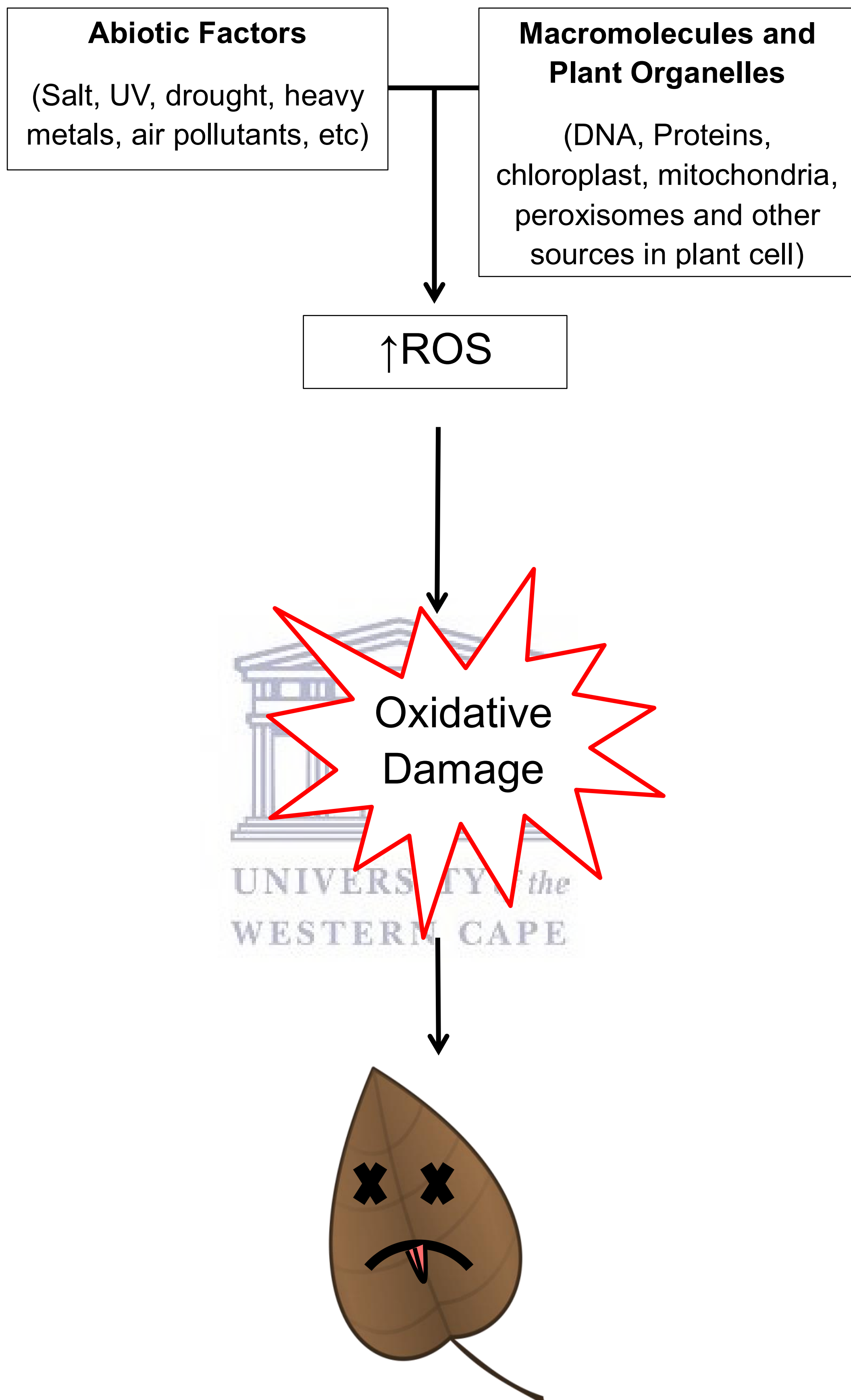

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Figure 1.1. Illustration depicting how abiotic factors ultimately result in oxidative damage in plants. The production of ROS within organelles and due to stressors lead to oxidative damage and cell death (adapted from Gill and Tuteja, 2010).

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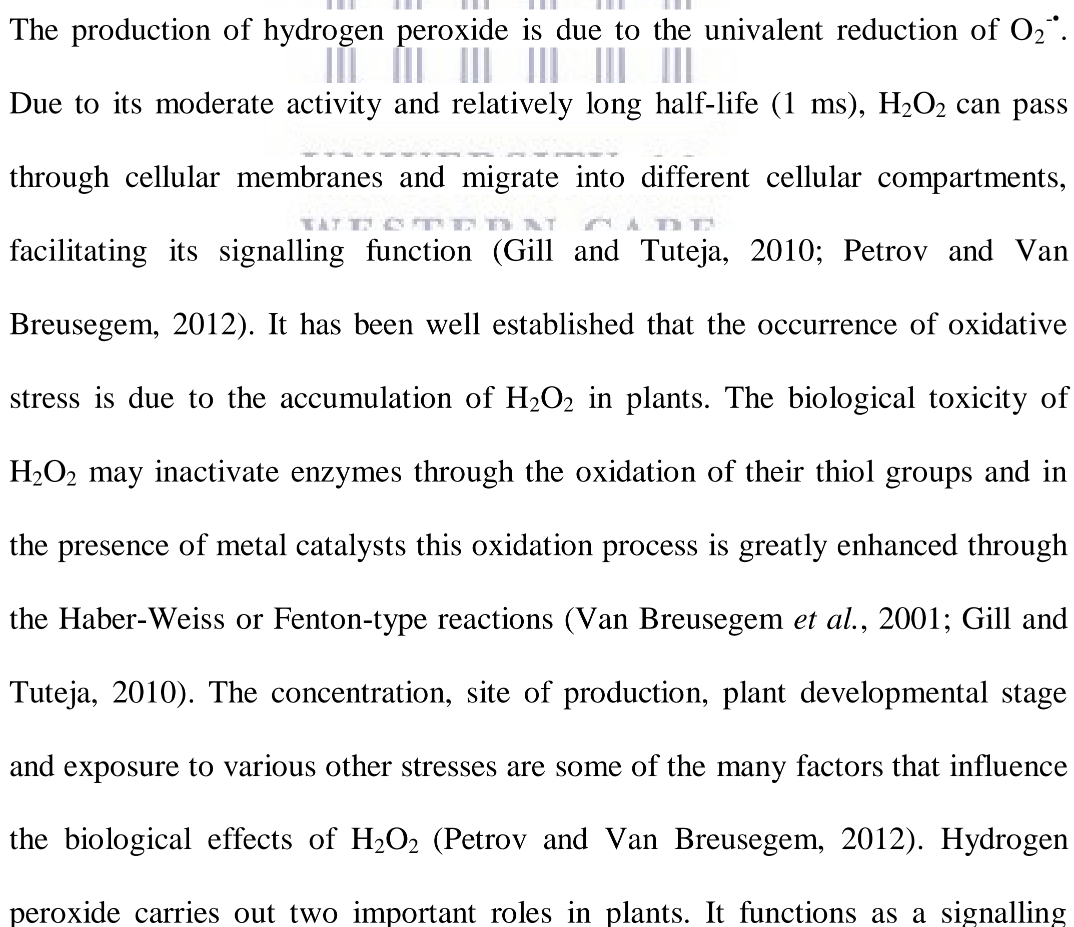
1.2.1. Superoxide radical ($O_2^{-\bullet}$)

The thylakoid membrane of Photosystem I is the main site for superoxide radical production. This radical has a half-life of approximately 2-4 µs and is the first ROS to be produced. Consequently this may trigger the production of other ROS such as the $OH^{\bullet}$ radical, and possibly singlet oxygen, both of which may bring about the peroxidation of membrane lipids and cellular weakening (Gill and Tuteja, 2010).

1.2.2. Hydrogen peroxide (H_2O_2)



The production of hydrogen peroxide is due to the univalent reduction of $O_2^{-\bullet}$. Due to its moderate activity and relatively long half-life (1 ms), H_2O_2 can pass through cellular membranes and migrate into different cellular compartments, facilitating its signalling function (Gill and Tuteja, 2010; Petrov and Van Breusegem, 2012). It has been well established that the occurrence of oxidative stress is due to the accumulation of H_2O_2 in plants. The biological toxicity of H_2O_2 may inactivate enzymes through the oxidation of their thiol groups and in the presence of metal catalysts this oxidation process is greatly enhanced through the Haber-Weiss or Fenton-type reactions (Van Breusegem *et al.*, 2001; Gill and Tuteja, 2010). The concentration, site of production, plant developmental stage and exposure to various other stresses are some of the many factors that influence the biological effects of H_2O_2 (Petrov and Van Breusegem, 2012). Hydrogen peroxide carries out two important roles in plants. It functions as a signalling

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molecule or second messenger at low concentrations, where it is responsible for signalling and eliciting tolerance to various abiotic and biotic stresses. Contrastingly, at high concentrations it induces PCD (Gill and Tuteja, 2010; Petrov and Van Breusegem, 2012). Programmed cell death is important for developmental processes and environmental responses such as leaf senescence and the hypersensitive response to pathogens as well as allelopathic plant–plant interactions (Petrov and Van Breusegem, 2012). Due to the vast impact of H_2O_2 on the normal functioning of a plant cell, its multi-functionality on the one hand and the dangers it poses at high concentrations on the other, necessitate H_2O_2 levels to be strictly regulated and maintained at levels that are not damaging to the plant.

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1.2.3. Hydroxyl radical (OH^-)

Out of all the ROS, the hydroxyl radical is the most reactive due to having a very strong reduction potential and relatively short half-life of 1 µs (Van Breusegem *et al.*, 2001; Gill and Tuteja, 2010). As a result it has a high affinity for macromolecules such as DNA, proteins and lipids at its site of production. In the presence of transition metals such as iron, OH^- is produced by means of the conversion of H_2O_2 and O_2^- through the fenton reaction (Gill and Tuteja, 2010). In vivo, hydroxyl radicals are thought to be the cause of oxygen toxicity and due to the absence of any enzymatic mechanisms for its removal, the accumulation of these radicals ultimately leads to cell death (Vranová *et al.*, 2002).

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1.3. Reactive oxygen species and cell biochemistry

1.3.1. Lipid peroxidation

Lipid peroxidation is the process whereby ROS cause the oxidative deterioration of lipids, which ultimately affects the structure and functioning of cellular membranes. Most often lipid peroxidation affects polyunsaturated fatty acids, because they contain carbon-carbon double bond(s) that are especially reactive with ROS (Vasilaki and McMillan, 2012; Ayala, Muñoz and Argüelles, 2014). As mentioned previously, increased ROS production occurs when a plant undergoes oxidative stress. This disrupts the plants' endogenous antioxidant mechanism subsequently leading to an increase in oxidative damage to membrane structure and function (Gokul, 2013; Ayala *et al.*, 2014). Malondialdehyde (MDA) is an endogenous cytotoxic by-product of lipid peroxidation and is used as a marker for the measurement of lipid peroxidation (Marnett and Hancock, 1999). The implications of lipid peroxidation include the deterioration of cellular membranes which affect the integrity and permeability of a cell and ultimately leads to the leakage of ions (Sinha and Saxena, 2005; Zhang *et al.*, 2006).

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1.3.2 Chlorosis of plant material

Chlorosis is a condition whereby plants produce insufficient amounts of chlorophyll, but can also be attributed to the degradation of chlorophyll. Chlorophyll is an important biomolecule that gives plants their distinct green colour and plays a crucial role in photosynthesis (Hörtensteiner and Kräutler, 2011). When a plant undergoes chlorosis the leaves of the plant take on a pale yellowish colour (Fatoba and Udoh, 2008; Yadav, 2010; Abadía *et al.*, 2011). The affected plant is thus unable to produce the necessary carbohydrates through photosynthesis and may die if the condition is not reversed. Chlorosis can be brought on by a number of factors which include mineral deficiencies in the soil, damaged and/or compacted roots, pathogens or oxidative stress (Koenig and Kuhns, 2010; Gerber *et al.* 2011). As chloroplasts are highly sensitive to oxidative stress, high concentrations of ROS might cause oxidative damage to the chloroplasts, which would result in a decrease in chlorophyll production and ultimately lead to increased chlorosis.

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1.4. Nitric oxide production in plants

Nitric oxide (NO) is a signalling gaseous molecule endogenously synthesized in plants via enzymatic and non-enzymatic processes (Keyster *et al.*, 2014). To date there are two enzymatic pathways responsible for the endogenous synthesis of NO (Wilson, Neill and Hancock, 2008; Corpas *et al.*, 2009; Palavan-Unsal and Arisan, 2009). The first pathway being an L-arginine dependant pathway that is catalysed by nitric oxide synthase (NOS, EC 1.14.23.39) converting L-arginine to L-

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citrulline and NO (Qiao and Fan, 2008; Corpas and Barroso, 2015). In the second pathway the biosynthesis of NO is based on the NADPH-dependant enzyme nitrate reductase (NR, EC 1.6.6.1), which utilizes nitrite/nitrate as a precursor for the production of NO (figure 1.2); (Yamasaki and Sakihama, 2000; Corpas and Barroso, 2015).

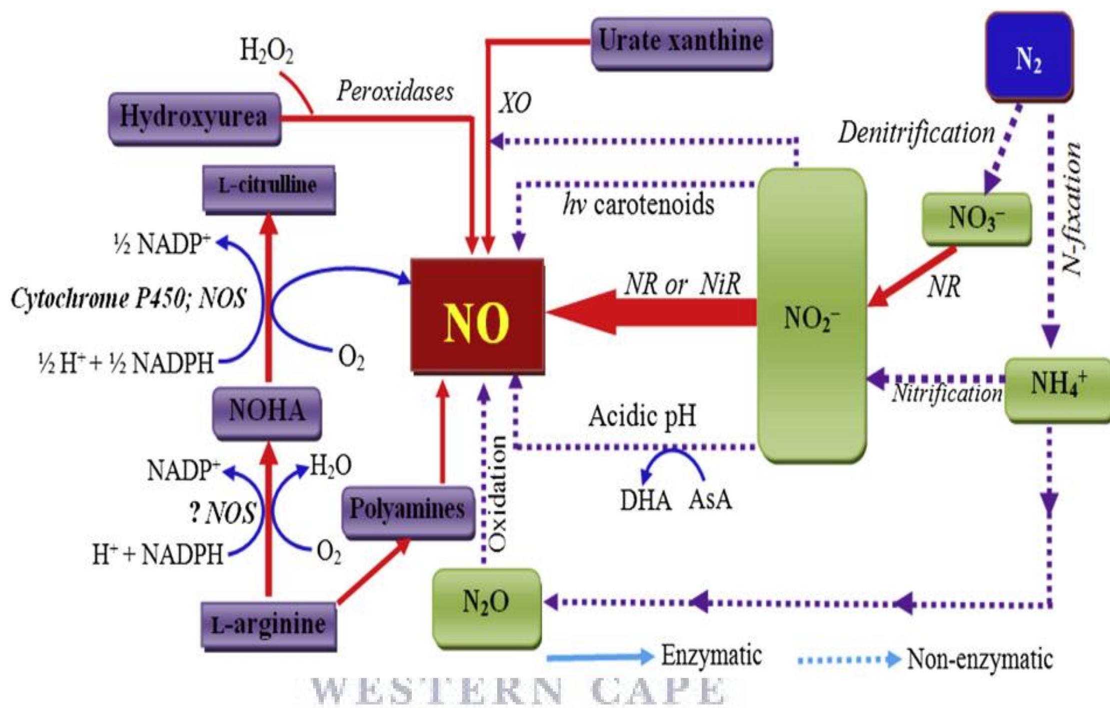

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Figure 1.2. Proposed NO biosynthesis pathways in plants (Gill *et al.,* 2013). AsA, ascorbic acid; DHA, dehydroxyascorbate; H2O2, hydrogen peroxide; NOHA, Nω-hydroxy-L-arginine; NOS, nitric oxide synthase; NADPH, nicotinamide adenine dinucleotide phosphate; NR, nitrate reductase; NR, nitrite reductase; N_2O, nitrous oxide; NO_2, nitrogen dioxide ion; NO_3^-, nitrate ion; NH_4^+, ammonium ion; XO, xanthine oxidase.

As a signalling molecule NO is known to regulate plant growth, development and response to biotic and abiotic stresses (Jack, 2012). It carries out a vital role in maintaining plant redox homeostasis as well as alleviating oxidative stress brought on by the accumulation of ROS (del Río *et al.,* 2003; Palavan-Unsal and

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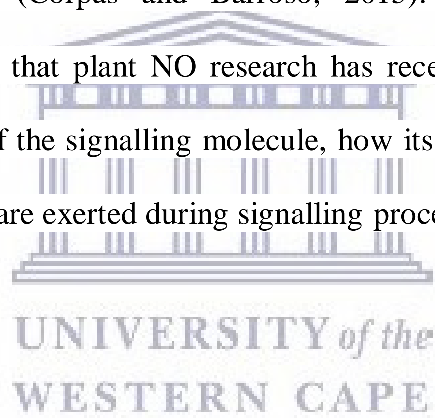
Arisan, 2009; Blokhina and Fagerstedt, 2010). Nitric oxide has a dual role depending on its rate of production. At low concentrations NO functions as a signalling molecule and at high concentrations it functions as a stress molecule (figure 1.3). This is thought to be associated with damage to carbohydrates, lipids, nucleic acids, and proteins caused by processes such as protein nitration (Corpas and Barroso, 2015). Reactive nitrogen species (RNS) are a family of NO-related molecules that give NO its many roles. Peroxynitrite ($ONOO^-$) and S-nitrosothiols (SNOs) are two examples of RNS that result from the reaction of NO with the superoxide radical and the reaction of NO with thiol groups, respectively (Corpas and Barroso, 2015). Regardless of the ever increasing popularity that plant NO research has received, not much is known about the synthesis of the signalling molecule, how its concentration is regulated and where its effects are exerted during signalling processes (Planchet and Kaiser, 2006).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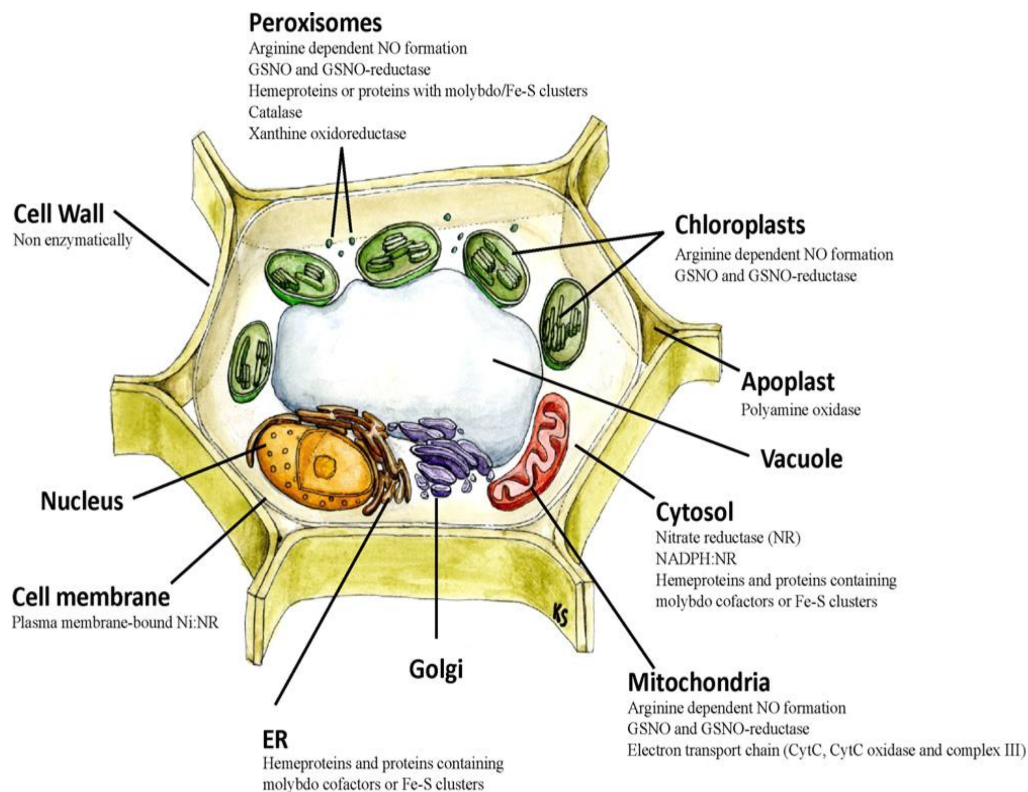

Figure 1.3. Proposed organelles for the site NO production. In some cases the origin of NO has been well studied (e.g. nitrate reductase), whilst in others the origin of NO needs more study (i.e. Arg-dependent NO formation); (Fröhlich and Durner, 2011).

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1.5. Plant antioxidant systems for ROS scavenging

Due to excessive amounts of ROS produced and the destructive nature of these reactive compounds, plants have developed ROS-scavenging mechanisms also known as the antioxidant defence system (figure 1.4); (Jack, 2012; Gokul, 2013). These antioxidant pathways are present in many cellular compartments where ROS are generated (Gill and Tuteja, 2010; Miller *et al.*, 2010). The ROS are then converted into less toxic compounds that are less harmful to the cellular integrity of the plant. Superoxide dismutase (SOD), ascorbate peroxidase (APX) and

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catalase (CAT) are considered to be the most prevalent antioxidant enzymes within plants (Blokhina, Virolainen and Fagerstedt, 2003; Sinha and Saxena, 2006; Lee *et al.*, 2007). The first scavenging antioxidant enzyme is SOD which catalyses the conversion of O_2^- to H_2O_2 and oxygen.

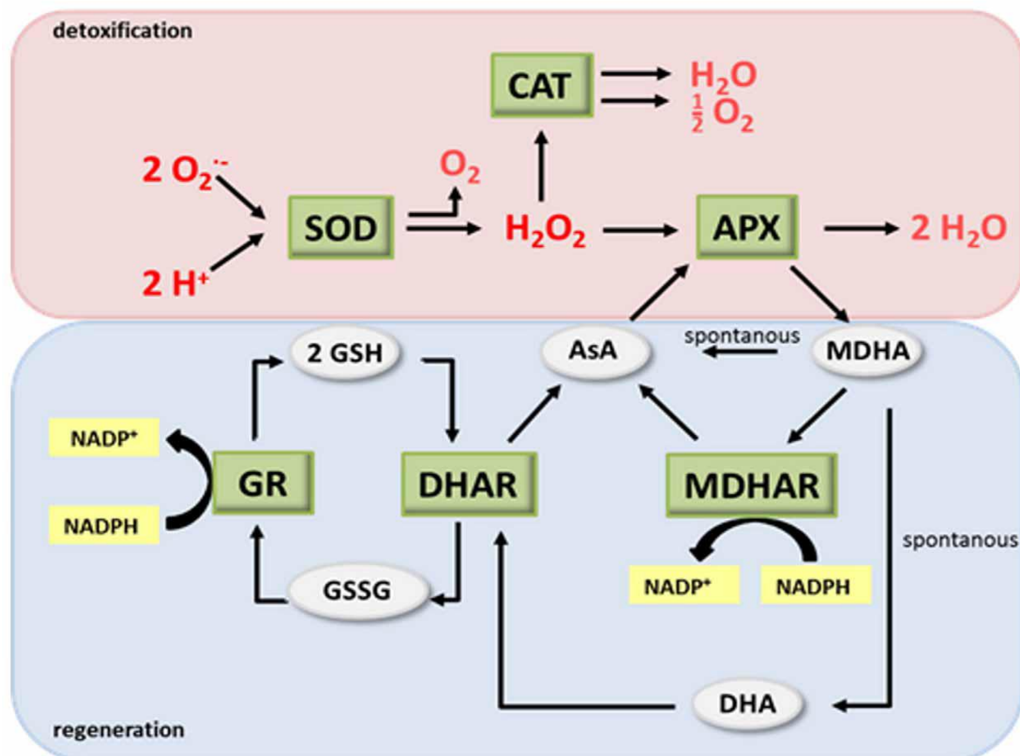

Figure 1.4. The antioxidant system (Foyer-Halliwell-Asada cycle); (Groß *et al.*, 2013). AsA, ascorbate; DHA, dehydroascorbate; SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; MDHA, monodehydroascorbate; MDHAR, MDHA reductase; DHAR, DHA reductase; GR, glutathione reductase; GSH glutathione; GSSG, glutathione disulphide.

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1.5.1. Superoxide dismutase (SOD)

In 1969 a blue copper-containing protein was isolated from bovine erythrocytes that catalysed the disproportionation of the superoxide radical (O_2^-) (McCord and Fridovich, 1969). The protein was termed superoxide dismutase (SOD). Superoxide dismutase plays a pivotal role within the antioxidant defence system, where it regulates the concentration of O_2^- and H_2O_2 ($O_2^- \xrightarrow{SOD} H_2O_2 + O_2$). All SODs contain metal co-factors that are directly bound to the protein and are thus metalloenzymes. These SODs can belong to one of three groups namely the copper zinc SODs (CuZnSODs), iron SODs (FeSODs) or manganese SODs (MnSODs) depending on the metal in the active site (DiGuiseppi, Fridovich and McCord, 1984). The latter is believed to be attributed to an evolutionary response to the availability of some metals (Mahanty *et al.*, 2012). Within the plant cell these isoforms are developmentally and environmentally regulated (Van Camp, Inzé and Van Montagu, 1997) and can be found in particular organelles within eukaryotic organisms. The CuZnSODs are the most prevalent isoforms within plants and are located within the cytosol (Gokul, 2013). The protective role superoxide dismutase carries out against oxidative stress and the cell damaging superoxide radical has been highly debatable due to the short half-life of O_2^- (Benov, 2001). It has then been proposed that damage to cells was not caused by O_2^- directly but rather due to the highly reactive hydroxyl radical (OH^-) that is given off when O_2^- participates in the iron-mediated Haber-Weiss reaction or superoxide-driven Fenton reaction (Benov, 2001). In this two-step reaction, the net result yields hydroxyl radicals that are produced from hydrogen peroxide and

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superoxide and as a result cause oxidative damage. This reaction is very slow and is catalysed by iron (Koppenol, 2013).

1.5.2. Ascorbate peroxidase (APX)

Ascorbate peroxidase (APX) is an enzyme that is crucial for the scavenging of H_2O_2 and as a result regulates ROS levels within plants (Kornyeyev *et al.*, 2003; Maruta *et al.*, 2012; Keyster *et al.*, 2014). The levels of H_2O_2 are regulated via the oxidation of ascorbate to reduce H_2O_2 to H_2O (Dalton *et al.*, 1986; Dalton *et al.*, 1993; Iturbe-Ormaetxe *et al.,* 2001; Matamoros *et al.*, 2006). Primarily, ascorbate peroxidase can be found throughout the cytosol and plays a fundamental role in the ascorbate–glutathione cycle (Sinha and Saxena, 2006). In this cycle the main substrate for the detoxification of H_2O_2 is ascorbate, which converts ascorbic acid to dehydroascorbate which ultimately lowers the concentration of H_2O_2 therefore alleviating oxidative stress (Noctor and Foyer, 1998; Sinha and Saxena, 2006). The APXs are hemoproteins as they contain a heme-cofactor which aids the enzyme in performing its respective function.

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1.5.3. Catalase (CAT)

Catalase is a ubiquitous enzyme found in all living organisms which are exposed to oxygen. This enzyme is responsible for catalysing the reaction of H_2O_2 to form H_2O and O_2 (Chelikani, Fita and Loewen, 2004). The role of catalase is to alleviate oxidative stress caused by ROS. When compared to the other enzymes it is interesting to note that catalase has one of the highest enzyme turnover rates, where approximately five million H_2O_2 molecules can be converted to H_2O and O_2 by one catalase molecule per minute (Goodsell, 2004). Catalase is a tetrameric molecule consisting of four polypeptide chains, each with over 500 amino acids in length (Kirkman and Gaetanit, 1984). It is located within the peroxisome and contains four porphyrin groups that enable the enzyme to react with H_2O_2 (Askarov *et al.*, 1984). These porphyrin groups are the reason catalase has such a high specificity for H_2O_2 (König *et al.*, 2002; Mhamdi *et al.*, 2010). Only two catalase isoforms have been identified in plants (Mhamdi *et al.*, 2010).

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1.6. Soybean an important leguminous food crop

Soybean (*Glycine max*) is one of the more economically important food crops worldwide. In South Africa, it is of high economic value as it is a growing component of the agricultural economy (Dlamini, Tshabalala and Mutengwa, 2013). It belongs to the *Fabaceae* family and is a leguminous vegetable that is native to Eastern Asia and is grown in tropical, subtropical, and temperate climates (International Institute of Tropical Agriculture, 2009; National Soybean

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Research Laboratory, 2009). This edible bean is of high nutritional value as it is a good source of protein, vitamins, minerals and dietary fibre that are essential for growth, development and overall nutritional well-being (Smith and Circle, 1972). Soybean has numerous applications within industry. Once processed the vegetable oil and the meal are separated. The oil can be used in food products such as salad dressing, cooking oil, mayonnaise, meat products and commercially baked goods or it can be used in industrial processes for the production of biodiesel (International Institute of Tropical Agriculture, 2009). The meal portion of soybean, which contains the protein, can be used for the production of animal feed or for human consumption where it is used in food products to produce soy milk, soy sauce and tempeh (National Soybean Research Laboratory, 2009).

1.6.1. Nutritional value of Soybean

The inability of the human body to synthesise all the essential amino acids makes soybean an excellent source of complete protein. Compared to other major grain and vegetable crops, soybean produces twice as much protein according to the US Food and Drug Administration (Henkel, 2000). Raw soybean consists of approximately 36% protein, 30% carbohydrates and 20% oil, making it an economically and nutritionally important food crop (figure 1.5); (International Institute of Tropical Agriculture, 2009). In many parts of Africa, malnutrition is a serious concern, as protein deficiencies such as kwashiorkor are very prevalent as animal protein is unaffordable. Thus, soybean being an inexpensive and rich

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source of protein comparable to meat, poultry and eggs, provides a means to overcome malnutrition in impoverished areas (International Institute of Tropical Agriculture, 2009).

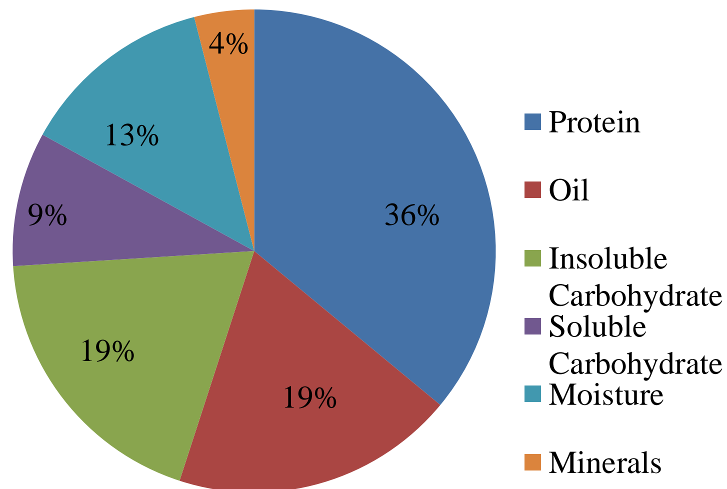

Figure 1.5. The chemical composition of soybean.

1.6.2. Economic importance of Soybean

It is estimated that approximately 323.7 million tonnes of soybean will be produced in 2016/2017 worldwide of which one million tonnes pertains to South Africa (Global Soybean Production, 2013). South Africa imports nearly as much soybean as it produces (International Institute of Tropical Agriculture, 2009) . It is one of the largest importers of soybean oilcake in Sub-Saharan Africa, which

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accounts for 72% of the import demand (Grain SA, 2016). The Free State and Mpumalanga are the two major provinces in the country where significant production of soybean occurs (Grain SA, 2016). Over the years, soybean production in South Africa has fluctuated, remaining mostly above the domestic demand (figure 1.6). According to Grain SA (2016), major economic losses within the soybean industry are mostly due to infections within soybean crops. It is believed that fungal infections will result in future economic losses to the global soybean industry. Thus it is of utmost importance to ensure that the major soybean producing areas are clear of infection by establishing effective control management strategies for already infected areas.

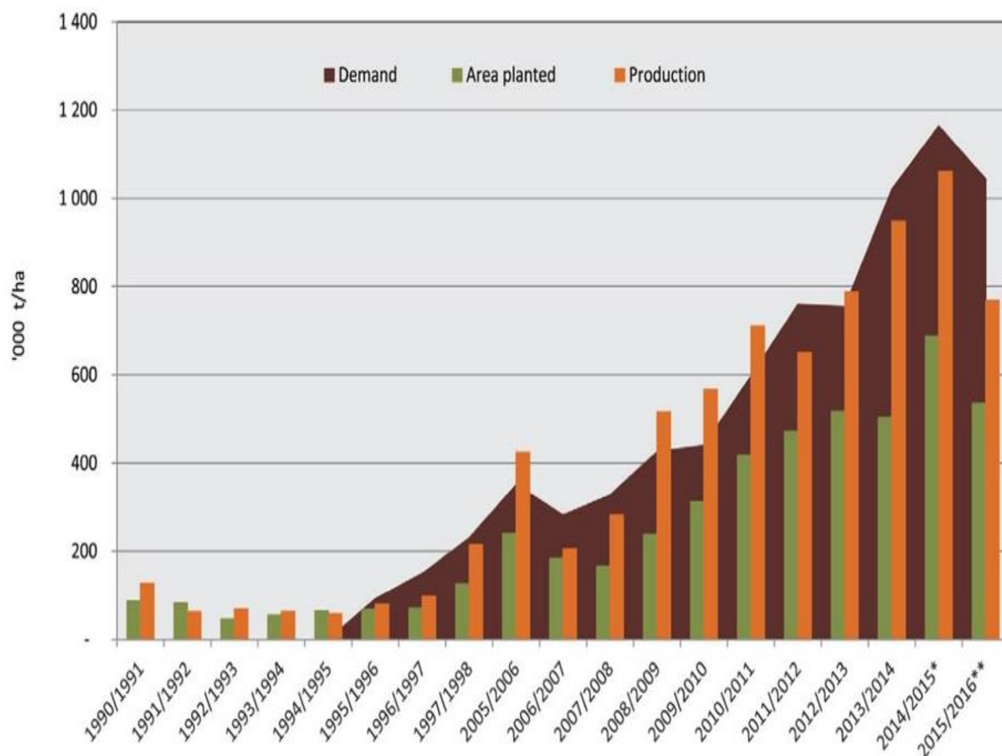

Figure 1.6. Annual South African soybean production (tons/hectare) (Grain SA, 2016).

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1.7. Aims and Objectives

The overall aim of the project is to provide information as to how the inhibition of NOS and scavenging of NO using a nitric oxide synthase inhibitor or a nitric oxide scavenger affects the antioxidant activities of superoxide dismutase, ascorbate peroxidase and catalase within soybean. The objective is to gain a better understanding as to how the inhibition of NOS, scavenging of NO and subsequently the changes in nitric oxide content affect the antioxidant capacities of a subset of antioxidants that are known to regulate the concentrations of ROS in soybean. Various biochemical tests will be done on the leaves of the soybean plant and thus determine the extent of oxidative damage caused by ROS. Lastly, the effect of the inhibition of nitric oxide biosynthesis has on ROS scavenging pathways, for example, the expression patterns of a subset of antioxidant enzymes isoforms, brought on by the use of a nitric oxide synthase inhibitor and nitric oxide scavenger will be investigated using in-gel activity profiling.

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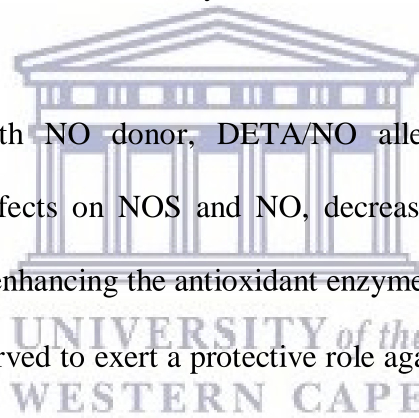
1.8. Highlights

- NOS inhibition resulted in an increase in oxidative stress within soybean.
- An increase in lipid peroxidation and H_2O_2 content was observed in response to the treatments with NOS inhibitor, L-NAME and NO scavenger, cPTIO.
- A decrease in chlorophyll and NO content was observed in conjunction with the increase in oxidative stress due to the above treatments.
- The inhibition of NOS had a detrimental effect on the scavenging capabilities of antioxidant enzymes, as a decrease in SOD, APX and CAT was observed.
- Treatment with NO donor, DETA/NO alleviated the inhibitory and scavenging effects on NOS and NO, decreasing the levels of oxidative stress, whilst enhancing the antioxidant enzyme activities.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- NO was observed to exert a protective role against oxidative stress. It acts as a signalling molecule, that is important for the regulation of the antioxidant system and the maintenance of plant redox homeostasis
- The aforementioned work was presented at an international conference
    - **Julian Le Keur**, Arun Gokul, Marshall Keyster, Ndiko Ludidi (2016) Modulation of soybean antioxidant activities by nitric oxide. The 6th Plant NO International Meeting in Granada, Spain.
- The inference from total antioxidant enzyme activity assays was observed in the antioxidant in-gel profiling.

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Chapter 2

Materials and Methods

2.1. Growth Parameters

Soybean (*Glycine max*) seeds were surface sterilized with 5% (v/v) sodium hypochlorite (NaOCl) solution for 30 minutes and thoroughly washed with deionised water. The seeds were transferred and germinated in a foil pan containing wet paper towel. Once the apical meristems of the seeds had developed, the germinated seeds were then carefully transferred and planted into 21 pots containing promix, a synthetic soil obtained from the Department of Plant Biotechnology of the University of the Western Cape. Each pot containing three germinated seeds was supplemented with nutrient solution and was allowed to grow until the plants reached the third trifoliate stage (V_3) of growth (three leaf stage). The nutrient solution was prepared by adding 10 ml each of the following macro- and micronutrients (100x stock solutions) namely, 0.5 mM potassium sulphate (K_2SO_4), 0.5 mM magnesium sulphate ($MgSO_4$), 8 mM monopotassium phosphate (KH_2PO_4), 1 mM calcium chloride ($CaCl_2$), 10 mM potassium nitrate (KNO_3), 2 mM ammonium nitrate (NH_4NO_3), 30 µM boric acid (H_3BO_3), 10 µM manganese sulphate ($MnSO_4$), 0.7 µM zinc sulphate ($ZnSO_4$), 0.2 µM copper sulphate ($CuSO_4$), 1 µM sodium molybdate ($NaMOO_4$), 0.05 µM cobalt chloride ($COCl_2$) and 50 µM ferric ethylenediaminetetraacetic acid [Fe(III)EDTA]. The nutrient solution was brought up to a total volume of 1000 ml to which 0.24% (w/v) of HEPES was added and the pH adjusted to 7.2.

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Treatments commenced at the V_3 stage of growth. In total five treatments were carried out in triplicate namely untreated, 100 µM 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), 100 µM cPTIO and 100 µM Diethylenetriamine/nitric oxide adduct (DETA/NO), 1 mM Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) and 1 mM L-NAME and 100 µM DETA/NO. Treatments were made up in nutrient solution and carried out over a period of five days, whereby each pot received 100 ml of their respective treatment every second day.

2.2. Determination of chlorophyll a and b

The concentration of chlorophyll a and b was determined according to a modified protocol of Oancea *et al.* (2005). The concentration of chlorophyll a and b was determined within the leaves for the five treatments mentioned in section 2.1. The assay comprised of 100 mg of frozen ground leaf material that was added to 1.5 ml Eppendorf tubes. The Eppendorf tubes were covered with foil to prevent the degradation of chlorophyll species. To each 1.5 ml tube ten volumes of 100% (v/v) acetone was added and briefly vortexed. Once mixed, the samples were transferred to glass cuvettes and their absorbance readings were measured at wavelengths 662 nm and 644 nm, respectively, on a spectrophotometer. The absorbance readings of the three experimental replicates were done in triplicate. A calculation was then used to determine the concentrations of the different chlorophyll species using the optical readings obtained (Oancea *et al.*, 2005).

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2.3. Protein extraction

Protein was extracted by adding 100 mg of frozen ground leaf material to 2 ml Eppendorf tubes. To each tube 0.5 ml of protein extraction buffer containing 4 mM phosphate buffer, 1 mM EDTA and 5% (w/v) PvP was added and vortexed for 3 minutes. Once adequately mixed, the homogenised plant material was subject to a centrifugation step at 12000 x g for 5 minutes. The supernatant was transferred to a clean Eppendorf tube. The protein concentrations were quantified using a Bradford assay (Bradford, 1976), after which the protein samples were stored at -20 °C. Protein quantitation was done in triplicate.

2.4. Quantification of malondialdehyde (MDA) as a measure of lipid peroxidation

An adapted protocol of Zhang *et al.* (2006) was used to measure lipid peroxidation levels for untreated and treated plants. Ground leaf material (100 mg) was added to 1.5 ml Eppendorf tubes, after which five volumes of 6% (w/v) trichloroacetic acid (TCA) was added to each tube. The Eppendorf tubes were briefly vortexed and subject to a centrifugation step at 13000 x g for 10 minutes. A volume of 400 µl of the supernatant was transferred to a new Eppendorf tube, to which 600 µl of 0.5% (w/v) thiobarbituric acid (TBA)/20% TCA was added. The solution was vortexed briefly and the tube caps wrapped in parafilm to ensure they do not open during incubation. The tubes were incubated on a heating block for 20 minutes at 90 °C. After incubation, the samples were centrifuged at 13000 x g for 5 minutes. Samples were then loaded in triplicate onto a 96-well microtitre

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plate and absorbance read on a spectrophotometer at wavelengths of 532 nm as well as 600 nm. The absorbance values at 600 nm were subtracted from the absorbance values at 532 nm to correct for non-specific turbidity. The extinction coefficient of 155 $mM.cm^{-1}$ was used to calculate the MDA values.

2.5. A spectrophotometric assay to determine the total NO content

Nitric oxide content was determined according to an adapted protocol of Ludidi (2013). To 25 ml of potassium phosphate buffer (pH 7.0), 5 g of G-25 sephadex beads were added and left to swell overnight at 4 °C. The matrix was then transferred to a chromatography column and the buffer was allowed to flow through the column until enough of the buffer was left to cover the beads. The matrix was washed twice with potassium phosphate buffer (pH 7.0). To a 15 ml conical tube containing 1 ml of 50 mM potassium phosphate buffer, 25 mg of methemoglobin was added. To another 15 ml tube that contained 1 ml of 50 mM potassium phosphate buffer, 25 mg methemoglobin and 1.5 mg of sodium hyposulfite was added and the solution was briefly vortexed until the solution became dark purple in colour. The methemoglobin and sodium hyposulfite solution was aerated by aspirating with a 1 ml pipette for 5 minutes. The solution was incubated at room temperature for 30 minutes allowing the blood to turn from dark purple to a light red indicating the conversion of methemoglobin to oxyhemoglobin.

The oxyhemoglobin was transferred to the chromatography column containing the sephadex beads and desalted to purify the oxyhemoglobin. In total, 4-5 fractions

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of the oxyhemoglobin (250 µl each) was collected in 1 ml Eppendorf tubes after the blood had passed through the column. A serial dilution of oxyhemoglobin was prepared namely 0x (undiluted), 10x, 20x, 50x, 100x. This dilution series was pipetted into a 96-well microtitre plate to generate a standard curve. The absorbance of each fraction was taken at 415 nm and the fraction containing the oxyhemoglobin will be the highest absorbance at that 415 nm. An extinction coefficient of 131 $mM^{-1}.cm^{-1}$ for oxyhemoglobin was used to calculate the concentration of the oxyhemoglobin fraction at 415 nm. To a 0.5 ml Eppendorf tube 50 µl of protein extract was incubated along with 0.2 µl of (500 units) superoxide dismutase and 5 µl of (1 U/ml) of catalase. To the same 96-well microtitre plate 200 µl of potassium phosphate buffer and 200 µl of methemoglobin were added individually to separate wells in triplicate. The methemoglobin served as the blank. To the remaining wells 150 µl of oxyhemoglobin was added in triplicate after which 50 µl of the protein extract/enzyme mixture was added to the oxyhemolobin making a total reation volume of 200 µl. The microtitre plate was then incubated on a shaker for 10 min. The absorbance was measured at 401 and 421 nm. The NO concentration was calculated by subtracting the absorbance at 421 nm from the absorbance at 401 nm and dividing the difference by 77 $mM^{-1}.cm^{-1}$.

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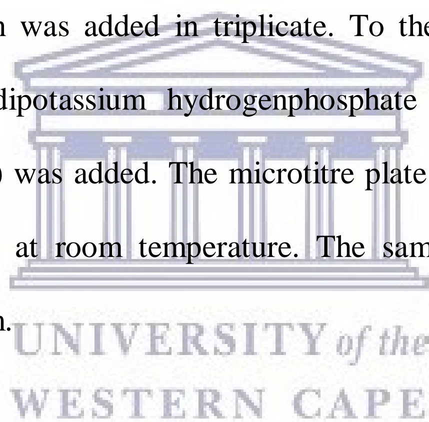
2.6. A spectrophotometric assay for the determination of hydrogen peroxide (H_2O_2) content

Hydrogen peroxide content in the leaf plant material was determined according to an adapted protocol of Velikova *et al.* (2000). A standard curve for the assay was prepared using the following standards namely 0, 5000, 10000, 15000, 20000 and 25000 nM. The standards were prepared by diluting an appropriate volume of H_2O_2 in distilled water. Standards were loaded onto a 96-well microtitre plate in triplicate. Samples were prepared by using TCA extraction on frozen ground plant material as described in the MDA assay in section 2.4. To the microtitre plate 50 µl of TCA extraction was added in triplicate. To the standards as well as the samples 1.25 mM dipotassium hydrogenphosphate (K_2HPO_4) and 250 mM potassium iodide (KI) was added. The microtitre plate was then incubated for 20 minutes on a shaker at room temperature. The samples were then read at a wavelength of 390 nm.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2.7. A spectrophotometric assay to determine the total superoxide dismutase (SOD) activity

Total SOD activity was determined according to an adapted protocol of Stewart and Bewley (1980). Protein was extracted from 100 mg of frozen ground leaf material and quantified using a Bradford Assay. Prior to adding 10 µl of protein sample to the 96-well microtitre plate, the protein had to be diluted to a concentration of 1 $mg.ml^{-1}$. Thereafter, to each of the appropriate wells containing 10 µl of protein the following was added, namely 20 mM phosphate buffer (pH

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7.8), 0.1 mM nitrotetrazolium blue chloride (NBT), 5 µM riboflavin, 10 mM methionine and 0.1 mM ethylenediaminetetraacetic acid (EDTA). The total reaction was made up to a volume of 200 µl using distilled water. The microtitre plate was then incubated at room temperature and exposed to white light for 20 minutes. After incubation, absorbance readings were taken at a wavelength of 560 nm. The superoxide dismutase activity was calculated by determining how much SOD was needed to inhibit a 50% decrease in the reduction of NBT to formazan.

2.8. A kinetic spectrophotometric assay to determine total ascorbate peroxidase (APX) activity

Total APX activity was determined according to an adapted protocol of Asada (1984). Protein was extracted from 100 mg of frozen ground leaf material and quantified by using a Bradford Assay. An appropriate volume of protein was aliquoted into 0.5 ml Eppendorf tubes containing 2 mM ascorbate for 5 minutes. To a 96-well microtitre plate 10 µl of protein, 71.43 mM K_2HPO_4 and 0.36 mM ascorbate was added in triplicate. Prior to measuring the absorbance of the samples, 0.714 mM H_2O_2 was added to the appropriate wells to initiate the reaction. Reactions were made up to a total volume of 200 µl with distilled water. Absorbance readings were measured at a wavelength of 290 nm. The ascorbate peroxidase activity was calculated using the extinction coefficient of 2.8 $mM.cm^{-1}$.

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2.9. A kinetic spectrophotometric assay to determine the total catalase (CAT) activity

Total CAT activity was determined according to an adapted protocol of Aebi (1984). A total reaction mixture of 1 ml was prepared and containing the following, 50 mM K_2HPO_4 (pH 7.0), 0.5 mM EDTA and 20 µl protein extract. To initiate the reaction, 1 mM H_2O_2 was added to the reaction mixture after which the absorbance was read at 240 nm every 5 seconds. An extinction coefficient of 39.4 $mM^{-1}.cm^{-1}$ was used to calculate the activity.

2.10. Superoxide dismutase in-gel PAGE activity profiling

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The superoxide dismutase activity profile for *G. max* was determined according to an adapted protocol of Beauchamp and Fridovich (1971). A 5% (v/v) stacking and 13% (v/v) separating native PAGE gel was prepared. The PAGE gel was then transferred to the gel tank containing native PAGE running buffer comprising of 192 mM glycine and 24 mM Tris base. A protein concentration of 100 µg was added to a total volume of 60 µl 4x native loading dye. The protein/loading dye mixture was then loaded onto the native PAGE gel. Electrophoresis was at 80 V until the loading dye had reached the bottom of the glass casting plate. The gel was then carefully removed from the tank as well as the casting plates and placed into a small container and rinsed with distilled water. All incubations were done in the dark. A washing solution of 50 mM potassium phosphate buffer (pH 7.0) was used to wash the PAGE gel, which was then put on a shaker for 20 minutes. Following the wash step, the solution was discarded and a second solution

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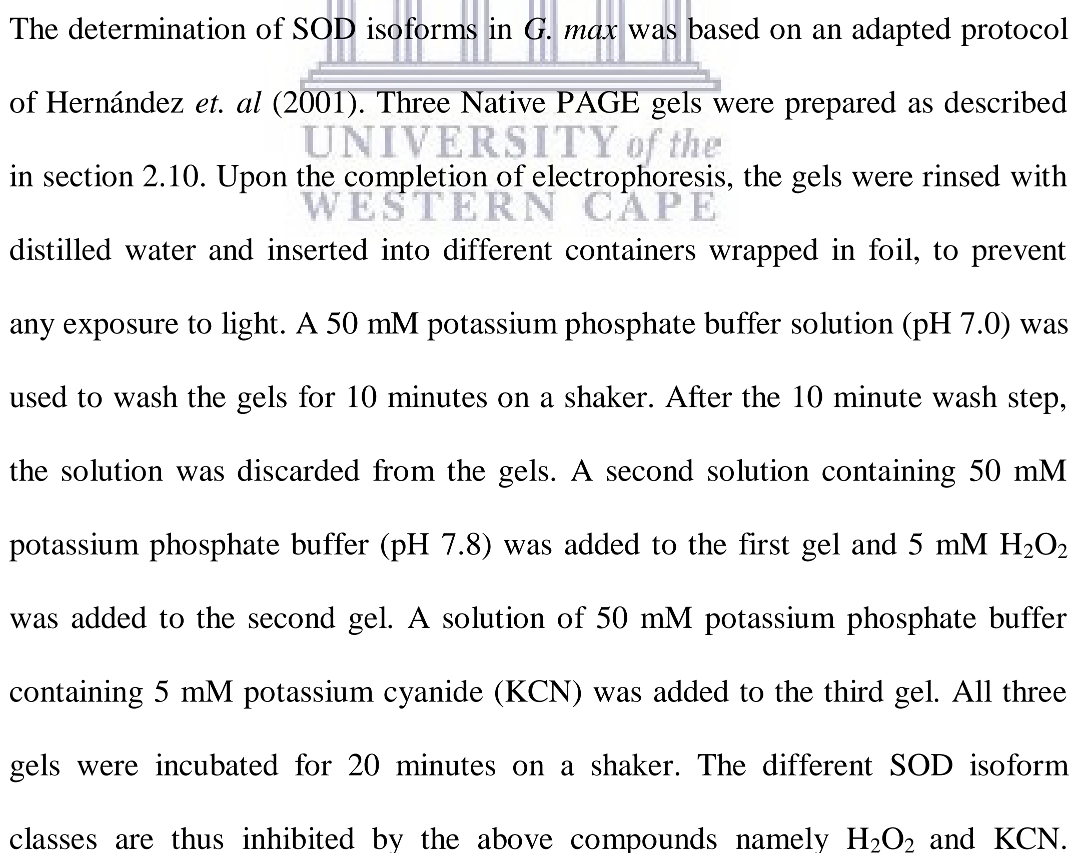
consisting of 50 mM potassium phosphate buffer (pH 7.8), 0.5 mM NBT was applied onto the gel, which was incubated on a shaker for 20 minutes. The second solution was discarded and a final solution of 50 mM potassium phosphate buffer (pH 7.8), 35.5 mM TEMED and 0.5 mM riboflavin was added to the gel and allowed to incubate on a shaker for 20 minutes. After 20 minutes the third solution was discarded and the gel was rinsed with distilled water. The gel was exposed to a light source within a white light box after which the activity of the bands had developed and were visualised.

2.10.1 Superoxide isoform class determination



The determination of SOD isoforms in *G. max* was based on an adapted protocol of Hernández *et. al* (2001). Three Native PAGE gels were prepared as described in section 2.10. Upon the completion of electrophoresis, the gels were rinsed with distilled water and inserted into different containers wrapped in foil, to prevent any exposure to light. A 50 mM potassium phosphate buffer solution (pH 7.0) was used to wash the gels for 10 minutes on a shaker. After the 10 minute wash step, the solution was discarded from the gels. A second solution containing 50 mM potassium phosphate buffer (pH 7.8) was added to the first gel and 5 mM H_2O_2 was added to the second gel. A solution of 50 mM potassium phosphate buffer containing 5 mM potassium cyanide (KCN) was added to the third gel. All three gels were incubated for 20 minutes on a shaker. The different SOD isoform classes are thus inhibited by the above compounds namely H_2O_2 and KCN.

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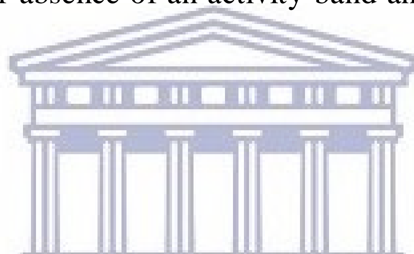
Hydrogen peroxide inhibits Cu/ZnSODs and FeSODs, whilst KCN inhibits MnSODs. The solutions in which the gels were incubated were discarded and a solution of 50 mM potassium phosphate buffer (pH 7.8) and 0.5 mM NBT was added to the individual gels. The gels were incubated for 20 minutes on a shaker after which the solution was discarded and a final solution was added containing 50 mM potassium phosphate buffer, 35.5 mM TEMED and 0.5 mM riboflavin to all the individual gels. The gels were allowed to incubate in this solution for 20 minutes. The gel without the inhibitor (Gel 1) was used as the reference gel and the other two gels were the inhibitor gels, which were compared to the reference gel for the presence or absence of an activity band and thus identifying the SOD class.



2.11. Ascorbate peroxidase in-gel PAGE activity profiling

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An adapted protocol of Seckin *et. al* (2010) was used to visualise the activity of the APX isoforms within *G. max*. A 5% (v/v) stacking and 13% (v/v) separating native PAGE gel was prepared. The PAGE gel was then transferred to the electrophoretic chamber containing native PAGE running buffer comprising of 192 mM glycine, 24 mM Tris base and 2 mM ascorbate. A protein concentration of 100 µg was added to a total volume of 60 µl 4x native loading dye. The protein/loading dye mixture was then loaded onto the native PAGE gel. Electrophoresis was performed at 80 V until the loading dye had reached the bottom of the glass casting plate. The gel was then carefully removed from the tank as well as from the casting plates and placed into a small container and rinsed

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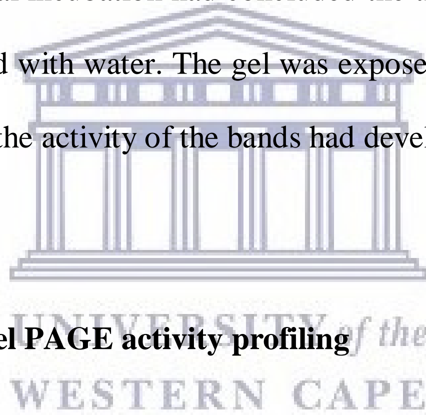
with water. All incubations were done in the dark. The first incubation of the gel occurred in a 50 mM potassium phosphate buffer (pH 7.0), to which 2 mM ascorbate was added onto the gel. The gel was incubated for 20 minutes on a shaker. After 20 minutes the first solution was discarded and a second incubation containing 50 mM potassium phosphate buffer (pH 7.8), 4 mM ascorbate and 2 mM H_2O_2 was added to the gel. Incubation was for 20 minutes on a shaker. After the incubation step the second solution was discarded and a third solution was added to the gel containing 50 mM potassium phosphate buffer (pH 7.8), 28 mM TEMED and 0.5 mM NBT. The final incubation was for approximately 20 minutes. Once the final incubation had concluded the third solution was discarded and the gel was rinsed with water. The gel was exposed to a light source within a light box after which the activity of the bands had developed and were visualised.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2.12. Catalase in-gel PAGE activity profiling

The catalase activity profile for *G. max* was determined according to an adapted protocol of Weydert and Cullen (2010). A 5% (v/v) stacking and 7% (v/v) separating native PAGE gel was prepared. The PAGE gel was then transferred to the gel tank containing native PAGE running buffer comprising of 192 mM glycine, 24 mM Tris base. A protein concentration of 100 µg was added to a total volume of 60 µl 4x native loading dye. The protein/loading dye mixture was then loaded onto the native PAGE gel. Electrophoresis was performed at 80V until the loading dye had reached the bottom of the glass casting plate. The gel was then carefully removed from the tank as well as the casting plates and placed into a

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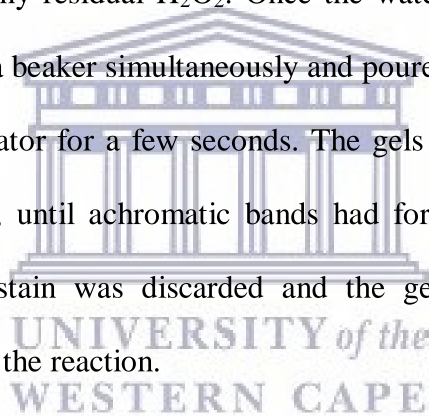
small container and rinsed with distilled water. All incubations were done in the dark. The gel was subjected to a washing step with distilled water for 30 minutes, changing the water every 10 minutes. The water was discarded and the gels were incubated in a solution of 0.003% H_2O_2 that was prepared by mixing 10 µl of 30% (v/v) H_2O_2 with 100 ml of distilled water and incubated on a shaker for 10 minutes. The H_2O_2 solution was then discarded and the relevant stains were prepared. In two separate 50 ml conical tubes 2% (w/v) Ferric chloride (FeCl) and 2% (w/v) potassium ferricyanide ($K_3Fe[CN_6]$) was prepared in 30 ml of distilled water. The gels were rinsed twice with distilled water for 5 min and incubated on a shaker to remove any residual H_2O_2. Once the water has been discarded both stains were added to a beaker simultaneously and poured over the gel and allowed to shake on an incubator for a few seconds. The gels were then transferred to a fluorescent light box, until achromatic bands had formed. Once the bands had become visible the stain was discarded and the gel extensively rinsed with distilled water to stop the reaction.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2.13. Densitometry analysis

The imaging software AlphaEaseFC™ (Alpha Innotech Corporation) was used for densitometry analysis. The imaging software was used in accordance with the manufacturer's specifications.

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2.14. Statistical analysis

The Duncan's multiple range test was used to perform statistical analysis, whereby a P-value of less than 0.05 denoted significance.

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Chapter 3

The effect the inhibition of nitric oxide biosynthesis has on the biochemical aspects of soybean

3.1. Abstract

Nitric oxide is a signalling gaseous molecule that is endogenously synthesized from various enzymatic and non-enzymatic sources in plants. It is known to play an important role in the regulation of plant physiological processes such as growth and development. Similarly, reactive oxygen species also act as signalling molecules controlling processes such as growth, differentiation and response to abiotic and biotic stresses. Due to the commonality between these two signalling molecules in regulating physiological processes, the effects of lowering nitric oxide content on the biochemical processes as well as the enzymatic activities of a subset of antioxidant enzymes in soybean were investigated in this chapter. The inhibition of NOS resulted in an increase in oxidative stress within soybean. Lipid peroxidation and H_2O_2 content increased in response to the treatments with NOS inhibitor, L-NAME and NO scavenger, cPTIO. Chlorophyll and NO content had also decreased in response to the increase in oxidative stress due to the latter treatments. The scavenging capabilities of a subset of antioxidant enzymes namely APX, SOD and CAT had also decreased in response to the inhibitory and scavenging effects of L-NAME and cPTIO, respectively. However, these inhibitory and scavenging effects on NOS and NO, respectively, were alleviated in the presence of a NO donor, DETA/NO, decreasing the levels of oxidative

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stress, whilst enhancing the antioxidant enzyme activities. Thus it was concluded that NO carries out a protective function against oxidative stress.

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3.2. Introduction

Due to the sedentary lifestyle of a plant, for example, the inability to move around; plants are exposed to abiotic and biotic stresses on a daily basis. These stressors can be beneficial or detrimental to an organism depending on their location (Anjum *et al.*, 2011). Abiotic stressors include both the chemical and physical parts of the environment (Chapin III *et al.*, 2011). They affect plants in terms of growth, health and reproduction. Abiotic stressors that affect plants include drought, extreme temperature as well as soil pH (Anjum *et al.*, 2011). As a result, the plant undergoes oxidative stress which is due to the accumulation of reactive oxygen species (ROS) which subsequently can be detrimental if not regulated (figure 1.1). Reactive oxygen species play important roles in plants, not only in toxicity but also as signalling molecules which controls processes such as growth, differentiation and responses to abiotic as well as biotic stresses (Pitzschke *et al.* 2006). Damage caused by oxidative stress include that of macromolecules as well as the deregulation, over-flow or even the disruption of electron transport chains (ETC) within mitochondria and chloroplasts (Jack, 2012; Groß *et al.*, 2013). Subsequently, various cellular mechanisms such as lipid peroxidation, protein oxidation and nucleic acid damage are activated in response to oxidative stress which ultimately lead to programmed cell death (PCD); (Jack, 2012). Reactive oxygen species that cause oxidative damage in plants include; the singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide radical ($O_2^{-\bullet}$) and hydroxyl radical ($OH^{-\bullet}$) (Apel and Hirt, 2004). To overcome ROS toxicity, plants have developed a ROS-scavenging mechanism also known as the antioxidant defence system (figure 1.4); (Gokul, 2013; Groß *et al.*, 2013). These antioxidant

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pathways exist in the cellular compartments where ROS are generated (Gill and Tuteja, 2010; Miller *et al.*, 2010). The ROS are converted into less toxic compounds that are less harmful to the cellular integrity of the plant. Superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) are considered to be the most prevalent antioxidant enzymes in plants (Blokhina *et al.,* 2003; Sinha and Saxena, 2006; Lee *et al.*, 2007).

Nitric oxide (NO) is a signalling gaseous molecule that is endogenously synthesized from various enzymatic and non-enzymatic sources in plants (figure 1.2) (Keyster *et al.*, 2014; Röszer, 2014). Nitric oxide is known to play an important role in the regulation of plant physiological processes such as growth, development as well as in the response to abiotic and biotic stresses (Jack, 2012). Due to the fact that both NO and ROS regulate physiological processes, the effects of the changes in NO content on the enzymatic activities of a subset of antioxidant enzymes that in turn regulate the levels of ROS would give us a better understanding as to how plants respond to biotic and abiotic stress. Furthermore, we can gain insight into the interplay between production and metabolism of NO and ROS.

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3.3. Results

3.3.1. The effect of NO on chlorophyll production in soybean

Nitric oxide (NO) is a signalling gaseous molecule which regulates plant physiological processes such as photosynthesis and photorespiration (Planchet and Kaiser, 2006). To date, not much research has been done on how endogenous NO content directly affects photosynthesis. However, studies have shown that NO donors differentially regulate the photosynthetic rate in plants (Hill and Bennett, 1970). The lowering of NO content can have detrimental effects on plant development. Therefore, this study evaluated the effect that changes in NO (inhibition or scavenging) had on the chlorophyll content in the leaves of *G. max* (soybean) using a NO synthase inhibitor (L-NAME) or a NO scavenger (cPTIO) as well as a combination with a NO donor (DETA/NO) (Table 3.1.).

When compared to the control no differences were observed for the combination treatment of cPTIO with DETA/NO for chlorophyll a and b. However, the treatment of L-NAME as well as its combination DETA/NO showed a decrease in chlorophyll a and b concentration when compared to the control and each other. A decrease in the concentration of chlorophyll a and b was also observed for the treatment with cPTIO when compared to the control. An overall decrease was observed for the total chlorophyll concentration even after the addition of the DETA/NO, compared to the control. However, the effects of NOS inhibition and NO scavenging using L-NAME and cPTIO respectively, were slightly alleviated with the addition of DETA/NO.

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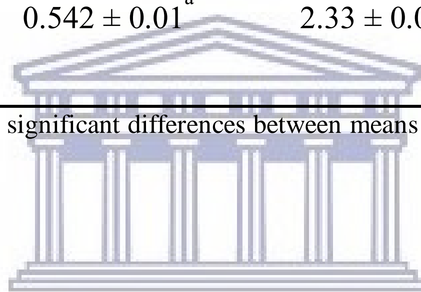
Table 3.1. The effect of lowering NO content on chlorophyll production in soybean

Treatments	Chlorophyll a	Chlorophyll b	Total Chlorophyll
Untreated	0.596 ± 0.01^{a}	2.51 ± 0.03^{b}	3.11 ± 0.04^{c}
L-NAME	0.355 ± 0.02^{d}	1.47 ± 0.07^{e}	1.83 ± 0.09^{f}
L-NAME + DETA/NO	0.385 ± 0.02^{g}	1.61 ± 0.2^{h}	2.00 ± 0.04^{i}
cPTIO	0.486 ± 0.02^{j}	2.03 ± 0.09^{k}	2.52 ± 0.1^{l}
cPTIO + DETA/NO	0.542 ± 0.01^{a}	2.33 ± 0.08^{b}	2.87 ± 0.09^{m}

The various letters signify significant differences between means at $P < 0.05$ (DMRT). Values are means ±S.E (n = 3).



3.3.2. The effect of NO on lipid peroxidation in soybean

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Toxic levels of reactive oxygen species (ROS) are the result of a plant undergoing oxidative stress. Lipid peroxidation is the process whereby ROS cause the oxidative deterioration of polyunsaturated fatty acids which subsequently affect the structure and functioning of cellular membranes (Vasilaki and McMillan, 2012; Ayala, Muñoz and Argüelles, 2014). Malondialdehyde (MDA) is an endogenous by-product of lipid peroxidation and is used as a marker for the measurement of lipid peroxidation (Marnett and Hancock, 1999). The reaction of lipid peroxides with thiobarbituric acid (TBA) can be used to measure MDA

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content (Sinha and Saxena, 2006). The extent of damage done to the membranes within the leaves of soybean due to NOS inhibition and NO scavenging was determined using the lipid peroxidation assay (Section 2.4). When compared to the control, plants treated with L-NAME showed a ~15% increase in MDA levels (figure 3.1.). A ~10% increase in MDA was observed for plants treated with cPTIO when compared to the control. No differences in MDA levels were observed for the two combination treatments with DETA/NO when compared to the control (figure 3.1.). When comparing the treatments of L-NAME to L-NAME + DETA/NO, MDA levels were observed to be ~15% higher for the L-NAME treatment. In contrast, the comparison of cPTIO to cPTIO + DETA/NO, MDA levels were observed to be ~10% higher for the cPTIO treatment (figure 3.1.).

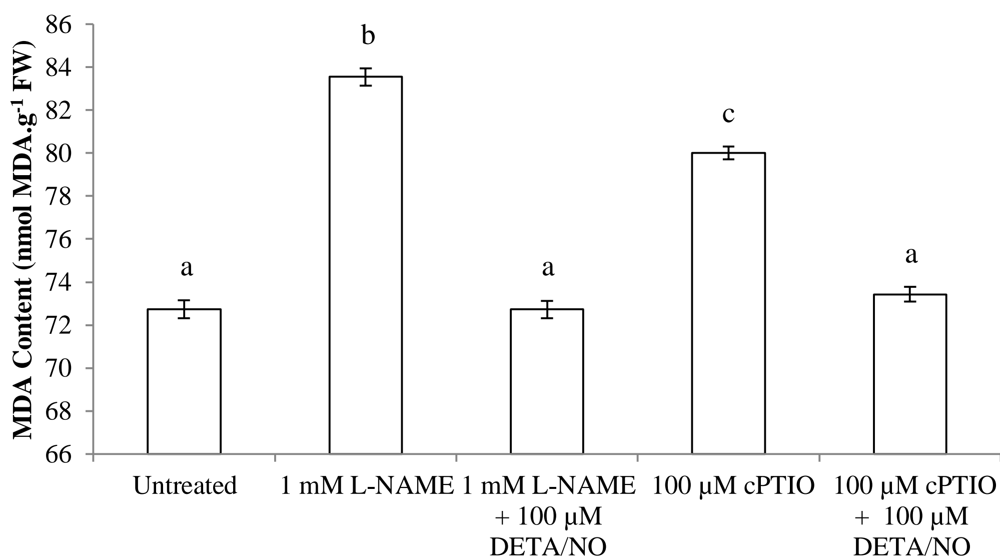

Figure 3.1. Leaf MDA content in response to treatment with 1 mM nitric oxide synthase inhibitor, 100 µM nitric oxide scavenger and combination with 100 µM DETA/NO in soybean. MDA is used as a biomarker for lipid peroxidation. The various letters signify significant differences between means at $P<0.05$ (DMRT). Error bars represent the mean (±SE; n = 3) from data that is representative of experiments done in triplicate.

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3.3.3. *Nitric oxide inhibition and scavenging decrease endogenous NO content in soybean*

Nitric oxide (NO) carries out a vital role in maintaining plant redox homeostasis as well as alleviating oxidative stress caused by the accumulation of ROS (del Río *et al.*, 2003; Palavan-Unsal and Arisan, 2009; Blokhina and Fagerstedt, 2010). Hemoglobin is an iron-containing metalloprotein that transports oxygen in red blood cells and plays an important role in the transport of NO. Hemoglobin binds to oxyhemoglobin and converts the oxyhemoglobin to methemoglobin. Thus, hemoglobin can be used as a measure of NO content in plants using the oxyhemoglobin method (Section 2.5).

In comparison to the control, soybean plants treated with L-NAME showed a ~44% decrease in NO content (figure 3.2.). A ~30% decrease in NO content was observed for plants treated with cPTIO when compared to the control (figure 3.2.). No differences in NO levels were observed for the two combination treatments with DETA/NO in comparison to the control (figure 3.2.). Comparing the treatments of L-NAME to L-NAME + DETA/NO, the NO content was observed to be ~66% higher for the DETA/NO treatment. In contrast, the comparison of cPTIO to cPTIO + DETA/NO, the NO content was observed to be ~26% higher for the DETA/NO treatment (figure 3.2.).

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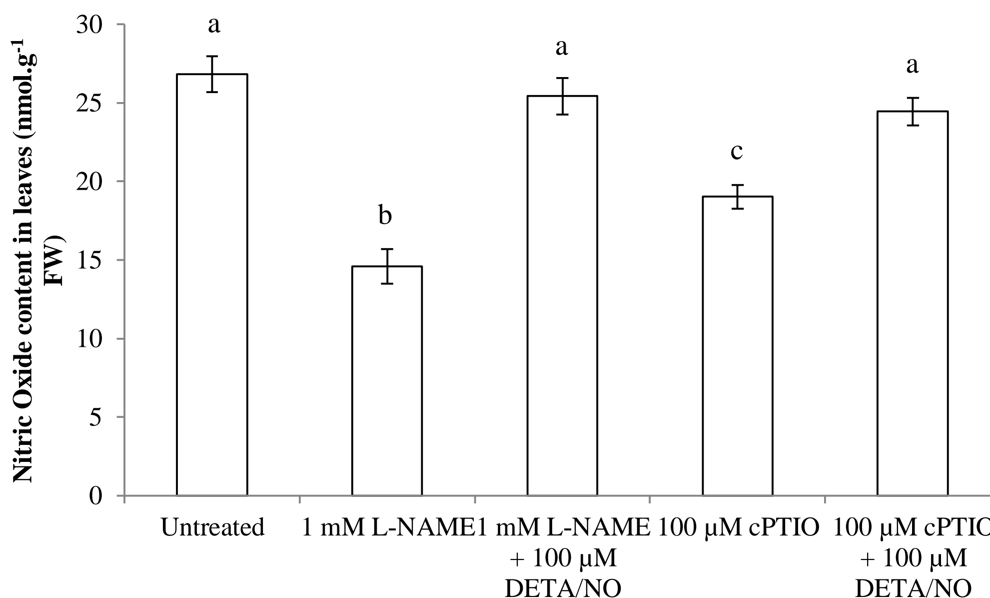

Figure 3.2. Nitric oxide content in soybean as measured after treatment with 1 mM nitric oxide synthase inhibitor, 100 µM nitric oxide scavenger and combination with 100 µM DETA/NO. The various letters signify significant differences between means at $P<0.05$ (DMRT). Error bars represent the mean (±SE; n = 3) from data that are representative of experiments done in triplicate.

3.3.4. The effect of NO inhibition and scavenging on hydrogen peroxide content in soybean

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It is well known that the occurrence of oxidative stress is in part due to the accumulation of hydrogen peroxide (H_2O_2) in plants. Hydrogen peroxide carries out two important roles in plants. It functions as a signalling molecule or second messenger at low concentrations and at high concentrations it induces programmed cell death (Gill and Tuteja, 2010; Petrov and Van Breusegem, 2012). Upon interaction with biomolecules such as enzymes, H_2O_2 may inactivate enzymes through the oxidation of thiol groups and in the presence of metal catalysts this oxidation process is greatly enhanced through the Haber-Weiss or

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Fenton-type reactions (Van Breusegem *et al.*, 2001; Gill and Tuteja, 2010). Hydrogen peroxide has an enormous impact on the functioning of a plant cell. Its multi-functionality on one hand and the danger it poses at high concentration on the other, make it a double edged sword. That being said, H_2O_2 levels need to be strictly regulated as not to cause severe damage to the plant itself. It was thus necessary to gain a better understanding as to how changes in NO content affects H_2O_2 content in soybean.

When compared to the control, both individual treatments namely L-NAME and cPTIO displayed an increase in H_2O_2 concentration of ~19% (figure 3.3.). No differences in H_2O_2 concentration were observed for the combination treatments with DETA/NO in comparison to the control (figure 3.3.). Comparing the DETA/NO treatments to their individual treatment counterparts, the concentration of H_2O_2 was observed to be ~16% lower for both DETA/NO treatments (figure 3.3.).

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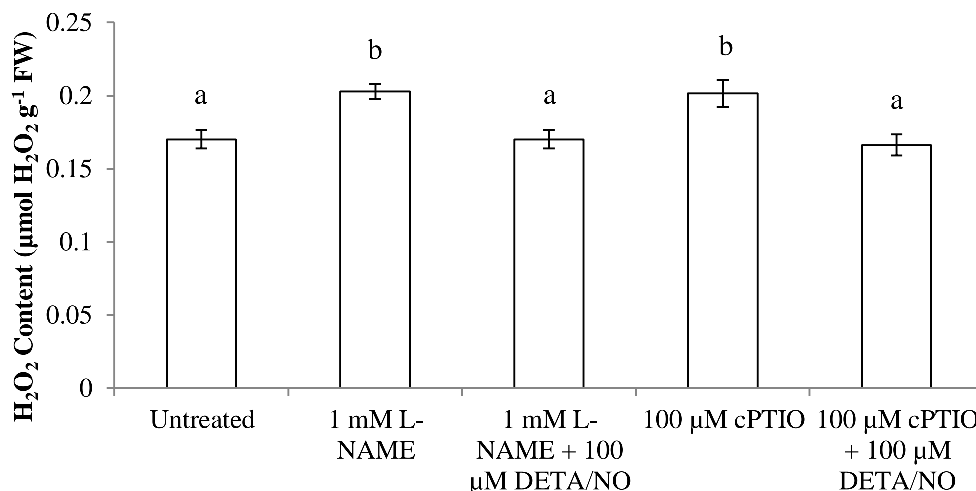

Figure 3.3. Leaf H_2O_2 content in response to treatment with 1 mM nitric oxide synthase inhibitor, 100 µM nitric oxide scavenger and combination with 100 µM DETA/NO in soybean. The various letters signify significant differences between means at P<0.05 (DMRT). Error bars represent the mean (±SE; n = 3) from data that is representative of experiments done in triplicate.

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3.3.5. Nitric oxide alters the enzymatic activity of superoxide dismutase

Superoxide dismutase (SOD) is a metalloprotein that catalyses the disproportionation of the superoxide radical (O_2^-) into oxygen and hydrogen peroxide (McCord and Fridovich, 1969). It plays a crucial role within the antioxidant defence system, because it regulates the concentrations of O_2^- and H_2O_2. All SODs are classified as metalloenzymes and can belong to one of three groups namely the copper zinc SODs (Cu/ZnSODs), iron SODs (FeSODs) or manganese SODs (MnSODs) depending on the metal in their active site (DiGuiseppi, Fridovich and McCord, 1984). Due to the short half-life of O_2^- the notion of SOD carrying out a protective role against oxidative stress has been debatable (Benov, 2001). Therefore, determining the effect NOS inhibition and NO scavenging had on the enzymatic activity of SOD within soybean was of importance.

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In comparison to the control, both individual treatments namely L-NAME and cPTIO displayed a ~39% decrease in SOD activity (figure 3.4.). No differences in SOD activity were observed for plants treated with the L-NAME + DETA/NO combination treatment in comparison to the control (figure 3.4.). A slight decrease in SOD activity of ~20% was observed for the plants treated with the cPTIO + DETA/NO combination compared to the control. Comparing the treatments of L-NAME to L-NAME + DETA/NO, the SOD activity was observed to be ~54% higher for the DETA/NO treatment. In contrast, comparing cPTIO to cPTIO + DETA/NO, the SOD activity was observed to be ~41% higher for the DETA/NO treatment (figure 3.4.).

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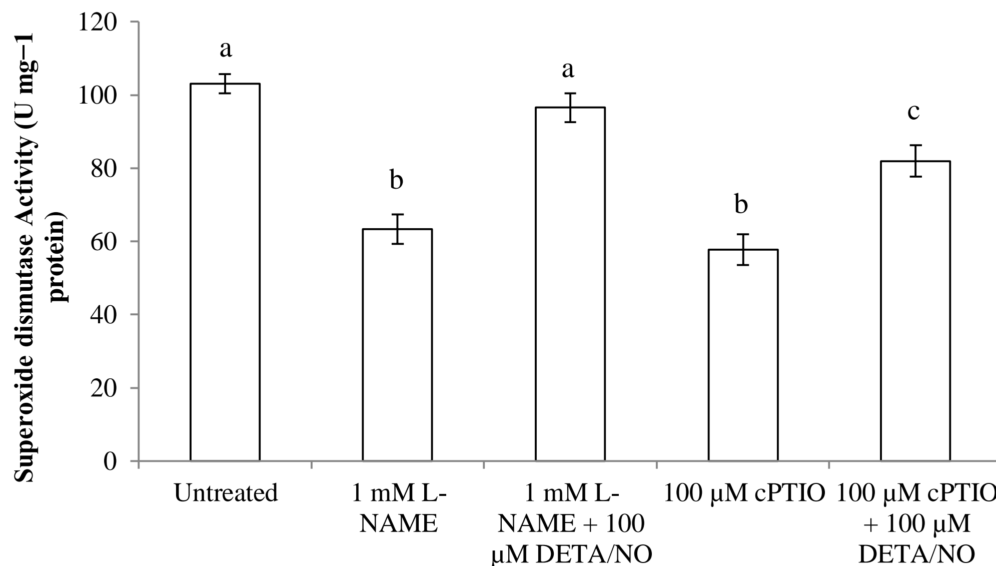
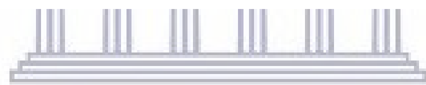


Figure 3.4. SOD total enzymatic activity in response to treatment with 1 mM nitric oxide synthase inhibitor, 100 µM nitric oxide scavenger and combination with 100 µM DETA/NO in soybean as measured by a spectrophotometric SOD assay. The various letters signify significant differences between means at $P<0.05$ (DMRT). Error bars represent the mean (±SE; n = 3) from data that are representative of experiments done in triplicate.



3.3.6. Nitric oxide alters the enzymatic activity of ascorbate peroxidase

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Ascorbate peroxidase (APX) is an enzyme that scavenges H_2O_2 and as a result regulates ROS levels within plants (Kornyeyev *et al.*, 2003; Maruta *et al.*, 2012; Keyster *et al.*, 2011). This regulation occurs via the oxidation of ascorbate to reduce H_2O_2 to H_2O (Dalton *et al.*, 1986, 1993; Noctor and Foyer, 1998; Iturbe-Ormaetxe *et al.*, 2001; Matamoros *et al.*, 2006; Sinha and Saxena, 2006). In this study, treatment with L-NAME displayed a ~47% decrease in APX activity in comparison to the control (figure 3.5.). In contrast, plants treated with cPTIO, compared to the control, showed a ~19% decrease in APX activity (figure 3.5.). Treatments with DETA/NO namely L-NAME, displayed an increase of ~103% in

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APX activity, whereas cPTIO increased by ~8% in comparison to the control. Comparing the treatments of L-NAME to L-NAME and DETA/NO, the APX activity was observed to be ~282% higher for the DETA/NO treatment. When comparing cPTIO to cPTIO and DETA/NO, the APX activity was observed to be ~33% higher for the DETA/NO treatment (figure 3.5.).

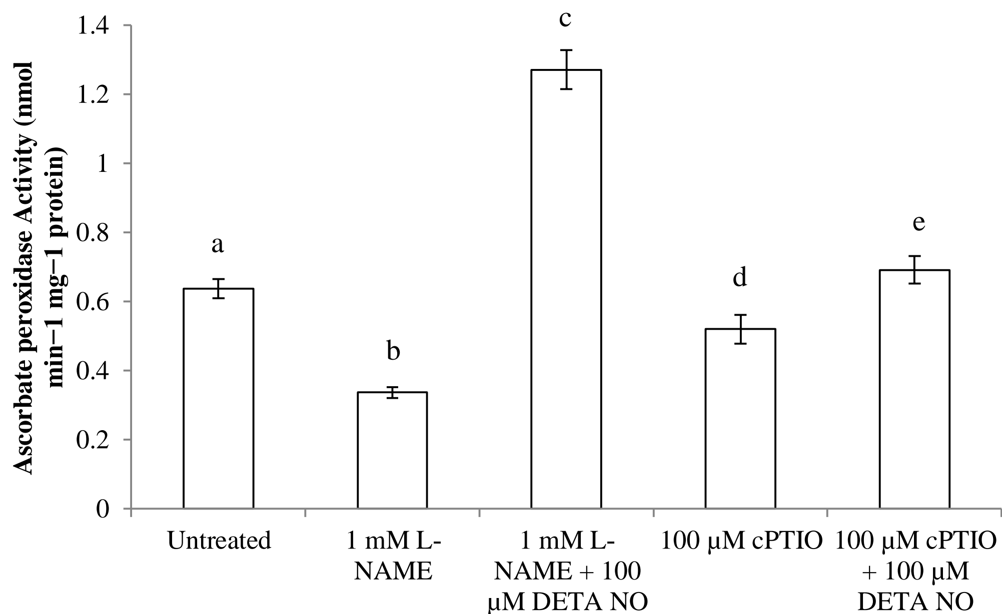

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Figure 3.5. APX total enzymatic activity in response to treatment with 1 mM nitric oxide synthase inhibitor, 100 µM nitric oxide scavenger and combination with 100 µM DETA/NO in soybean as measured by a kinetic spectrophotometric APX assay. The various letters signify significant differences between means at P<0.05 (DMRT). Error bars represent the mean (±SE; n = 3) from data that are representative of experiments done in triplicate.

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3.3.7. Nitric oxide alters the enzymatic activity of catalase

Catalase is an enzyme present in all living organisms exposed to oxygen and is responsible for the conversion of H_2O_2 to H_2O and O_2 (Chelikani *et al.* 2004). The role of catalase is to alleviate oxidative stress caused by ROS. Compared to the other enzymes, catalase has one of the highest enzyme turnover rates (Goodsell, 2004). Catalase contains four porphyrin groups that enable the enzyme to react with H_2O_2 and are the reason as to why catalase has such a high specificity for H_2O_2 (Askarov *et al.*, 1984; König *et al.*, 2002; Mhamdi *et al.*, 2010). It was therefore important to study the effect of NO on the total enzymatic activity of catalase. In comparison to the control, treatment with L-NAME displayed a ~55% decrease in CAT activity (figure 3.6.). In contrast, plants treated with cPTIO, compared to the control, showed a ~51% decrease in CAT activity (figure 3.6.). Treatments with DETA/NO namely L-NAME displayed a decrease of ~19% in CAT activity, whereas cPTIO decreased by ~11% in comparison to the control. When comparing the treatments of L-NAME to L-NAME and DETA/NO, the CAT activity was observed to be ~81% higher for the DETA/NO treatment. Similarly, comparing cPTIO to cPTIO and DETA/NO, the CAT activity was observed to be ~82% higher for the DETA/NO treatment (figure 3.6.).

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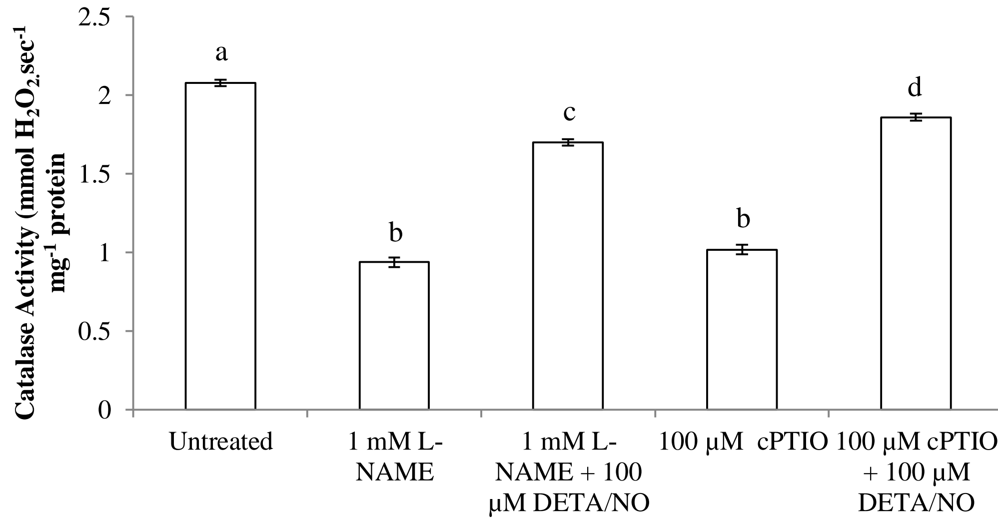

Figure 3.6. CAT total enzymatic activity in response to treatment with 1 mM nitric oxide synthase inhibitor, 100 µM nitric oxide scavenger and combination with 100 µM DETA/NO in soybean as measured by a kinetic spectrophotometric CAT assay. The various letters signify significant differences between means at $P<0.05$ (DMRT). Error bars represent the mean (±SE; n = 3) from data that are representative of experiments done in triplicate.

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3.4. Discussion

The study investigated the inhibition of nitric oxide synthase (NOS) and the implications this had on the antioxidant profiles within soybean using a NOS inhibitor (L-NAME) as well as a NO scavenger (cPTIO). Furthermore, a NO donor (DETA/NO) was used to assess whether the inhibitory and scavenging effects on NOS and NO, respectively, could be reversed. These included changes/fluctuations in chlorophyll content, H_2O_2 concentration, NO content, the degree of lipid peroxidation as well as changes in antioxidant activity namely superoxide dismutase, ascorbate peroxidase and catalase.

Chlorosis is a process within plants whereby an inadequate amount of chlorophyll is produced, but can also be attributed to the degradation of chlorophyll itself. Chlorophyll gives plants their distinct green colour and enables the plant to photosynthesise (Hörtensteiner and Kräutler, 2011). A key feature of chlorosis is the yellowing of the leaves, which was observed in three particular treatments namely with the nitric oxide synthase inhibitor (L-NAME), nitric oxide scavenger (cPTIO) and the combination of L-NAME with the NO donor, DETA/NO (table 3.1). This suggests that the affected plants were unable to sufficiently photosynthesise and produce the necessary carbohydrates as they were most likely undergoing oxidative stress. The DETA/NO treatment for cPTIO showed no difference in overall chlorophyll content (a and b) compared to the control. In comparison, to cPTIO, it appears that the DETA/NO treatment slightly alleviated the scavenging effect on NO. However, the treatments involving L-NAME and the combination of L-NAME and DETA/NO both showed a decrease in

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chlorophyll content compared to the control and each other, thus the overall chlorophyll content was much lower for these treatments (Table 3.1.).

It is interesting to note that an overall decrease was observed for the total chlorophyll concentration even after the addition of the DETA/NO across the treatments. However, the effects of NOS inhibition and NO scavenging were slightly alleviated with the addition of DETA/NO. The reduced chlorophyll content within the leaves can be attributed to the inhibitory as well as the scavenging effect on NOS and NO brought on by L-NAME and cPTIO, respectively. As previously stated, NO is known to play an important role in the regulation of various physiological processes in plants (figure 1.2); (Gill et al., 2013). Being a signalling molecule, it maintains plant redox homeostasis as well as alleviates oxidative stress caused by the accumulation of ROS (del Río et al., 2003; Palavan-Unsal and Arisan, 2009; Blokhina and Fagerstedt, 2010). Due to the significance of NOS and ultimately the synthesis of NO, the inhibition of its production may result in the poor expression of chlorophyll levels within the plant. This was observed in a study by Liu *et al.* (2013) who investigated whether NO regulated chlorophyll catabolism during dark-induced leaf senescence. Nitric oxide deficient mutants were used namely *nos1/noa1*. The study reported that during dark-induced leaf senescence a loss in chlorophyll had occurred in the *nos1/noa1* NO deficient mutants. The decrease was likely attributed to the up-regulation of genes responsible for chlorophyll catabolism. Furthermore, the *nos1/noa1* mutants had the pheophorbide *a* oxygenase (PAO) gene knocked out, which prevented the loss of chlorophyll during dark-induced leaf senescence. Thus, it was suggested that knocking out *PAO* from the mutants inhibited the

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degradation of chlorophyll as it caused an accumulation of pheophorbide *a*, a chlorophyll catabolite. The study concluded that NO acted as an anti-senescence signalling molecule that supressed the transcriptional activation of the genes responsible for chlorophyll senescence. Thus NO is seen to play a crucial role in the regulation of plant physiological processes such as the ability to photosynthesise. This is further supported by a study of Laxalt *et al.* (1997) who investigated the effect the inhibition of NOS had during the development of plant disease and its supposed role in preventing infection. The study focussed on the effect a NO donor had on the chlorophyll content in potato leaves inoculated with *Phytophthora infestans.* These inoculated plants were either treated with water (control), 100 µM NNLA (N-nitro-L-arginine) a NOS inhibitor, 100 µM SNP (sodium nitroprusside) a NO donor or a combination of 100 µM SNP and 100 µM NNLA. It was observed that inoculated plants maintained in water as well as inoculated plants treated with NNLA showed a decrease in chlorophyll content over a period of two weeks. Infected plants treated with SNP however seemed to alleviate these effects and showed an increase in chlorophyll content. This was also observed for the combination treatment of SNP with NNLA. The increase in chlorophyll content due to the addition of SNP was not, however, modified by NNLA. This was most likely due to using too low of a concentration of NNLA. It was thus concluded that NO is pivotal in regulating chlorophyll levels in infected potato leaves. Therefore, the findings of these studies are in support of the results of the current study where the inhibitory and scavenging effects on NOS and NO, respectively, were seen to be alleviated by the addition of DETA/NO.

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Malondialdehyde (MDA) is an endogenous cytotoxic compound used as a marker for the measure of lipid peroxidation (Marnett and Hancock, 1999). The implications of lipid peroxidation include the deterioration of cellular membranes which affect the integrity and permeability of a cell, ultimately leading to the leakage of ions (Sinha and Saxena, 2005; Zhang *et al.*, 2006). Malondialdehyde concentrations can be quantified by means of the thiobarbituric acid reactive substance (TBARS) assay. This particular assay uses the concentration of MDA to estimate the extent of damage caused by lipid peroxidation and was thus used for this chapter. The results of this study showed that the application of a NOS inhibitor (L-NAME) and NO scavenger (cPTIO) caused the peroxidation of membrane lipids, as an increase in MDA content was observed in the leaves of soybean (figure 3.1.). However, the application of the NO donor (DETA/NO) alleviated the effects of lipid peroxidation as the levels of MDA decreased close to that of the control plant.

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Similarly a study by Egbichi *et al.* (2013) investigated whether the toxic effects of salt stress could be alleviated through the application of NO donor, DETA/NO. Treatments included 150 mM NaCl (alone), 150 mM NaCl in combination with 10 µM DETA/NO and 150 mM NaCl in combination with 10 µM DETA. It was observed that salt stress resulted in an increase in MDA content. Interestingly, the addition of the DETA/NO was observed to alleviate the toxic effect of salt stress on lipid peroxidation by ~24%. This alleviating effect was also observed with the combination treatment of DETA/NO + NaCl. Thus, it was suggested that the application of DETA/NO protected plants against oxidative damage brought on by

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lipid peroxidation, as the exogenous application of NO reduced the levels of MDA caused by the salt stress.

Furthermore, a study by Arasimowicz-Jelonek *et al.* (2009) investigated the effect water deficit had on the nitric oxide (NO) content in cucumber seedling roots. Treatments of plants included water (control), two NO donors namely 100 mM sodium nitroprusside (SNP) and 100 mM S-nitrosoglutathione (GSNO), 200 mM cPTIO (NO scavenger) and a combination of 100 mM SNP with 200 mM cPTIO. Plant root tips subjected to water deficit displayed increased NO content. However, with addition of the NO scavenger, (cPTIO) this water-deficit NO synthesis was hindered. This was also partially observed with the application of NOS-like inhibitors namely L-NAME (100 µM). Lipid peroxidation was measured using Schiff's reagent, which showed intense pink staining in the control root seedlings. In comparison to the control, treatment with SNP showed weaker staining throughout the root seedlings, whereas GSNO caused pink staining mostly in the subapical portion of the roots. The study proposed that degree of lipid peroxidation brought on by water deficit was lowered with the addition of exogenous NO (NO donors). This was confirmed as the scavenging effect of NO using cPTIO resulted in an increase in TBARS content (MDA) a by-product of lipid peroxidation. Thus these findings are in support of the results of the current study. It can be seen that NO as a signalling molecule plays crucial role in maintaining redox homeostasis in plants as it alleviates the effects caused by oxidative stress for example by regulating membrane integrity. Literature has shown NO to be an effective inhibitor against lipid peroxidation, whereby it scavenges peroxyl radicals (Simontacchi *et al.*, 2015). Thus, the poor expression

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of NO levels in plants due to the inhibitory and scavenging effects on NOS and NO, respectively, would be detrimental, as NO will no longer be able to exert a protective role effect against oxidative stress.

As previously mentioned, NO is known to regulate and maintain plant redox homeostasis by alleviating the effects caused by the accumulation of ROS (del Río *et al.*, 2003; Palavan-Unsal and Arisan, 2009; Blokhina and Fagerstedt, 2010). Nitric oxide can be quantified by means of the oxyhemoglobin based assay. This assay was used for this study and is based on the reaction of NO and oxyhemoglobin (HbO_2), yielding methemoglobin (metHb) and nitrate (Murphy and Noack, 1994). The results of this chapter showed that the application of the nitric oxide synthase inhibitor, L-NAME and the nitric oxide scavenger, cPTIO caused a decrease in NO content in response to the inhibitory and scavenging effects on NOS and NO, respectively (figure 3.2.). However, the application of exogenous NO using the NO donor, DETA/NO, reversed the effects caused by the above mentioned treatments as it alleviated the inhibitory and scavenging effects on NOS and NO, respectively, thus increasing the NO content. The above findings are supported by the following studies done by Graziano *et al.* (2007), Freschi *et al.* (2010) and Keyster (2011).

The study by Graziano *et al.* (2007) observed that tomato plants grown in iron-deficient conditions, resulted in enhanced NO production. Plants were grown in media containing either 0.1 µM FeNaEDTA (iron deficient) or 50 µM FeNaEDTA (iron surplus). Treatments were as follows, iron-sufficient plants were treated with either 1 mM cPTIO (NO scavenger) or 1 mM L-NAME (NOS inhibitor), whereas iron-deficient plants were treated with 50 µM GSNO (NO

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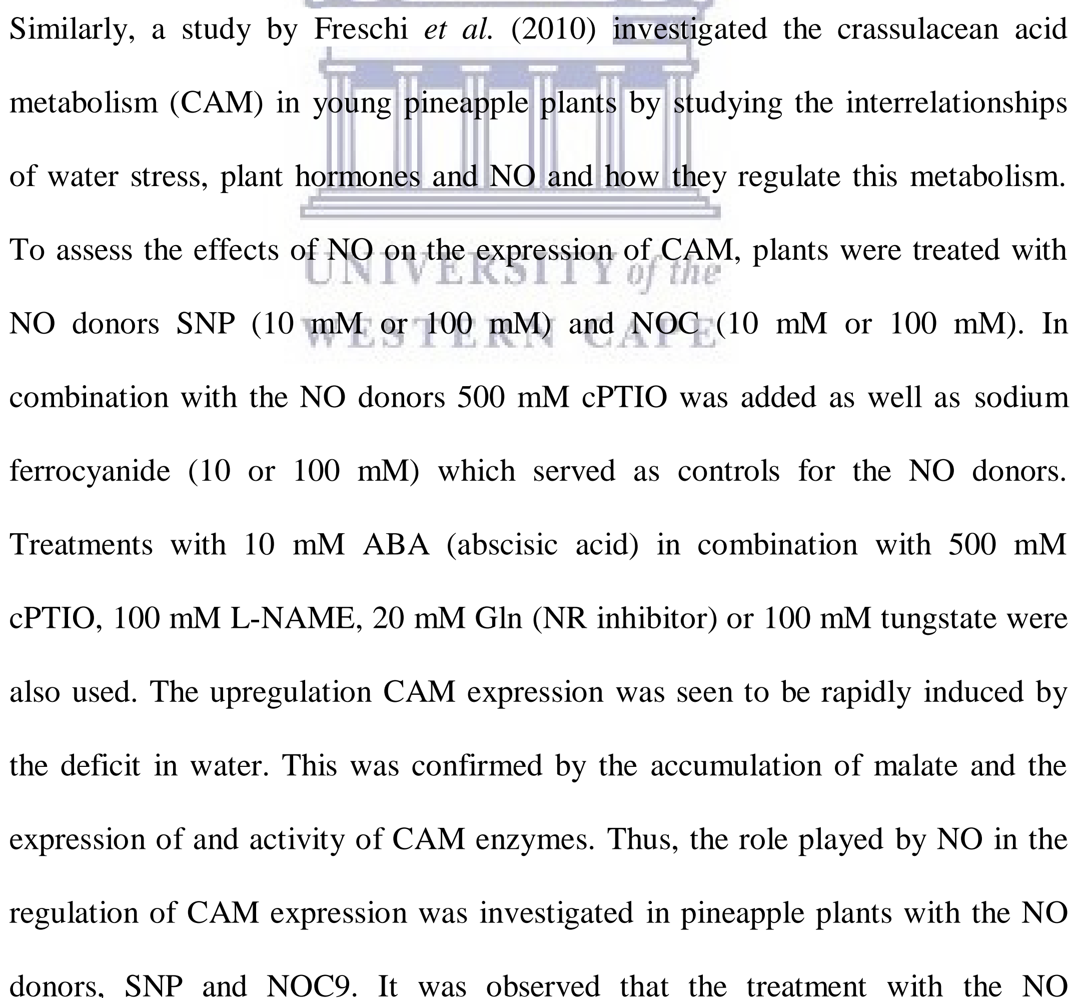
donor). It was reported that the NO scavenger, cPTIO, prohibited the iron-deficiency induced upregulation of transcription factor FER, LeFRO1 (ferric-chelate reductase) and LeIRT1 [Fe(II) transporter] genes. In contrast, the exogenous application of NO using NO donor, GSNO, alleviated the inhibition of iron-deficiency induced upregulation of FER, LeFRO1 and LeIRT1 in iron-deficient plants. The treatment with 1 mM L-NAME had little to no effect on NO content in iron-deprived plants and was unable to prevent iron-deficiency induced gene expression. Thus the study proposed that NO plays a crucial role in regulating iron uptake, but ultimately oxidative stress to maintain plant homeostasis.



Similarly, a study by Freschi *et al.* (2010) investigated the crassulacean acid metabolism (CAM) in young pineapple plants by studying the interrelationships of water stress, plant hormones and NO and how they regulate this metabolism. To assess the effects of NO on the expression of CAM, plants were treated with NO donors SNP (10 mM or 100 mM) and NOC (10 mM or 100 mM). In combination with the NO donors 500 mM cPTIO was added as well as sodium ferrocyanide (10 or 100 mM) which served as controls for the NO donors. Treatments with 10 mM ABA (abscisic acid) in combination with 500 mM cPTIO, 100 mM L-NAME, 20 mM Gln (NR inhibitor) or 100 mM tungstate were also used. The upregulation CAM expression was seen to be rapidly induced by the deficit in water. This was confirmed by the accumulation of malate and the expression of and activity of CAM enzymes. Thus, the role played by NO in the regulation of CAM expression was investigated in pineapple plants with the NO donors, SNP and NOC9. It was observed that the treatment with the NO

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scavenger, cPTIO, inhibited the upregulation of CAM expression, whereas NOS inhibitor L-NAME was observed to have no effect. It was also observed that control treatments with sodium ferrocyanide, had no effect on CAM expression. In contrast, the combination of SNP with NO scavenger, 500 mM cPTIO, inhibited CAM expression entirely, suggesting that the effect of SNP relied on the release of NO. The study proposed that NO acts as a key component in the ABA-dependent signalling pathway when signalling CAM expression.

In addition to the findings of the above studies, the study by Keyster *et al.* (2011) investigated the effect the inhibiton of NOS and application of exogenous NO had on root nodule functioning. What was observed in the study was that treatments with the NOS inhibitor L-NNA (Nω-nitro-L-arginine) caused a decrease in NO content. However, the combination of L-NNA with DETA/NO reversed the inhibitory effects on NOS, restoring NO content close to that of the control. These findings are supported by the results of the current chapter. According to literature the inhibition of NOS is caused by the use of L-arginine analogue, L-NAME as well as L-NNA, which has been used for many plant NO studies (Graziano and Lamattina, 2007). The fact that plants treated with L-NAME caused a decrease in NO content within the current study suggests that L-NAME is indeed a sufficient inhibitor of NOS. This is strongly supported by the above studies of Graziano *et al.* (2007), Freschi *et al.*, (2010) and Keyster (2011) as well as the fact that the exogenous application of NO using DETA/NO reversed the inhibitory and scavenging effects on NOS and NO, respectively.

Despite an increase in lipid peroxidation, changes in NO are also associated with an increase in H_2O_2. When H_2O_2 accumulates within plants, the plant induces a

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hypersensitive response, which is the equivalent to the innate immune system found in mammals, which is a form of cell death (Mur *et al.*, 2008). Hydrogen peroxide is a highly abundant signalling molecule that is produced in many cellular organelles such as chloroplasts, peroxisomes, plasma membranes and the extracellular matrix to name a few (figure 1.3); (Fröhlich and Durner, 2011; George, 2014). According to literature, higher concentrations of H_2O_2 are present in the leaves of plants, as cellular organelles are most abundant in the leaves (Ślesak *et al.*, 2007). The above mentioned is consistent with the findings of the current study where high concentrations of H_2O_2 were observed in response to changes in NO content (figure 3.3.). The results of this study showed that the application of a NOS inhibitor (L-NAME) and NO scavenger (cPTIO) caused an increase in H_2O_2 in response to the inhibition and scavenging of NOS and NO, respectively. However, the exogenous application with the NO donor (DETA/NO) decreased the levels of H_2O_2 close to that of the untreated control plant, whilst NO content increased. The results of this study are supported by a study done by Sang *et al.* (2008) that investigated the sources of NO production in maize under water stress and the role NO plays in the water stress-induced hydrogen peroxide (H_2O_2) accumulation and subcellular activities of antioxidants. Plants were pre-treated with 200 µM cPTIO (NO scavenger), 200 µM L-NAME (NOS inhibitor), 5 mM PBITU (NOS inhibitor), 100 µM NaN_3 (NR inhibitor), 2 mM KCN (NR inhibitor) after which each treatment was exposed to poly(ethylene glycol) (PEG) . It is known that ROS accumulate in response to oxidative stress. That being said, Sang *et al.* (2008), showed that NO acts as a scavenger of H_2O_2, decreasing its concentration induced by water stress. Treatment with the NO donor, SNP, was

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observed to significantly lower the levels of H_2O_2 that were detected using $CeCl_3$ staining, which is a cytochemical technique for the detection of H_2O_2. As a control for SNP, Fe(III)CN was used to determine the role of NO in the experiments, and was shown to have no effect on water stress-induced H_2O_2 accumulation in the maize plants. Treatments with cPTIO and L-NAME were used to clarify the physiological effects of endogenous NO-mediated H_2O_2 under water stress and were observed to have no effect on the reduction of accumulated H_2O_2 in maize under water stress. Thus, the study suggested that NO has the ability to decrease the H_2O_2 concentration in plants under water stress. As NO was shown to decrease the levels of H_2O_2 due to water stress, it was suggested that low NO concentrations partially protect plants from oxidative damage brought on by water stress. In order to understand how NO facilitates the accumulation of H_2O_2 in the leaves of maize; the authors investigated the effect of NO on water stress-induced activities of antioxidant enzymes in the leaf chloroplast and cytosol. It was observed that the antioxidant activities of SOD, APX and GR, were enhanced in the presence of NO donor, SNP, compared to the untreated control plants. This suggested that NO alleviates the effects brought water stress-induced H_2O_2 and thus enhances antioxidant enzyme activity. The study concluded that NOS and NR are sources for water stress-induced NO production and that NO acts as a ROS scavenger, when the antioxidant defence system in plants is induced, to overcome oxidative stress such as the accumulation of H_2O_2. The findings of Sang *et al.* (2008) are consistent with the results of the current study. The high level of H_2O_2 in the soybean leaves suggest that the plants were undergoing oxidative stress. This was most likely attributed to the imbalance in the rate of production and

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removal of ROS, subsequently down regulating the antioxidant activity, ultimately perturbing redox homeostasis within soybean. Therefore, it was of utmost importance to determine the antioxidant activity of superoxide dismutase, ascorbate peroxidase and catalase that are known to be associated with the scavenging of H_2O_2.

The superoxide radical is the first ROS produced and is highly toxic if not properly regulated. The toxicity is attributed to the formation of highly reactive hydroxyl radicals that are a result of the superoxide radical interacting with hydrogen peroxide (Tsang *et al.*, 1991). However, superoxide has a relatively short half-life and is removed through its dismutation by the antioxidant superoxide dismutase into oxygen and hydrogen peroxide. Superoxide dismutase is a key enzyme in the antioxidant defence system as it is the first line of defence against ROS that regulates the concentration of the superoxide radical and hydrogen peroxide (Alscher, Erturk and Heath, 2002). Thus this study investigated the effect NOS inhibition and scavenging of NO, had on the total superoxide dismutase (SOD) activity in soybean leaves by means of a spectrophotometric assay for SOD. The application of the NOS inhibitor, L-NAME and NO scavenger, cPTIO, resulted in a decrease in total SOD activity (figure 3.4.). The exogenous application of the nitric oxide donor, DETA/NO, was observed to increase the total SOD activity close to that of the untreated control. The findings of this study are supported by a study done by Liphoto (2010) that investigated the effect changes in NO had on the modulation of root nodule antioxidant systems. It was observed that a decrease in NO caused a decrease in the total activity of SOD using an inhibitor of NOS and scavenger of NO.

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However, the inhibitory and scavenging effects on NOS and NO were reversed with the application of a NO donor thus increasing the total SOD activity more than that of the control. These findings correlate with the results of the current study. According to literature copious amounts of superoxide can deter the production of NO, subsequently decreasing the antioxidant capacity of SOD (Chávez *et al.*, 2007). Superoxide dismutase competes with NO for removal of the superoxide radical. When NO and superoxide interact it results in the formation of peroxynitrite ($ONOO^-$) a strong oxidising agent. Peroxynitrite is highly reactive and if not regulated through the removal of superoxide by SOD it can cause damage to macromolecules as well as lead to lipid peroxidation (Cejas *et al.*, 2004). Thus, the availability of NO is important for maintaining redox homeostasis in plants allowing SOD to efficiently scavenge superoxide.

Following the dismutation of the highly reactive superoxide radical, hydrogen peroxide and oxygen are the end result. As previously mentioned, it is well known that the accumulation of H_2O_2 is the reason for oxidative stress in plants. If not regulated, H_2O_2 can be detrimental to the plant resulting in the inactivation of enzymes such as ascorbate peroxidase (APX), an antioxidant responsible for the scavenging and thus removal of H_2O_2 (Van Breusegem *et al.*, 2001; Gill and Tuteja, 2010). Due to the ability of H_2O_2 to traverse cellular membranes, it makes sense that APX can be found throughout the cytosol. Ultimately the concentration of H_2O_2 is lowered and the effects of oxidative stress are alleviated (Noctor and Foyer, 1998; Sinha and Saxena, 2006). In this study the application of the NOS inhibitor, L-NAME and NO scavenger, cPTIO, resulted in a decrease in the total activity of APX compared to the untreated control (figure 3.5.). This decrease in

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APX activity could be attributed to the inhibitory and scavenging effects on NOS and NO, respectively, which decreased the levels of NO as seen in figure 3.2. Without nitric oxide the plant is unable to regulate and maintain plant redox homeostasis and as a result limits the plants ability to induce its antioxidant system adequately. This is clear in the study, as the decrease in NO due to the above mentioned treatments caused a decrease in APX activity which resulted in an increase in lipid peroxidation and H_2O_2 content within the leaves, as the plant was under oxidative stress (see figures 3.1. and 3.3.). However, the total APX activity increased when NO was exogenously applied using NO donor, DETA/NO, alleviating the oxidative stress due to the accumulation of H_2O_2. The findings of this study are supported by the studies done by Shi *et al.* (2007) and Li *et al.* (2008).

The study by Li *et al.* (2008) investigated the changes in antioxidant enzyme activity, ferritin accumulation as well as the protective role of NO on barley leaves under salt stress. Treatments included a control of which no SNP and NaCl were added along with three other treatments namely 50 µM SNP; 50 mM NaCl as well as combination of 50 µM SNP and 50 mM NaCl. The study observed an increase in lipid peroxidation and ion leakage due to salt stress in the barley leaves. Treatment with 50 µM SNP (NO donor) decreased MDA content and ion leakage, H_2O_2 and carbonyl content, as well as increased the accumulation of ferritin at the protein level, alleviating the damage brought on by salt stress. In addition, the SNP was observed to enhance the activities of SOD, APX and CAT. Similarly, the study by Shi *et al.* (2007) investigated the effects of exogenous NO on both ROS metabolism in mitochondria and functions of plasma membrane and

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tonoplasts in cucumber seedlings under salt stress. Treatments included a control of which no SNP and NaCl were added along with five other treatments namely 50 mM SNP (NO donor); 100 mM NaCl, combination of 50 mM SNP and 100 mM NaCl; combination of 100 mM NaCl, 50 mM SNP and 50 mM sodium ferrocyanide; and lastly a combination of 100 mM NaCl, 50 mM SNP and 0.1% hemoglobin (NO Scavenger). The study observed an increase in H_2O_2 content in response to salt stress, which led to an increase in MDA content in cucumber mitochondria. The application of 50 mM SNP (NO donor) enhanced the activity of the antioxidant enzymes, which reduced the concentration of H_2O_2 in the mitochondria of cucumber roots, causing a decrease in MDA content. The application of SNP was also observed to reverse the inhibition of H^+-ATPase and H^+-PPase in plasma membranes and or tonoplasts by NaCl. Ferrocyanide was used as a control for the NO donor, SNP, and had no effect. In addition the effects of SNP were reverted with the addition of hemoglobin (NO scavenger). The APX activity in the mitochondria of cucumber root was observed to be enhanced in response to salt stress after 4 days. The exogenous application of NO had no effect on the APX activity. However, after 8 days of treatment, APX activity was observed to be significantly inhibited by salt stress and the addition of SNP was observed to alleviate the inhibition of APX activity. The application of the NO scavenger, hemoglobin and the SNP control, ferrocyanide, had little to no effect on the APX activity in combination with SNP. Thus, both studies suggested that NO effectively exerts a protective function in plants undergoing oxidative stress, increasing the antioxidant enzyme activities and thus alleviating the effects caused by an increase in ROS.

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In addition to the removal of H_2O_2 by APX, catalase (CAT) also aids in the scavenging and detoxification of H_2O_2 resulting in the release of water and oxygen. In this study the application of the NOS inhibitor, L-NAME and NO scavenger, cPTIO, showed a decrease in the total activity of CAT in response to the inhibition and scavenging of NOS and NO, respectively, compared to the untreated control (figure 3.6.) Similarly to APX, the decrease in CAT activity could be attributed to the low levels of NO content (figure 3.2.). As a result, the plant cannot regulate the levels of ROS and thus affect the plants ability to induce the antioxidant system adequately. This is clear in the current study as the decrease in NO due to the above mentioned treatments caused a decrease in total CAT activity and as a result increased lipid peroxidation and H_2O_2 content within the leaves of soybean (see figures 3.1. and 3.3.). However, when exogenous NO was applied using the NO donor, DETA/NO, the total CAT activity was observed to increase, alleviating the oxidative stress brought on by the high H_2O_2 concentration. The findings of this study are supported by the studies done by Shi *et al.* (2007) and Li *et al.* (2008) as mentioned previously with APX. To briefly summarise, both studies by Shi *et al.* (2007) and Li *et al.* (2008) observed that plants subject to salt stress resulted in an increase in oxidative stress. However, the application of a NO donor alleviated the effects of oxidative damage and subsequently enhanced the antioxidant enzyme activities of SOD, APX and CAT.

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In conclusion throughout the chapter it was observed that the application of a NOS inhibitor and NO scavenger had a detrimental effect on the NO content within soybean. These effects were shown to affect both the plants physiological as well as biochemical processes. The inhibition of NOS and scavenging of NO

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was shown to affect the photosynthetic capabilities within soybean and result in chlorosis. An increase in ROS was also observed in response to the inhibition of NOS and the scavenging of NO, resulting in an increase in both lipid peroxidation and H_2O_2 content. These increases are most likely attributed to the effect the inhibition of NOS and scavenging of NO had on the antioxidant capacities within soybean and thus the plant was unable to induce its antioxidant system adequately. However, when exogenous NO was applied all of the above effects were reversed suggesting that NO functions as an antioxidant, as it carries out a protective role against oxidative stress. In addition, as a signalling molecule, NO is observed to be crucial for the regulation of the plants antioxidant system which in turn maintains redox homeostasis within plants.

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Chapter 4

The inhibition of nitric oxide biosynthesis alters the expression of antioxidant isoform activity in soybean

4.1. Abstract

Soybean is an economically important food crop worldwide. In the South African agricultural industry, it is of high economic and nutritional value making it an excellent source of protein. According to the US Food and Drug Administration, soybean produces twice as much protein compared to other major grain and vegetable crops, making it an interesting crop to study (Henkel, 2000). It is well known that nitric oxide and reactive oxygen species both act as signalling molecules, controlling processes such as growth, differentiation and response to biotic and abiotic stresses. Based on this commonality, it was of utmost importance to investigate the effect of the inhibition of nitric oxide biosynthesis on the isoform expression patterns of a subset of antioxidant enzymes. These antioxidants play a crucial role in the ROS scavenging pathways of soybean. Profound changes were observed in the in-gel activity assays for SOD, APX and CAT, as the expression patterns for their respective isoforms decreased when nitric oxide content was lowered by either using a nitric oxide synthase inhibitor (L-NAME) or a nitric oxide scavenger (cPTIO). This inhibitory and scavenging effect on NOS and NO was observed to decrease the isoform activities for SOD, APX and CAT as a decrease in band intensity was observed. However, these changes in isoform expression were either partially or fully alleviated by the

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application of a nitric oxide donor, DETA/NO. Thus, we conclude that endogenously synthesized nitric oxide is a crucial component of plant signalling to induce ROS scavenging pathways.

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4.2. Introduction

The accumulation of reactive oxygen species (ROS) is known to cause oxidative stress in plants when not properly regulated (figure 1.1). However, when regulated, ROS are known to be fundamental in the regulation of plant physiological processes such as growth, differentiation and response to biotic and abiotic stresses. Thus, ROS are considered to be signalling molecules (Pitzschke, Forzani and Hirt, 2006). In the same way, nitric oxide (NO) an endogenously synthesised signalling molecule is essential for the regulation of plant physiological processes such as growth, development and response to biotic and abiotic stresses (figure 1.2; figure 1.3); (Jack, 2012; Keyster *et al.*, 2014). Thus, it is important to better understand the relationship that NO and ROS share in the regulation of physiological and biochemical processes. This understanding would give better insight into how plants respond to biotic and abiotic stresses mediated through changes in the production and metabolism of these two signalling molecules. Furthermore, this shared commonality between NO and ROS would also aid in understanding how these changes affect the antioxidant activities of a subset of antioxidant enzymes responsible for regulation of ROS concentrations. It is well known that antioxidant enzymes regulate ROS homeostasis within plants, where they exert a protective function against oxidative stress (Sinha and Saxena, 2006; Lee *et al.*, 2007). If an imbalance in ROS production and antioxidant enzyme concentration occurs in plants, the plant is said to be experiencing oxidative stress. Thus the antioxidant system within plants may contain many isoforms of the same antioxidant enzyme (Sarowar *et al.,* 2005). The antioxidant enzyme activities can be affected by various stresses such as

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drought, salt stress, soil pH etc., either down- or upregulating the expression pattern of the isoforms or completely inhibiting the isoform activities. In chapter 3 we concluded that endogenously synthesized nitric oxide is crucial for the regulation of physiological as well as biochemical processes in plants. Thus, it is of utmost importance to investigate the effect of the inhibition of nitric oxide biosynthesis on ROS scavenging pathways, for example, the antioxidant enzymes activities (isoform expression patterns), brought on by the use of a nitric oxide synthase inhibitor and nitric oxide scavenger.

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4.3. Results

4.3.1. Nitric oxide modulates antioxidant activities in soybean

The first scavenging antioxidant enzyme is SOD, which catalyses the conversion of O_2^- to H_2O_2. Superoxide dismutase was first discovered by McCord and Fridovich (1969) that isolated the blue copper-containing protein from bovine erythrocytes. All SODs contain metal co-factors and are thus metalloenzymes. These SOD isoforms can be classified as being copper zinc SODs (CuZnSODs), iron SODs (FeSODs) or manganese SODs (MnSODs) depending on the metal in their active site (DiGuiseppi, Fridovich and McCord, 1984). The requirement for different metals by metalloenzymes could be attributable to an evolutionary response to the availability of some metals (Mahanty *et al.*, 2012). The objectives of this chapter were to identify soybean antioxidant isoform expression patterns in response to the inhibition and scavenging of NOS and NO, respectively, starting with SOD.

4.3.1.1. Identification of SOD classes and the effect of NO on the activity of SOD isoforms in soybean

In total 13 SODs were identified in the leaves of soybean of which two isoforms are MnSODs, three are FeSODs and eight are CuZnSODs (figure 4.1). The inhibition pattern of SOD isoforms determine the class each SOD belonged to

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(figure 4.2); (Section 2.10.1). When compared to the control (untreated), a decrease in band intensity for the FeSODs and MnSODs was observed for the individual treatments with either the NOS inhibitor, L-NAME or NO scavenger, cPTIO. However, an increase in band intensity for the FeSODS and MnSODs was observed in the two treatments with DETA/NO close to that of the control. When comparing both individual treatments namely L-NAME and cPTIO to their DETA/NO counterparts, the band intensities for the FeSODS and MnSODs were observed to be much higher in the treatments with DETA/NO.

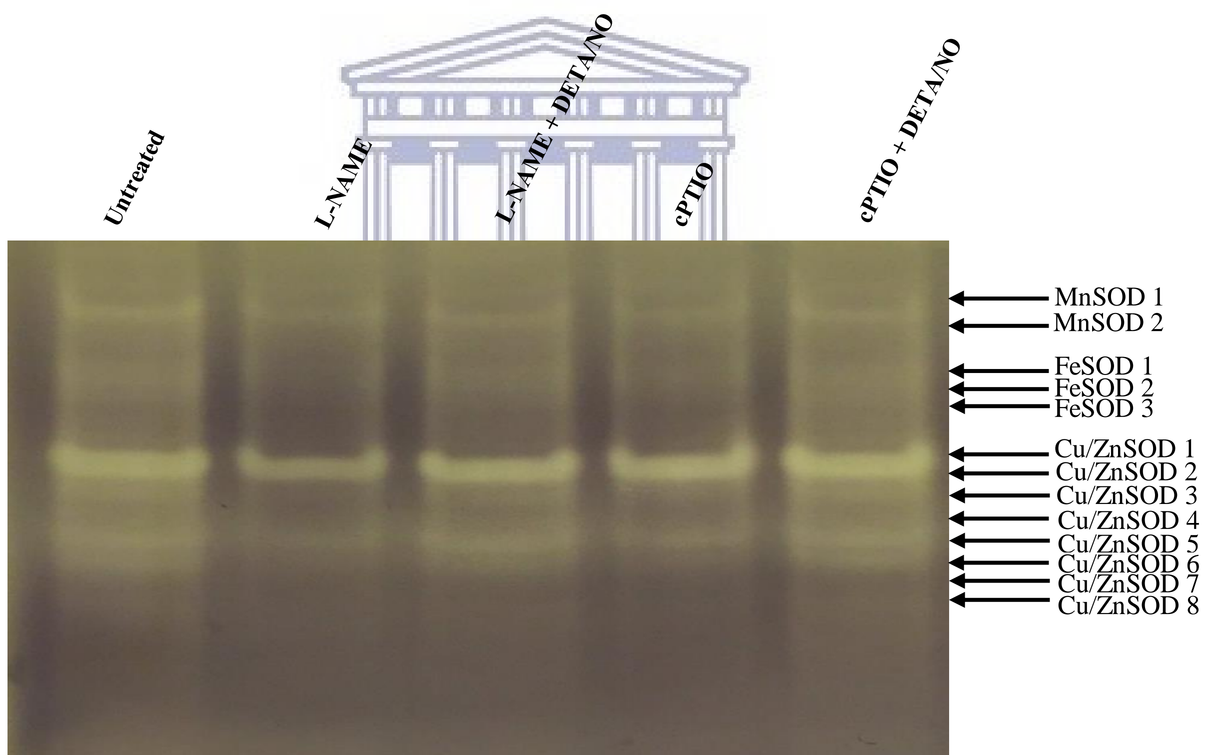

Figure 4.1. The effect 1 mM L-NAME, 100 µM cPTIO and combination treatments with 100 µM DETA/NO on the activity profile of superoxide dismutase in soybean. SOD in-gel activity profiling was conducted on plant material which was treated with the above, respectively. This in-gel assay was conducted to understand how changes in nitric oxide content influenced SOD isoforms. The gel illustrates the effect nitric oxide content has on the SOD isoforms.

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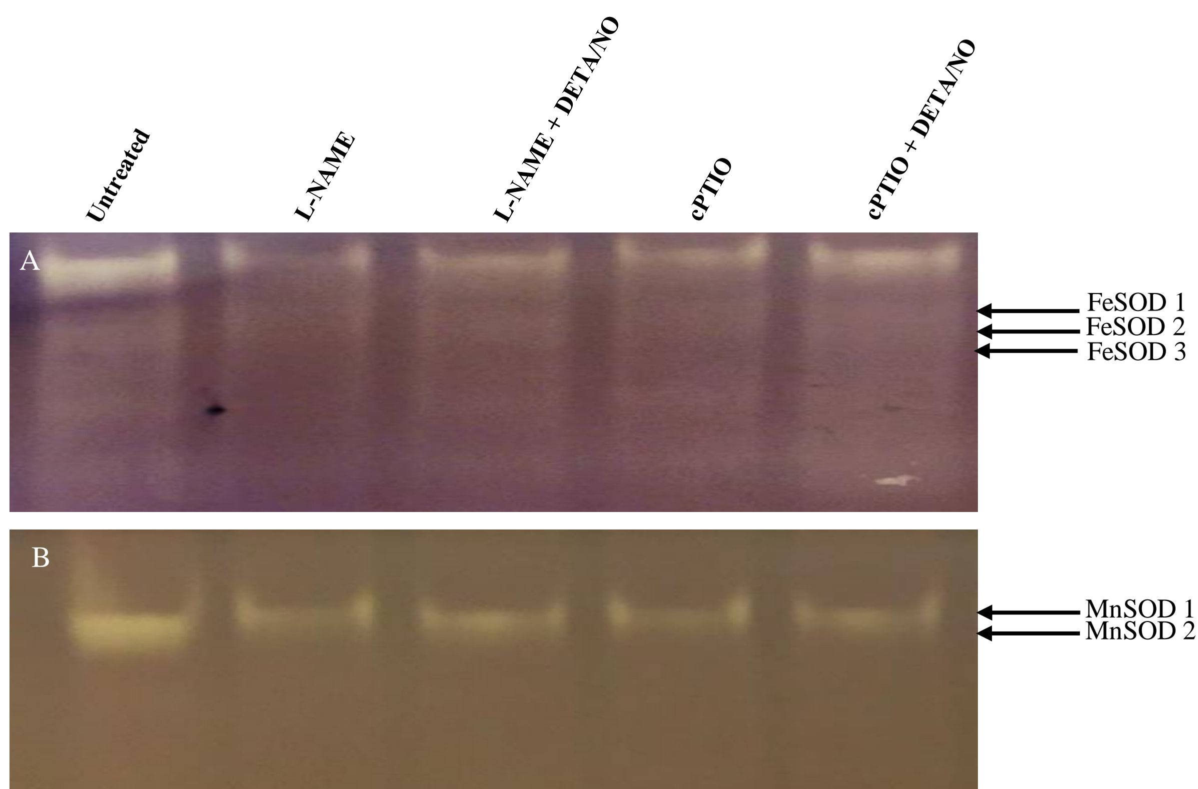

Figure 4.2. SOD class Identification. SODs were classified according to which compounds were able to inhibit their activity; the compounds used were (A) potassium cyanide (KCN) and (B) hydrogen peroxide (H_2O_2).

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Densitometry analysis was performed to determine the change in isoform activities due to the inhibition and scavenging of NOS and NO, respectively (table 4.1). The following results were grouped according to their respective isoform classes, namely MnSODs (MnSOD 1 and 2), FeSODs (FeSOD 1, 2 and 3) and CuZnSODs (CuZnSODs 1-8). The intensity values (AU) were calculated as percentage increases or decreases and given as a percentage range across the SOD classes. The individual intensity values for each isoform are shown in table 4.1. The MnSODs account for two of the 13 isoforms. When compared to the control, the treatment with L-NAME showed a ~20-30% decrease in intensity units for MnSODs. Treatment with cPTIO showed a ~20-26% decrease in intensity units for MnSODs when compared to the control. A ~11-25% decrease in intensity

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units for MnSODs was observed for the combination treatment of L-NAME and DETA/NO when compared to the control. The combination treatment of cPTIO and DETA/NO displayed a decrease in intensity units for MnSODs of ~8-10% in comparison to the control. When comparing the intensity units of MnSODs, the treatments of L-NAME to L-NAME and DETA/NO showed a ~8-10% increase for the DETA/NO treatment. The comparison of treatments, cPTIO to cPTIO and DETA/NO showed an increase in intensity units for MnSODs of ~14-21% for the DETA/NO treatment (table 4.1).

The FeSODs account for three of the 12 isoforms. When compared to the control, the treatment with L-NAME showed a ~38-53% decrease in intensity units for FeSODs. The treatment with cPTIO showed a ~26-34% decrease in intensity units for FeSODs when compared to the control. The treatment in combination with DETA/NO showed a ~20-21% decrease in intensity units for FeSODs for L-NAME and DETA/NO compared to the control. In contrast, the combination of cPTIO and DETA/NO caused a ~6-11% increase in intensity units for FeSODs when compared to the control. When comparing the intensity units of FeSODs for the treatments of L-NAME to L-NAME and DETA/NO, a ~29-68% increase was observed for the DETA/NO treatment. In contrast, comparing cPTIO to cPTIO and DETA/NO a ~54-68% increase in intensity units for FeSODs was observed for the DETA/NO treatment. The Cu/ZnSODs account for the remaining eight isoforms. When compared to the control, the treatment with L-NAME showed a decrease in intensity units for Cu/ZnSODs of ~29-60% was observed. Treatment with cPTIO showed a decrease in intensity units for Cu/ZnSODs of ~2-37% when compared to the control. A ~6-43% decrease in intensity units for Cu/ZnSODs

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was observed for the combination treatment of L-NAME and DETA/NO when compared to the control. The combination treatment of cPTIO and DETA/NO displayed a ~1-12% increase in intensity units for Cu/ZnSODs 1-6, whereas Cu/ZnSODs 7-8 showed a 10-18% decrease compared to the control (table 4.1). When comparing the intensity units of Cu/ZnSODs, the treatments of L-NAME to L-NAME and DETA/NO, an increase of ~32-43% is observed for the DETA/NO treatment. The comparison of treatments, cPTIO to cPTIO and DETA/NO showed a ~15-27% increase in intensity units for Cu/ZnSODs 1-8 for the DETA/NO treatment (table 4.1).

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Table 4.1. Densitometry analysis for SOD native PAGE activity gels (AU).

SOD Isoforms	Untreated	L-NAME	L-NAME + DETA/NO	cPTIO	cPTIO + DETA/NO
MnSOD 1	61 ± 1.58[a]	49 ± 1.35[c]	54 ± 1.35[g]	49 ± 1.33[c]	56 ± 1.33[g]
MnSOD 2	69 ± 1.16[b]	48 ± 1.94[c]	52 ± 1.50[c]	51 ± 1.14[c]	62 ± 1.57[a]
FeSOD 1	50 ± 1.14[c]	31 ± 1.69[d]	40 ± 1.36[f]	37 ± 1.85[f]	53 ± 1.69[g]
FeSOD 2	51 ± 1.10[c]	29 ± 1.56[d]	40 ± 1.44[f]	35 ± 1.75[f]	54 ± 1.13[g]
FeSOD 3	47 ± 1.4[c]	22 ± 1.35[e]	37 ± 1.84[f]	31 ± 1.70[d]	52 ± 1.44[c]
Cu/ZnSOD 1	70 ± 1.65[b]	28 ± 1.94[d]	40 ± 1.65[f]	45 ± 1.35[i]	71 ± 1.85[b]
Cu/ZnSOD 2	84 ± 1.75[d]	60 ± 1.6[a]	79 ± 1.40[h]	82 ± 1.65[d]	94 ± 1.59[j]
Cu/ZnSOD 3	63 ± 1.4[a]	32 ± 1.15[d]	48 ± 1.50[c]	49 ± 1.69[c]	65 ± 1.43[a]
Cu/ZnSOD 4	59 ± 1.14[a]	29 ± 1.55[d]	44 ± 1.81[i]	42 ± 1.59[i]	62 ± 1.85[g]
Cu/ZnSOD 5	57 ± 1.55[a]	30 ± 1.64[d]	44 ± 1.31[i]	43 ± 1.51[i]	60 ± 1.31[a]
Cu/ZnSOD 6	60 ± 1.69[a]	38 ± 1.10[f]	50 ± 1.91[c]	47 ± 1.44[c]	63 ± 1.81[a]
Cu/ZnSOD 7	62 ± 1.94[a]	38 ± 1.85[f]	54 ± 1.31[g]	40 ± 1.40[f]	56 ± 1.62[g]
Cu/ZnSOD 8	57 ± 1.07[a]	33 ± 1.2[d]	47 ± 1.4[c]	37 ± 1.08[f]	47 ± 1.50[c]

The various letters signify significant differences between means at $P<0.05$ (DMRT). Values are means ± S.E ($n = 3$).

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4.3.2. The effect of NO on the enzymatic activity of ascorbate peroxidase in soybean

In total three APX isoforms were observed in the soybean leaves on the native PAGE gel (figure 4.3). When compared to the control (untreated), a decrease in band intensity for APX 1, APX 2 and APX 3 was observed for the individual treatments with either the NOS inhibitor, L-NAME or NO scavenger, cPTIO. However, an increase in band intensity was observed in the two treatments with DETA/NO close to that of the control. When comparing both individual treatments namely L-NAME and cPTIO to their DETA/NO counterparts, the band intensities were observed to be much higher in the treatments with DETA/NO.

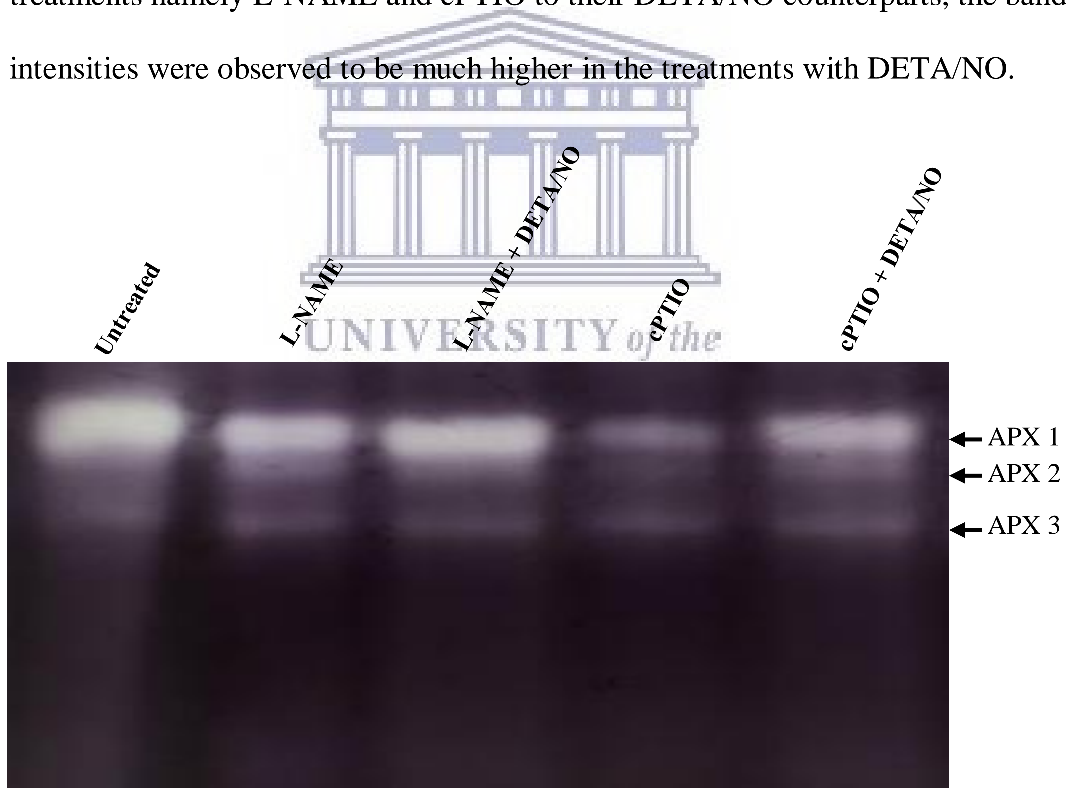

Figure 4.3. The effect 1 mM L-NAME, 100µM cPTIO and combination treatments with 100 µM DETA/NO on the activity profile of ascorbate peroxidase in soybean. APX in-gel activity assays were conducted on plant material which was treated with the above, respectively. This in-gel assay was conducted to understand how changes in nitric oxide content influenced the levels of APX. The gel illustrates the effect changes nitric oxide content has on the APX isoforms.

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Densitometry analysis determined the change in isoform activities due to the inhibition and scavenging of NOS and NO, respectively. When compared to the control, the treatment with L-NAME showed a ~6% decrease in intensity units for the APX 1 isoform. Treatment with cPTIO showed a ~11% decrease in intensity units for the APX 1 isoform compared to the control. A ~3% increase in intensity units for the APX 1 isoform was observed for the combination treatment of L-NAME and DETA/NO when compared to the control. The combination treatment of cPTIO and DETA/NO displayed a ~6% decrease in intensity units for the APX 1 isoform compared to the control. Comparing the intensity units for the APX 1 isoform, the treatments of L-NAME to L-NAME and DETA/NO, an increase of ~9% was observed for the DETA/NO treatment. The comparison of treatments, cPTIO to cPTIO and DETA/NO showed a ~6% increase in intensity units for the APX 1 isoform for the DETA/NO treatment (table 4.2). When compared to the control, the treatment with L-NAME showed a ~3% decrease in intensity units for the APX 2 isoform. Treatment with cPTIO showed a ~15% decrease in intensity units for the APX 2 isoform compared to the control. A ~4% increase in intensity units for the APX 2 isoform was observed for the combination treatment of L-NAME and DETA/NO when compared to the control. The combination treatment of cPTIO and DETA/NO displayed a ~7% decrease in intensity units for the APX 2 isoform compared to the control. When comparing the intensity units for the APX 2 isoform, the treatments of L-NAME to L-NAME and DETA/NO, an increase of ~7% is observed for the DETA/NO treatment. The comparison of treatments, cPTIO to cPTIO and DETA/NO showed a ~9% increase in intensity units for the APX 2 isoform for the DETA/NO treatment (table 4.2). When

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compared to the control, the treatment with L-NAME showed a ~27% decrease in intensity units for the APX 3 isoform. Treatment with cPTIO showed a ~5% decrease in intensity units for the APX 3 isoform compared to the control. A ~23% decrease in intensity units for the APX 3 isoform was observed for the combination treatment of L-NAME and DETA/NO when compared to the control. The combination treatment of cPTIO and DETA/NO displayed a ~9% increase in intensity units for the APX 3 isoform compared to the control. When comparing the intensity units for the APX 3 isoform, the treatments of L-NAME to L-NAME and DETA/NO, an increase of ~6% was observed for the DETA/NO treatment. The comparison of treatments, cPTIO to cPTIO and DETA/NO showed a ~14% increase in intensity units for the APX 2 isoform for the DETA/NO treatment (table 4.2).

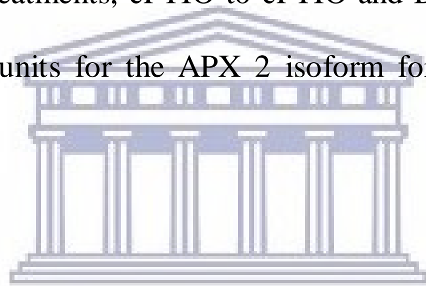

Table 4.2. Densitometry analysis for APX native PAGE activity gels (AU).

	Untreated	L-NAME	L-NAME + DETA/NO	cPTIO	cPTIO + DETA/NO
APX 1	141 ± 1.45^{a}	133 ± 1.45^{h}	145 ± 1.55^{a}	125 ± 1.59^{e}	133 ± 1.51^{h}
APX 2	75 ± 1.63^{b}	76 ± 1.55^{b}	78 ± 1.35^{b}	64 ± 1.55^{f}	70 ± 1.53^{i}
APX 3	44 ± 1.30^{c}	32 ± 1.85^{d}	34 ± 1.25^{d}	42 ± 1.53^{g}	48 ± 1.42^{c}

The various letters signify significant differences between means at $P<0.05$ (DMRT). Values are means ± S.E (n = 3).

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4.3.3. The effect of NO on the enzymatic activity of catalase in soybean

In total two CAT isoforms were observed in the leaves of soybean on the native PAGE gel (figure 4.4). When compared to the control (untreated), a decrease in band intensity for both CAT 1 and CAT 2 were observed for the individual treatments with either the NOS inhibitor, L-NAME or NO scavenger, cPTIO. However, an increase in band intensity was observed in the two treatments with DETA/NO close to that of the control. When comparing both individual treatments namely L-NAME and cPTIO to their DETA/NO counterparts, the band intensities were observed to be much higher in the treatments with DETA/NO.

Figure 4.4. The effect 1 mM L-NAME, 100 µM cPTIO and combination treatments with 100 µM DETA/NO on the activity profile of catalase in soybean. CAT in-gel activity profiling was conducted on plant material which was treated with the above, respectively. This in-gel assay was conducted to understand how changes in nitric oxide content influenced the levels of CAT. The gel illustrates the effect changes in nitric oxide content have on CAT.

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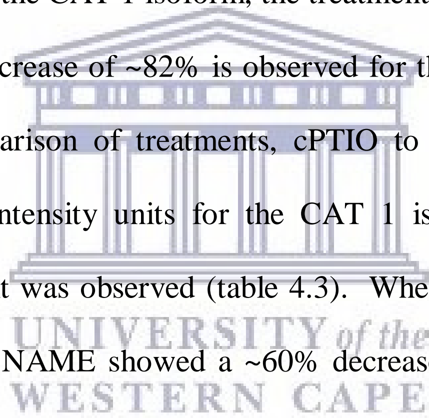
Densitometry analysis was used to determine the change in isoform activities due to the inhibition and scavenging of NOS and NO, respectively. When compared to the control, the treatment with L-NAME showed a ~61% decrease in intensity units for the CAT 1 isoform. Treatment with cPTIO showed a ~71% decrease in intensity units for the CAT 1 isoform compared to the control. A ~29% decrease in intensity units for the CAT 1 isoform was observed for the combination treatment of L-NAME and DETA/NO when compared to the control. The combination treatment of cPTIO and DETA/NO displayed a ~35% decrease in intensity units for the CAT 1 isoform compared to the control. When comparing the intensity units for the CAT 1 isoform, the treatments of L-NAME to L-NAME and DETA/NO, an increase of ~82% is observed for the DETA/NO combination treatment. The comparison of treatments, cPTIO to cPTIO and DETA/NO, a ~125% increase in intensity units for the CAT 1 isoform for the DETA/NO combination treatment was observed (table 4.3). When compared to the control, the treatment with L-NAME showed a ~60% decrease in intensity units for the CAT 2 isoform. Treatment with cPTIO showed a ~56% decrease in intensity units for the CAT 2 isoform compared to the control. A ~27% decrease in intensity units for the CAT 2 isoform was observed for the combination treatment of L-NAME and DETA/NO when compared to the control. The combination treatment of cPTIO and DETA/NO displayed a ~47% decrease in intensity units for the CAT 2 isoform compared to the control. When comparing the intensity units for the CAT 2 isoform, the treatments of L-NAME to L-NAME and DETA/NO, an increase of ~82% was observed for the DETA/NO combination treatment. The comparison of treatments, cPTIO to cPTIO and DETA/NO, showed a ~19%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increase in intensity units for the CAT 2 isoform for the DETA/NO treatment (table 4.3).

Table 4.3. Densitometry analysis for CAT native PAGE activity gels (AU).

	Untreated	L-NAME	L-NAME + DETA/NO	cPTIO	cPTIO + DETA/NO
CAT 1	28 ± 1.58[a]	11 ± 1.65[c]	20 ± 1.60[d]	8 ± 1.70[c]	18 ± 1.65[d]
CAT 2	70 ± 1.19[b]	28 ± 1.36[a]	51 ± 1.21[e]	31 ± 1.11[a]	37 ± 1.85[f]

The various letters signify significant differences between means at $P<0.05$ (DMRT). Values are means ± S.E (n = 3).

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4.4. Discussion

In the previous chapter we investigated the inhibition of nitric oxide synthase (NOS) and the subsequent implications on the antioxidant profiles within soybean leaves using a NOS inhibitor as well as a NO scavenger. Furthermore, a NO donor was used to assess whether the inhibitory and scavenging effects on NOS and NO, respectively, could be reversed. It was observed that the inhibition of NOS and scavenging of NO had a detrimental effect on the biochemical and physiological aspects of soybean. This was most likely attributed to the inadequate scavenging of the antioxidant enzymes brought on by the inhibitory and scavenging effects on NOS and NO, respectively, which elicited an increase in oxidative stress. It was observed that with the addition of the NO donor, the inhibitory and scavenging effects on NOS and NO, respectively, was alleviated. Thus, the current chapter aimed at investigating the effect of NOS inhibition and NO scavenging on the antioxidant isoform profiles in soybean.

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The accumulation of the superoxide radical can be detrimental to plants as it is known to cause damage to macromolecules. Thus, plants have developed a highly effective ROS scavenging mechanism known as the antioxidants. There are three classifications of SOD isoforms namely the MnSODs, FeSODs and Cu/ZnSODs depending on the metal in the active site (DiGuiseppi, Fridovich and McCord, 1984). The concentration of superoxide dismutase tends to increase when plants undergo oxidative stress. Thus, the abundance of SOD isoforms allows the plant to effectively overcome oxidative stress (Sen Raychaudhuri and Wang Deng,

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2000). In this study, protein extracts obtained from the leaves of soybean were used to determine the SOD isoform profile (figure 4.1). The identification of the SOD isoforms were performed using hydrogen peroxide (H_2O_2) and potassium cyanide (KCN) which are known to inhibit particular SOD classes (figure 4.2). Potassium cyanide inhibits the CuZnSODs, whilst hydrogen peroxide inhibits both CuZnSODs and MnSODs. A study by (Talukdar and Talukdar, 2013) identified SOD classes using KCN and H_2O_2 within common bean subjected to metal stress. In this chapter, a similar inhibition pattern to that of Talukdar and Talukdar (2013) was observed and determined the class each SOD belonged to. In total, 13 SOD isoforms were identified of which two are MnSODs, three are FeSODs and eight are CuZnSODs (figure 4.1). In a study by Klein (2012) it was interesting to note that only 11 SODs were identified in soybean of which two were MnSODs, three were FeSODs and six were CuZnSODs. This was not consistent with the results of this chapter as two extra CuZnSODs are present. The latter could be due to the difference in gel percentage. The study by Klein (2012) used a 10% polyacrylamide gel, whereas this current study used a 13% polyacrylamide gel and thus the higher percentage gel i.e. higher resolution, could better differentiate between the SOD isoforms due to better separation.

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The cPTIO and DETA/NO treatments showed an increase in SOD isoform activity for Cu/ZnSOD 1-6 when compared to the control; however the Cu/ZnSOD 7 and 8 show a decrease in activity in comparison to the control. This decrease could be attributed to one of two reasons. First being that the Cu/ZnSOD 7 and 8 might be sensitive to the inhibitory and scavenging effects on NOS and NO, respectively. The second being that this might be an artefact due to technical

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difficulties experienced when conducting the experiment such as the migration time on the PAGE gel, the concentrations of the reagents that made up the gel, improper or overstraining that could be masking the activity of the isoform bands as well as the duration the gels were exposed to light (table 4.1). However, the application of NO donor, DETA/NO, was observed to alleviate the inhibitory and scavenging effects of NOS and NO, respectively.

After the disproportionation of superoxide to hydrogen peroxide, ascorbate peroxidase (APX) scavenges H_2O_2 and as a result regulates ROS levels within plants (Kornyeyev *et al.*, 2003; Maruta *et al.*, 2012; Keyster *et al.*, 2014). Ascorbate is oxidised by APX to reduce H_2O_2 to H_2O (Dalton *et al.*, 1986; Dalton *et al.*, 1993; Iturbe-Ormaetxe *et al.*, 2001; Matamoros *et al.*, 2006). In this chapter, only three APX isoforms were identified. It was observed that the inhibition of NOS and scavenging of NO using L-NAME and cPTIO, respectively, affected the antioxidant activity of APX, resulting in a decrease in APX activity (figure 4.3) (table 4.2). However, the application of the NO donor, DETA/NO, reversed these affects thus enhancing pixel intensities of the isoforms as well as the scavenging capability of APX. These findings are supported by the study of Egbichi *et al.* (2013) which investigated whether the application of the nitric oxide donor, DETA/NO, could reduce the toxic effect resulting from short-term salt stress. Treatments included 150 mM NaCl (alone), 150 mM NaCl in combination with 10 μM DETA/NO and 150 mM NaCl in combination with 10 μM DETA. To summarise briefly, the application of DETA/NO decreased the levels of H_2O_2 and MDA content, alleviating the effects of oxidative stress. Furthermore, the study observed an increase in total APX activity in response to

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salt stress, when compared to the control. The increase in response to salt stress, as well as the increase in MDA and H_2O_2 content suggests that the plants antioxidant system was unable to function adequately. However, the addition of NO donor, DETA/NO, enhanced the antioxidant activities. Similarly to our findings in chapter 3 for the total APX activity, the addition of the NO donor, DETA/NO alleviated the inhibitory and scavenging effects on NOS and NO, respectively. This trend was also observed with the native gel staining of APX isoform activity in the current chapter, where the application of exogenous NO increased the pixel intensities of the APX isoforms.

Similarly to APX, catalase also scavenges hydrogen peroxide aiding in its detoxification and resulting in the release of water and oxygen. Catalase plays a crucial role in the alleviation of oxidative stress and has a high affinity for H_2O_2 (Kirkman and Gaetanit, 1984; König *et al.*, 2002; Chelikani *et al.*, 2004; Mhamdi *et al.*, 2010). Only two catalase isoforms have been identified in plants (Mhamdi *et al.*, 2010). When comparing the results of SOD and APX with CAT, a similar trend is observed, where the inhibition of NOS and scavenging of NO caused a decrease in CAT activity and the addition of a NO donor reversed these effects (figure 4.4); (table 4.3). These findings are supported by a study done by Li *et al.* (2008), who observed that salt stress caused an increase in oxidative stress. The application of a NO donor (SNP) however alleviated the effects caused by oxidative stress and was observed to increase the accumulation of ferritin. Furthermore, the addition of SNP also enhanced the antioxidant activities of SOD, APX and CAT. Similarly to the findings in chapter 3 for the total CAT activity, the addition of DETA/NO alleviated the inhibitory and scavenging effects on

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NOS and NO, respectively. A similar trend was observed with the native gel staining of CAT isoform activity in this chapter, where the application of exogenous NO increased the pixel intensities of the CAT isoforms. Overall the application of exogenous NO enhanced the SOD, APX and CAT activities and thus their scavenging capabilities, alleviating oxidative stress and regulating the levels of superoxide and hydrogen peroxide in soybean and ultimately promoting better growth.

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General Conclusion and Future prospects

Throughout the study it was observed that the application of a NOS inhibitor and NO scavenger had a detrimental effect on the NO content within soybean. Due to the inhibition of NOS and scavenging of NO, an increase in ROS was observed which subsequently affected the scavenging capabilities of the antioxidant enzymes. However, the exogenous application of NO reversed these effects enhancing the activities of the antioxidant enzymes allowing them to efficiently scavenge superoxide and hydrogen peroxide. Thus, the results of this study show that NO can function as an inducer of the antioxidant system and plays a protective role against oxidative stress. Thus, as a signalling molecule, NO is crucial for the regulation of the plant antioxidant system which in turn maintains redox homeostasis within plants. In future transcriptomics could be an option for investigating total gene responses to either an increase or decrease in NO. Proteomic analysis is also an option as it would be of utmost importance to identify the proteins, via proteomic profiling, that are unique to the changes in NO content imposed by the use of the nitric oxide synthase inhibitors or NO donors. This would ultimately give us a better understanding as to how plants respond to biotic and abiotic stress that is mediated through changes in the production and metabolism of reactive oxygen species and nitric oxide.

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